AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

Pharmaceutical Development and Evaluation of Liposomal Drug Delivery System for Azacitidine

Sreedhar Bandari[1] *, Seshasai Marella[2],Mule Shanmukha[2]

1. Jawaharlal Nehru Technological University,Hyderabad, Andhra Pradesh, India 500085

2. Hospira Healthcare India Pvt.Ltd, SIPCOT, Sriperumbudur (T.K), Tamilanadu, India. 602105.

ABSTRACT

Azacitidine and its deoxy derivative are used in the treatment of myelodysplastic syndrome. These types of drugs were first synthesized in Czechoslovakia as potential chemotherapeutic agents for cancer. Conventional compositions of Azacitidine are available as powder product or as a solution of Azacitidine in water. Both these products have been associated with a number of toxicities when administered intravenously. To overcome these problems, in the present study, inclusion of Azacitidine in liposomal formulation has proved to be good approach to eliminate the toxicities and improve drug antitumor activity. We formulated Azacitidine liposomes containing Hydrogenated soy phosphatidylcholine, 1,2-Distearoyl-*sn*-glycero-3[Phospho-rac-(1-glycerol) (Sodium Salt) [DSPG-Na] and Cholesterol by dried thin film hydration technique. Particle size analysis, zeta potential, %free drug are strongly affected by the different lipid concentration and result shown F5 formulation have the optimum % free drug, Particle size and F2 formulation shown highest Percent drug release when compared to the F4 & F5 formulations. The release kinetics of F2, F4 and F5 formulations were studied. All the formulations follow zero-order release kinetics and follow case-II transport when it applied to the Korsmeyer-Peppas model for the mechanism of drug release. The stability of the F2, F4 & F5 formulations were studied at 5±3^{o}c and at room temperature for duration of 3 months. Hence it can be concluded that the liposomes along with Hydrogenated soy phosphatidylcholine, DSPG-Na and Cholesterol are suitable carriers for the preparation of Azacitidine liposomes.

Key words: Azacitidine, Liposomes, Hydrogenated soy phosphatidylcholine, 1,2-Distearoyl-*sn*-glycero-3[Phospho-rac-(1-glycerol.) [DSPG-Na], cholesterol.

*Corresponding Author Email: sreebandari@yahoo.co.in

Received 01 October 2012, Accepted 08 October 2012

Please cite this article in press as: Bandari S. *et al.*, Pharmaceutical Development and Evaluation of Liposomal Drug Delivery System for Azacitidine . American Journal of PharmTech Research 2012.

INTRODUCTION

The goal of any drug delivery system is to provide a therapeutic amount of drug to the proper site in the body, to achieve promptly and then maintain the desired drug concentration. Liposomes are Microscopic, Fluid-filled pouch, whose walls are made up of layers of Phospholipids identical to the Phospholipid that makes up cell membrane[1].

Azacitidine ($C_8H_{12}N_4O_5$) with Molecular weight 244.205 gm/mol, an synthesized in Czechoslovakia as potential chemotherapeutic agents for cancer. The mechanism of action of Azacitidine (5-azacytidine) is a chemical analogue of the cytosine nucleoside used in DNA and RNA. Azacitidine is thought to induce anti-neoplastic activity via two mechanisms; inhibition of DNA methyltransferase at low doses, causing hypomethylation of DNA, and direct cytotoxicity in abnormal hematopoietic cells in the bone marrow through its incorporation into DNA and RNA at high doses, resulting in cell death. As azacitidine is a ribonucleoside, it incoporates into RNA to a larger extent than into DNA. The incorporation into RNA leads to the dissembly of polyribosomes, defective methylation and acceptor function of transfer RNA, and inhibition of the production of protein. Its incorporation into DNA leads to a covalent binding with DNA methyltransferases, which prevents DNA synthesis and subsequent cytotoxicity. In clinical trials Azacitidine is successfully used, mostly against myelodysplastic syndrome and myelodysplastic syndrome, the main side effects of this drug are cardiac toxicity and Infusion reactions[2, 3].

To overcome these problems, an alternative approach is needed. In the present study Azacitidine liposomes are formulated using various Phospholipids to check effect of drug loading and particle size. These liposomes appeared to reduce some of the toxic effects caused by the release of their contents, but have new toxic effects appeared like skin toxicity generally known as “Hand-Foot Syndrome” and the presence of large molecules on the liposomal surface may reduce the interaction of liposomal with cells & hinder entry of liposomes in to tumor tissue[4, 5, 6].

Thus, these remains a need for stable, long circulating liposomes that do not cause such deleterious effects such as the “Hand-Foot Syndrome” as well as methods of manufacturing such liposomes & composition based on them. The present formulation meets this need, and testing the effect of stabilizers on particle size analysis, percent free drug, Assay, *In-vitro* drug release studies, release kinetics & stability studies.

The main objective of the study was designed to prepare and evaluate the neutral and charged Azacitidine liposomes and study the effect of various stabilizers based on the Physicochemical and *in-vitro* release studies.

MATERIALS AND METHODS

Azacitidine, Hydrogenated soy phosphatidylcholine, 1,2-Distearoyl-sn-glycero-3[Phospho-rac-(1-glycerol.) (Sodium Salt) [DSPG-Na] and Cholesterol were obtained as a sample from SRC laboratories Pvt ltd , Kukatpally, Hyderabad. The laboratory grade chemicals used for the work are Histidine, Chloroform, Sodium hydroxide, Triton x-100, orthophospharic acid and methanol were purchased from Merck Chemicals Pvt., Ltd. Mumbai.

Preformulation study

General Procedure for the Preparation of calibration curve by HPLC

A stock solution of standard drug was prepared, later required dilutions were made with a buffer pH 6.8. To a series of 10ml volumetric flasks aliquots standard solutions were taken and the volume was made up using a phosphate buffer pH 6.8. The absorbance of these solutions was measured at respective wave length of maximum absorbance, using column by HPLC. Absorbance values were plotted (Figure 1) against respective concentration to obtain standard calibration curve.

Compatibility Studies

IR spectroscopy can be used to investigate and predict any physicochemical interactions between different components in a formulation and therefore it can be applied to the selection of suitable chemically compatible excipients. The aim of the present study was to test, whether there are any interactions between the carriers and drug; The following IR spectroscopy was recorded.

Procedure for the preparation of Azacitidine liposome[7, 8, 9]

Liposomes were prepared by dried thin film hydration technique using rotary evaporator. Accurately weighed quantities of HSPC, DSPG-Na and cholesterol are dissolved in chloroform and rotated in a rota-vap by applying vacuum of about 25mmHg at 25^0c, until it forms a thin film. Required quantity of L-Histidine was dissolved in WFI and it is added to the above thin film in R.B flask and rotated until it forms a milky white suspension. The above solution is homogenized for 15 cycles to reduce particle size of liposomes. Drug solution is prepared by adding the required quantity of Drug in a WFI and pH is adjusted to 6.4 to 6.7 and this drug solution is added to the solution in a R.B flask (lipid solution) and rotated for 1hr.

In-process Checks during formulation of Azacitidine liposomes

RPM: 70-80rpm (Film formation), 50-60rpm (Hydration), 60-65rpm (Drug Loading).

Temperature: 35-40°c (Film formation), 60-70°c (Hydration), 60°c (Drug Loading).

Physical characterization of liposomes [10, 11, 12]

Determination of particle size, Zeta potential and SEM analysis was carried out by using the Malvern Zeta Sizer and Scanning Electron Microscopy in Indian Institute of Chemical technology, for the optimized formulations.

Percent free drug [13]

Measure the absorbance of solution at 280nm using Histidine solution as blank. Transferred 0.1ml of sample to a 20ml stoppered test tube, add 8ml of Histidine solution to it, mix well, measure the absorbance at 280nm using calibrated UV spectrophotometer. Transfer the solution from the cell to test tube (A_1).To the above test tube containing solution, added 1ml sodium hydroxide solution, mix well measure the absorbance at 280 nm using UV transfer the solution from the cell to test tube (A_2).To the above test tube containing solution, add 1ml of Triton X-100 solution, mix well measure the absorbance at 280 nm using calibrated UV (A_3).

$$\text{Percent Free Azacitidine} = [(A_2 \times 1.125) - A_1/A_3 \times 1.25] \times 100$$

Azacitidine Assay by HPLC method [13]

A standard and sample solution were prepared, Inject separately 20 microlitre of the standard and sample solution in chromatographic condition and record the chromatogram. Calculate the content of drug per ml in liposomal injection as follows.

$$\text{Assay} = A_i \times S_w \times 200 \times P / A_s \times 20 \times 2 \times LC$$

Where,

A_i = Area corresponding to Azacitidine in sample.

A_s = Area corresponding to Azacitidine in working standard.

P = % purity of Azacitidine in working standard.

LC = Label claim of Azacitidine (mg/vial).

S_w = Weight of working standard in mg.

***In vitro* dissolution studies of Azacitidine liposome** [14]

The *in vitro* release of drug from the liposomal formulation was carried out by using dialysis membrane employing in two sides open ended cylinder.4 ml of liposomal suspension containing known amount of drug was placed in a dialysis membrane previously soaked overnight. The two sides open cylinder was placed in 200ml of BS (pH 6.8), maintained at 37^o C and stirred with the help of a magnetic stirrer. Aliquots (4ml) of release medium were withdrawn at different time intervals and the sample was replaced with fresh BS (pH 6.8) to maintain constant volume. 1 ml of methanol was added to each aliquot to precipitate the lipids and dissolve the entrapped Azacitidine and then the samples were analyzed by HPLC chromatographic conditions.

RELEASE KINETICS [15, 16]

To analyze the *in vitro* release data various kinetic models were use to describe the release kinetics. The zero order rate Eq. (2) describes the systems where the drug release rate is independent of its concentration. The first order Eq. (3) describes the release from system where release rate is concentration dependent. Higuchi (1963) described the release of drugs from insoluble matrix as a square root of time dependent process based on Fickian diffusion.

Zero order kinetics:

Zero order release would be predicted by the following equation:

$$A_t = A_0 – K_0t$$

When the data is plotted as cumulative percent drug release versus time, if the plot is linear then the data obeys Zero – order kinetics and its slope is equal to Zero order release constant K_0**.**

First order kinetics:

First - order release could be predicted by the following equation:

$$\text{Log } C = \log C_0 – K_t / 2.303$$

When the data plotted as log cumulative percent drug remaining versus time, yields a straight line, indicating that the release follow first order kinetics. The constant 'K_1' can be obtained by multiplying 2.303 with the slope value.

Higuchi's model:

Drug release from the matrix devices by diffusion has been described by following Higuchi's classical diffusion equation.

$$Q = [D_Є / \tau(2A – ЄCs\ )\ Cst]^{1/2}$$

When the data is spitted according to equation i.e. cumulative drug release versus square root of time yields a straight line, indicating that the drug was released by diffusion mechanism. The slope is equal to 'K' (Higuchi's 1963).

Korsmeyer equation / Peppa's model:

To study the mechanism of drug release from the liposomal solution, the release data was also fitted to the well-known exponential equation (Korsmeyer equation/ Peppa's law equation), which is often used to describe the drug release behavior from polymeric systems.

$$M_t / M_\alpha = Kt^n$$

Table1: Diffusion exponent and solute release mechanism for cylindrical shape

S.No	Diffusion	Exponent (n) Overall solute diffusion mechanism
1.	0.45	Fickian diffusion
2.	0.45<n<0.89	Anomalous (non-Fickian) diffusion
3.	0.89	Case-II transport
4.	n>0.89	Super case-II transport

Short term stability studies

The stability of a pharmaceutical delivery system may be defined as the capability of a particular formulation, in a specific container. The short-term stability was conducted to monitor physical and chemical stabilities of the liquid form of Azacitidine liposomal formulations at 5±3^{o}c and room temperature for up to three months. The stability parameter, such as Assay was determined as function of the storage time.

RESULTS AND DISCUSSION

Standard calibration curve of Azacitidine in HPLC:

The areas of Azacitidine standard solution in different concentration (mg/mL) of drug in buffer, pH 76.8 showed linearity at λ max 280nm. The linearity was plotted for area against concentration with R2 value 1.0 and with the slope equation y=680756x. The absorbance values and standard curve shown in Figure 1.

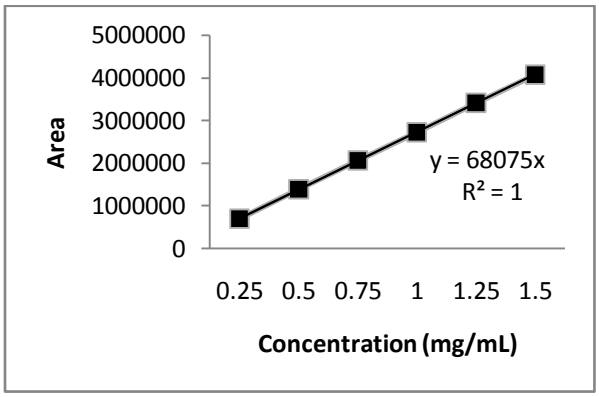

Figure1: Standard graph of Azacitidine in phosphate buffer of pH 7.4.

Compatibility studies:

The compatibility between the drug and the selected lipid and other excipients was evaluated using FTIR peak matching method. There was no appearance or disappearance of peaks in the drug-lipid mixture, which confirmed the absence of any chemical interaction between the drug, lipid and other chemicals.

Azacitidine liposomal formulation:

The Liposomes were prepared by dried thin film hydration technique using rotary evaporator with drug and carrier (HSPC)**.**The formulation containing Azacitidine was prepared with lipids like HSPC, DSPG-Na and cholesterol and all other parameters like temperature, vacuum and RPM were kept constant. The composition and ratios of compounds showed in Table 2. among those compositions 5 Formulations are selected as optimized batches for further evaluation as showed in Table 3.

Table 2: The composition and concentrations of HSPC: cholesterol: DSPG-Na for optimize the formula.

S.No.	Name of placebo liposome formulations	Concentration of lipids (mg/mL)		
		HSPC	Cholesterol	DSPG-Na
1	P-1	2.0	0.5	0.5
2	P-2	3.0	1	1
3	P-3	4.0	1.5	1.5
4	P-4	5.0	2.0	2.0
5	P-5	6.0	2.5	2.5
6	P-6	7.0	3.0	3.0
7	P-7	8.0	3.5	3.5
8	P-8	9.0	4.0	4.0
9	P-9	10.0	4.5	4.5
10	P-10	10.5	4.5	4.5
11	P-11	9.0	4.5	4.5
12	P-12	9.0	3.5	3.5
13	P-13	9.0	3.0	3.0

Table 3: The composition and ratios of Drug, HSPC, DSPG-Na and Cholesterol for optimized batches.

Formulation code	Drug (mg/ml)	HSPC (mg/ml)	DSPG-Na (mg/ml)	Cholesterol (mg/ml)
F1	2	8.0	3.5	-
F2	2	9.0	4.0	-
F3	2	9.0	4.5	1
F4	2	9.0	3.5	1
F5	2	9.0	3.0	-

Physicochemical characterization

Particle size distribution:

The particle size distribution was analyzed for F2, F4, F5 formulations of Azacitidine Liposomes by wet method. The particle size was optimum in F5 Formulation, When compared to F2 and F4, The results were shown in Table 4 and Figure 2, 3 & 4.

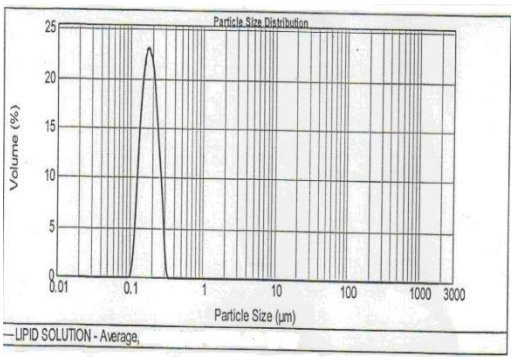

Figure 2: Particle size distribution by wet method of Azacitidine liposomal solution for F2 formulation.

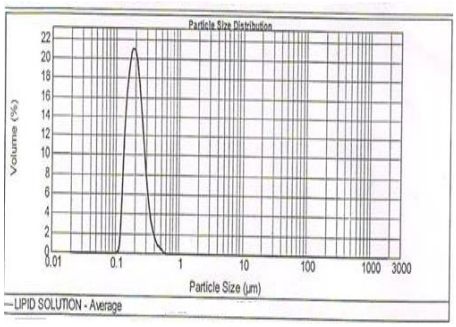

Figure 3: Particle size distribution by wet method of Azacitidine liposomal solution for F4 formulation.

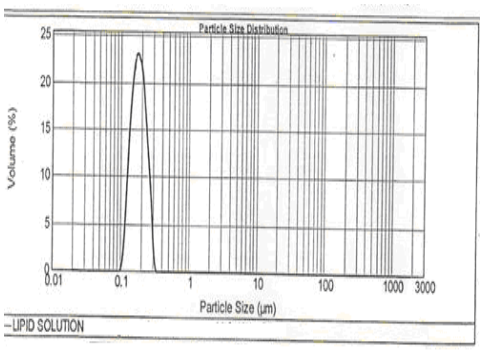

Figure 4: Particle size distribution by wet method of Azacitidine liposomal solution for F5 formulation.

Scanning Electron Microscopy:

The Morphology and surface appearance of Liposomes were examined by using SEM. The SEM photographs of F2 and F5 formulation showed that the particles have smooth surface. The SEM images were shown in Figure 5 and 6.

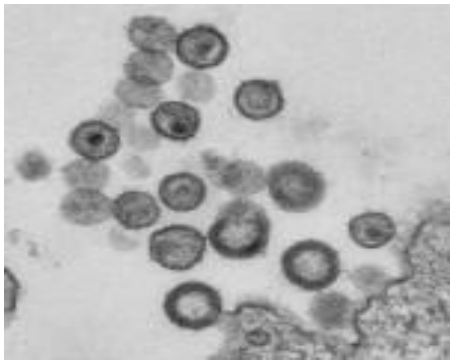

Figure 5: SEM of F2 formulation.

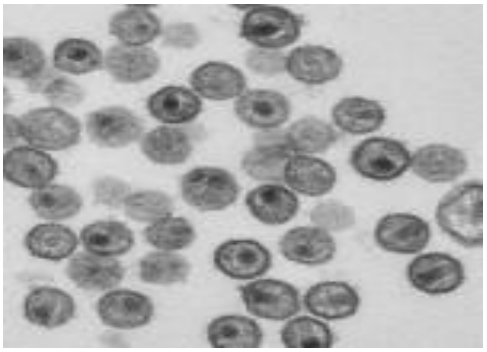

Figure 6:SEM of F5 formulation

Zeta Potential analysis:

The zeta potential report of liposomal solution for F2, F4, F5 formulations are -23.4mV, -20.9mV, -19.6mV which lies near to the arbitrary value. The report shows good stability value for formulated liposomal solution, the results were shown in Table 4.

Table 4: Physicochemical characteristics of Azacitidine Liposomes for Optimized Batches.

S.No	Formulation code	Average vesicular size (nm)	Zeta Potential(mV)	Poly dispersive index (Pdi)
1.	F2	356nm	-23.4	0.635
2.	F4	564nm	-20.9	0.762
3.	F5	317nm	-19.6	0.645

***In Vitro* Characterization**

Percent free drug:

The percent free drug is determined for all the formulations F1to F5. The free drug percentage was less in F5 formulation when compared with F1-F4; result was shown in the Table 5.

Assay:

The assay value is determined for all the formulations from F1to F5. The assay value is within the limit (>90%) for all the formulations, the results were shown in the Table 5.

Table 5: Percent free drug and Assay of Azacitidine for F1, F2, F3, F4 and F5.

S. No	Formulation code	Percentage of free drug	Assay
1.	F1	16.68±4.1%	95.7±0.32%
2.	F2	11.36±1.1%	98.5±0.65%
3.	F3	11.56±2.6%	97.1±0.41%
4.	F4	10.36±1.3%	97.3±0.96%
5.	F5	9±3.9%	98.2±0.58%

***In vitro* Dissolution data:**

The *in vitro* dissolution profile was determined by membrane diffusion method. The dissolution was carried out for a period of 24 hrs in 6.8 pH phosphate buffer. The cumulative percent release of F2, F4 and F5 formulations at various time intervals was calculated. The cumulative percent drug release in all formulations was plotted against time in Figure 7. The Maximum percent of drug release was found in F2 formulation which contains maximum drug entrapment.

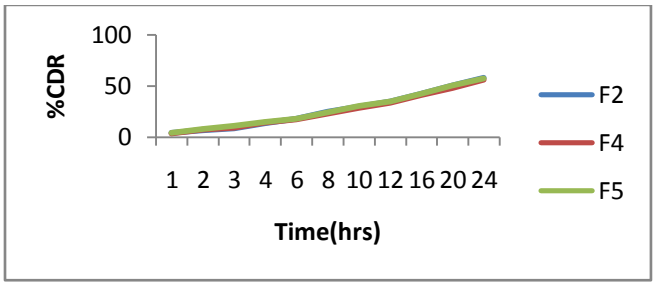

Figure 7: *in -vitro* release studies for optimized formulations F2, F4 and F5.

Release Kinetics:

The release kinetics of F2, F4, F5 formulations were studied. All formulations follow Zero order release kinetics and follow case II transport when it applied to the Korsmeyer-Peppa's Model for mechanism of drug release. The results are shown in Figure 8, 9, 10 and 11.

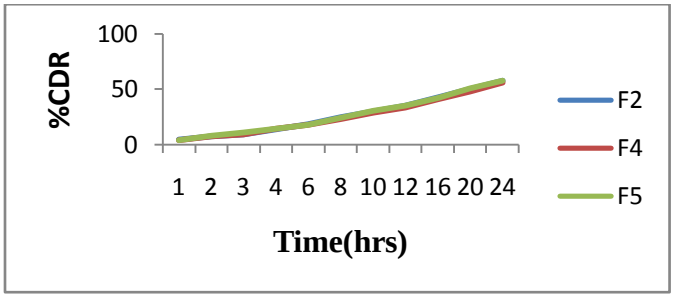

Figure 8: Zero order release studies for optimized formulations F2, F4 and F5.

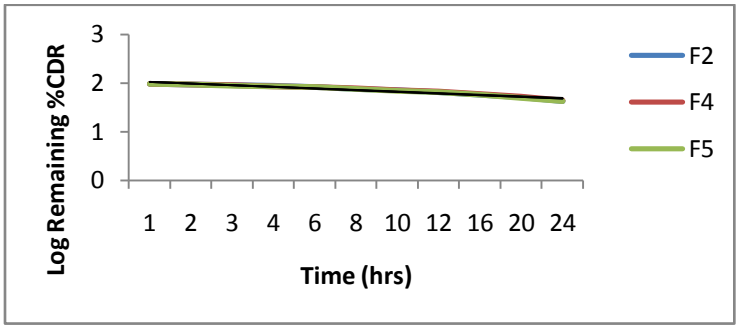

Figure 9: First order release studies for optimized formulations F2, F4 and F5.

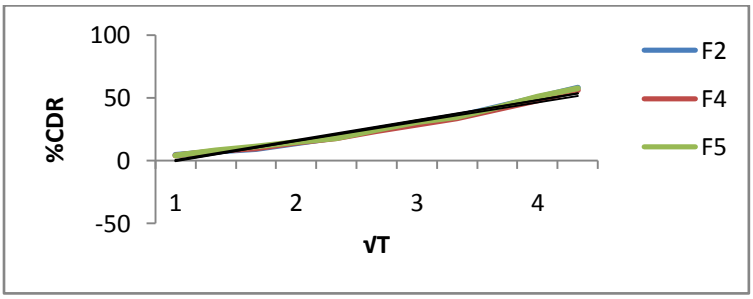

Figure 10: Higuchi's order plot for optimized formulations F2, F4 and F6.

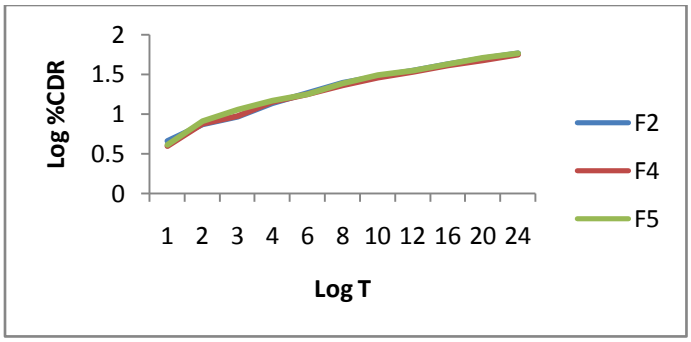

Figure 11: Korsmeyer –Peppa's model for optimized formulations F2, F4 and F6.

Stability data:

The stability of the Azacitidine Liposomes was evaluated after storage at 5 ± 3^0c and room temperature for 3months. The assays of the samples were determined as a function of the storage time. The Liposomes stored at 5 ± 3^0c were found to be stable for duration of 3months. The results were showed in Table 6 and 7.

Table 6: Effect of temperature on Assay of Azacitidine at 5±3°c.

Formulation code	Effect of stability on Assay at 5±3°c			
	Initial	1 M stability	2 M stability	3M stability
F2	98.5±0.53%	98.1±0.2%	99±1.0%	97.6±0.6%
F4	97.3±0.46%	96.6±1.12%	96.8±0.48%	96.2±0.67%
F5	98.2±0.57%	96.7±0.47%	96.2±1.63%	95.7±1.38%

Table 7: Effect of temperature on Assay of Azacitidine at room temperature

Formulation code	Effect of stability on Assay at room temperature			
	Initial	1 M stability	2 M stability	3M stability
F2	98.5±0.53%	97.4±0.72%	94±1.3%	95.2±0.65%
F4	97.3±0.46%	97.0±1.12%	95.8±0.43%	95.2±0.96%
F5	98.2±0.57%	97.7±0.9%	96.2±0.63%	95.7±1.3%

CONCLUSION

From the executed experimental results, it could be concluded that the lipids Hydrogenated soy phosphatidylcholine, 1,2-Distearoyl-*sn*-glycero-3[Phospho-rac-(1-glycerol...) [DSPG-Na] and cholesterol were suitable carrier for the preparation of Azacitidine liposomes. Though the preliminary data based on *in-vitro* dissolution profile, release kinetics and stability studies proved that the suitability of such formulations, Still a thorough experiment will be required based on the animal studies.

REFERENCES

1. Chein YM, editor, Novel drug delivery systems, 2nd edition, New York; Marcel Dekker Inc, 1992; 50:1-2.
2. www.drugbank.com
3. Gautam Vinod , Srikanth Annappa, Sangeetha, Hanurmesh Rivankar., Non-Pegylated Long-Circulating Liposomes- Patent application.
4. www.dailymed.com
5. Vyas SP. Sihorkar V., In: Advance in liposomal therapeutics, (Eds.), CBS Publishers, New Delhi, 2001; 230
6. Vyas SP, Dixit V., In: Advances in liposomal therapeutic, 1st ed., CBS Publishres, New Delhi, 2001;1: 230-243.
7. Ganesh GNK, Gowthamarajan K, Suresh Kumar R Senthil V1, Jawahar N1. Formulation and evaluation of liposomal drug delivery system for anti-cancer drug. International J Pharma Res Deve. 2011; 3(3): 27 -37.
8. Gautam Vinod , Srikanth Annappa, Sangeetha, Hanurmesh Rivankar., Non-Pegylated Long-Circulating Liposomes- Patent application.

9. Xue Ming Li, LiYan Ding, Yuanlong Xu, Yonglu Wang, QiNeng Ping., International Journal of Pharmaceutics 2009; 373: 116-123.
10. Luigi Cattel, Immordino MN, Silvia Arpicco, Flavio Rocco. Preparation, Characterization, Cytotoxicity and pharmacokinetics of liposomes containing lipophilic gemcitabine prod rugs. J Control Rel. 2004; 100: 331-346.
11. Jorge JCS, Perez-Soler R, Morais JG, Cruz MEM. Liposomal plamitoyl-L-asparaginase: Characterization and biological activity. Can Chemother Pharmcology 1994; 34: 230-234.
12. Timothy D. Heath, Ninfa G. Lopez. The effects of liposome size and surface charge on liposome- mediated delivery of methrotrexate aspirate to cells in vitro. Biochimica et Biophysica Acta 1985; 820: 74-84.
13. Howard G, Barath, Albert Z. Conner Journal of chromatography 1977; 131: 375-381.
14. Ahmad et al., Antibody-Targeted Delivery of Azacitidine. Entrapped in Statically Stabilized Liposomes Can Eradicate Lun Cancer in Mice. Cancer Res *1993;* 53: 1484–1488.
15. Harris Shoaib M, Jaweria Tazeen, Hamid A. Merchant. Evolution drug release kinetics from Ibuprofen Matrix tablets using HPMC. Pak J Pharm Sci 2006; 19(2): 119-124.
16. Higuchi. T., Mechanism of sustained action, medication; Theoretical analysis of rate of release of solid drug dispersed in solid matrix. J Pharm Sci 1963; 1145-1149.