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DEVELOPMENT OF CYCLOPHOSPHAMIDE NANO INJECTABLE LIPOSOMAL FORMULATION, CHARACTERIZATION AND STABILITY EVALUATION

***Rajasekhara Reddy S[1], Anjaneyulu Vinukonda[2], Neelesh Chaubey[3], Harish Pandey[3]**

[1]Alembic Pharmaceuticals Ltd, Vadodara, India
[2]Gland Pharma Ltd, Visakhapatnam, India
[3]Department of pharmacy; Sri Satya Sai University of Technology and Medical Sciences, Sehore, India
*Corresponding author Email: **srrpharma@gmail.com**

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ABSTRACT

Cyclophosphamide is an alkylating agent extensively used as an anticancer chemotherapeutic agent. However, the genotoxic and mutagenic effect of Cyclophosphamide is still the primary limitation for wide application. Conventional chemotherapy Cyclophosphamide used to treat various types of cancers and few autoimmune disorders. Liposomes, a phospholipid bilayer vesicular system is extensively being used and studied for drug delivery applications in cancer therapy. The reason behind is advantages that liposomes offer such as their biocompatible and versatility of efficiently encapsulating both hydrophilic and hydrophobic drugs. Liposomes have been used to improve the therapeutic index of new or established drugs by modifying drug absorption, reducing metabolism, prolonging biological half-life or reducing toxicity. Drug distribution is then controlled primarily by properties of the carrier and nolonger by physico-chemical characteristics of the drug substance only. Therefore, in the present study the Cyclophosphamide loaded liposomes were developed by passive loading method using lipids Phospho lipids and cholesterol, which is able to encapsulate Cyclophosphamide. The prepared Cyclophosphamide Liposomes physicochemical parameters characterized for shape, percent drug content, % entrapment efficiency, particle size, zeta potential, Osmolality, In-vitro drug release and stability studies of the Liposomes. The Cyclophosphamide Liposomes morphology by TEM showed spherical shape with a mean average size of 84 µm. These results revealed that, Liposomal formulation could be a valuable carrier for the delivery for chemotherapy. The stability studies performed as per ICH guidelines.

INTRODUCTION

First-line therapy of solid tumors is based on surgery, radiotherapy and/or chemotherapy. For metastasized tumors, or for lesions, which cannotbe removed surgically, chemotherapy is among the very few treatment options available. Unfortunately, however, the therapeutic potential of classical chemotherapeutic drugs Is limited and they generally cause severe side effects. Advances in nanotechnology and in chemical/pharmaceutical engineering have led to the development of many different drug delivery systems. These systems aim to improve the bio-distribution and target site accumulation of chemotherapeutic drugs. Examples of drug delivery systems are polymer conjugates, micelles and liposomes,

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which typically have sizes ranging from 5 to 200 nm. These so called nanomedicine formulations have shown promising results in preclinical trials, and some of them are already routinely used in clinical practice

By means of improved circulation times, nanomedicines can accumulate in tumors via the so called Enhanced Permeability and Retention (EPR) effect, which was first described by Matsumura and Maeda in 1986. EPR relies on specific pathophysiological characteristics of tumors vs. healthy tissues. In healthy tissues, low-molecular-weight drugs easily extravasate out of blood vessels, while nanomedicines are unable to do so, because of their size. Conversely, in tumors, the abnormally wide fenestrations in the blood vessels allow for the extravasation of materials with sizes up to several hundreds of nanometers. This, together with the absence of lymphatic drainage, leads to a relatively effective and selective accumulation of Nano medicines in tumors. Conventional chemotherapy is based on low molecular weight drugs (generally less than 1000 Da). Due to their small size, chemotherapeutic agents, such as doxorubicin, Cyclophosphamide and gemcitabine, have unfavorable pharmacokinetics and a suboptimal biodistribution, as exemplified by a short blood half-life and prominent off-target accumulation in multiple healthy organs. This, together with the unspecific mechanism of action of chemotherapeutic drugs and their large volume of distribution, causes severe side effects, such as myelosuppression, immunosuppression, mucositis, neurotoxicity, Bone Marrow failure, nausea and vomiting. By increasing the size of systemically administered anticancer agents to at least 5-10 nanometers in diameter (i.e. exceeding the renal clearance threshold of ~40000 Da), kidney excretion can be reduced, blood half-lifes prolonged, and target site accumulation improved. As an example, the encapsulation of doxorubicin into liposomes (Caelyx®/Doxil®) results in an increase in plasma half-life from 5-10 minutes for the free drug, to 2-3 days for the liposome-encapsulated drug . In this specific case, as in many other liposomal and micellar nanomedicine formulations, surface modification with the stealthy polymer polyethyleneglycol (PEG) decreases aggregation and opsonization with plasma proteins, contributing to the prolonged circulation half-life. Paclitaxel Albuminprotein-bound Nano particles for injectable suspension (Abraxene) half-life increased with Nano formulation compared with conventional liquid injection, this enables effective delivery of albumin bound compound to target sites while minimizing systemic toxicity. Currently, a number of passively targeted nanoparticles are in clinical use including, Abraxene, Doxil, Marqibo, Myocet, and DuanoXome. Many other nanoparticles have shown promising therapeutic efficacy in clinical trials.Major drawbacks of passive targeting include the inability to distinguish between healthy and diseased tissues, inadequate tumor accumulation, intra- and inter tumoral as well as inter-individual tumor heterogeneity.

Active targeting was at first employed to enhance the EPR-based drug delivery as acomplementary approach with passively targeted drugs to improve tumor accumulationby nanoparticles to increase targeting efficiency and enhance their retention at targeted tumors. Passively targeted drugs, which are dependent on the EPR effect, maynot be sufficient to achieve effective targeting at target sites. Active targeting approaches are necessarily much more complex than a passive one.

For both passive and active targeting approaches, the development of companion diagnostic imaging technologies to evaluate the targeting efficiencies is very important. Selection of suitable patients and modifying treatments for specific patients may improve tumor accumulation, efficacy, and therapeutic outcome reducing the adverse effects, unnecessary treatments, and overall health expenses. Finally, active targeting can be used to complement passive targeting for better treatment results. Liposomes made their successful entry into the market in 1995 with the development of the PEGylated liposomal formulation Doxil®. Since its entry, there has been no looking back for these delivery systems, which have been explored for various diseases ranging from cancer

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treatment to pain management. The main advantages of liposomes include, control over pharmacokinetics' and pharmacodynamics' properties, improved bioavailability and limited toxicity. Together these confer on liposomes the ability to overcome the limitations of conventional therapy. Different typesof liposomes, e.g., PEGylated liposomes (Lipodox), temperature sensitive liposomes (ThermoDox),Cationic liposomes (EndoTAG-1) and liposomal vaccines (Epaxal and Inflexal V), demonstrate the intense research on liposomes. Several liposomes successfully translated into the clinic and other liposomal formulations are in different phases of clinical investigation. Although many of these products proven beneficial in preclinical trials, but only formulations that show efficacy in clinical trials will make their way into the clinic. In summary, the liposomes currently in clinical trials may provide benefits to the diversified patient population for various therapeutic applications. Cyclophosphamide is a white crystalline powder with the molecular formula of C7H15Cl2N2O2P•H2O and a molecular weight of 279.1. Cyclophosphamide is soluble in water, saline, or ethanol. Currently Cyclophosphamide for Injection dry powder filled vials available in market for Intravenous injection/Infusion. The current research work focused to develop Cyclophosphamide Nano Liposomal formulation for injectable use. Cyclophosphamide loaded In Liposomes for Intravenous can act as drug reservoirs and modification of their composition or surface can adjust the drug release rate and/or the affinity of the target site or by Enhanced Permeability and Retention (EPR) effect. Cyclophosphamide loaded Liposomes were developed by passive loading technique. Hydrogenated soy phosphatidylcholine (HSPC) and Distearoylphosphatidylglycerol (DSPG) are essential Lipid components for liposomal membrane and Cholesterol added additionally to improve stability of liposomal membrane. The Cyclophosphamide loaded liposomes evaluated for percent drug content, % entrapment efficiency, particle size, zeta potential, Osmolality, in vitro drug release, and stability studies.

MATERIALS AND METHODS

Materials: Cyclophosphamide- MSN Labs, Hydrogenated Soy Phosphatidyl choline (HSPC) [LIPOID S PC-3] - Lipoid GmBH, Distearoylphosphatidylglycerolsodium salt ((DSPG-Na)[LIPOID PG 18:0/18:0] - Lipoid GmBH, Cholesterol - Dishman India, Alpha Tocopherol - DSM Netherland, Sucrose – Merck, Disodium Succinate Hexahydrate - Finar Ltd. India, Ethanol – Merck, Hydrochloric Acid & Sodium Hydroxide – Merckwere provided as gift sample by Alembic Pharmaceuticals Ltd. Vadodara, Gujarat, India.

Preparationof Liposome drug using Passive loading technique:

Initially both Active and passive loading techniques were evaluated for loading of drug. Due to hydrolytic degradation of Cyclophosphamide, molecule is not stable in a long time at aqueous environment for gradient loading liposomes. HencePassive loading technique selected as a suitable method for the formulation of Liposomes asit is suitable for encapsulation of organic solvent soluble drugs and to avoid hydrolytic degradation of Cyclophosphamide in water, higher encapsulation efficiencies can be achieved by this method,based on extensive survey of literature and initial formulation feasibility with entrapment efficiency trials.The major steps as follows.

Lipid phase preparation: Organic bulk prepared by dissolving lipids,Alpha Tocopherol and API.

Aqueous phase preparation & preparation of mlv liposomal suspension: Collect water, dissolve sucrose and in it Transfer the Lipid phase solution into sucrose solution maintained at 65±5°C in using syringe injection method with high pressure using Pre filled syringe.

Hydration of lipids: Hydrate lipids at 65±3 ℃. After hydration, cool down the dispersion

Particle size reduction (mlv's to ulv's) using high-pressure homogenization: Homogenize the above dispersion at a pressure NMT 800 bar until achieving desired particle size.

Tangential flow filtration for removal of free drug and residual solvents:

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a. Concentration mode: Note down the initial weight of the liposomal dispersion sample, set the concentration value to 30% of initial weight (in kg)

b. Constant mode: Continue the diafiltration process till conductivity reaches below the set point and stop Tangential flow filtration conductivity set point is reaches.

Buffer preparation and volume adjustment: Disodium succinate hexahydrate added after dissolving in water and batch volume adjustment based on API Potency.

Filtration & filling: Sterilization of liposomes is a complicated process. Because it is unstable in heat and certain methods of radiation. The only method is a membrane filter that is capable to filter liposomes of size <0.2μm.Fill the liposomal dispersion in to vials and partially stopper the vial.

LYOPHILIZATION:

Load partially stoppered vials on lyophilizer and freeze dry the vials with optimized cycle. After sealing of vials storesame in 2-8 °C.

Identification of critical tempratures for lyophilization:

Freeze-drying (Lyophilisation) is the process of preserving a product by removing ice from a product via sublimation. Each product has a critical temperature for freeze drying above which it will have a poor appearance, loss of activity or stability.

The freeze drying microscope will allow observation of the sample structure during drying and heating so that the exact point of collapse can be determined. Impedance analysis provides indication of molecular mobility of a sample in its frozen state which may be attribute to changes within the formulation, that are pertinent to lyophilization

Freeze-Dry Microscopy (FDM):

Historically DSC was routinely used to determine the glass transition temperature of the maximally freeze concentrated solute (Tg'), information which was then applied to freeze-drying process design. Recently due to technological advancement, Freeze-Dry Microscopy (FDM) considered / employed to determine an even more representative critical temperature: the collapse temperature (Tc).

Lyophilization cycle was developed and optimized using FDM analysis data and SMART RUN of Lyostar-3 Lyophilizer.The knowledge of a product's collapse behaviour is vital over the lyophilisation process. It has performed FDM analysis using Lyostat2 to determine freezing temperature and collapse temperature of formulation.

Optimization of drug lipids ratio and Short time Stability life assessment:

It is very difficult to achieve stability of liposomal formulation due to chemical and physical degradation. Chemically, they are prone to oxidation and hydrolysis and they can physically fuse forming larger vesicles. It can be prevented addition of anti-oxidant(Alpha Topherol) and addtion of cholesterol to avoid fusion and stabilise the liposomal membrane.

Percent entrapment efficiency

The percent entrapment efficiency (% EE) is an important parameter to assess the drug delivery potential of the system. The %EE of the Liposomes was determined by using ultra centrifugation method. The Liposomal %EE value varied from 39.5 to 98.5 % respectively. From the results of optimized formula of lipids, it was assumed that the inner core of the vesicle is large enough to accommodate the drug Cyclophosphamide(i.e100 mg to 500 mg). The vesicle size and %EE are the basic parameters of vesicular systems based on which the formulations were optimized. As the decrease of HSPC, there was a significant decrease in %EE and drug release.HSPC optimal level needed for high drug entrapment. The optimum levels of HSPC and cholesterol produced higher percentage entrapment by showed lower vesicle size. The low size may be helpful in providing a large surface area to encapsulate Cyclophosphamide in the Liposomal lipid membrane. Data represent formulation 5 is having high %EE compare to other formulations.

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Table 1: Clinically used Liposome based Products

S. No	Clinical Products (Approval Year)	Administration	Active Agent	Indication	Company
1.	Doxil®(1995)	i.v.	Doxorubicin	Ovarian, breast cancer, Kaposi's sarcoma	Sequus Pharmaceuticals
2.	DaunoXome®(1996)	i.v.	Daunorubicin	AIDS-related Kaposi's sarcoma	NeXstarPharmaceuticals
3.	Depocyt®(1999)	Spinal	Cytarabine/Ara-C	Neoplastic meningitis	SkyPharma Inc.
4.	Myocet®(2000)	i.v.	Doxorubicin	Combination therapy with cyclophosphamide in metastatic breast cancer	Elan Pharmaceuticals
5.	Mepact®(2004)	i.v.	Mifamurtide	High-grade,resectable, non-metastatic osteosarcoma	Takeda Pharmaceutical Limited
6.	Marqibo®(2012)	i.v.	Vincristine	Acute lymphoblastic leukaemia	Talon Therapeutics, Inc.
7.	Onivyde™ (2015)	i.v.	lrinotecan	Combination therapy with fluorouracil and leucovorin in metastatic adenocarcinoma of the pancreas	Merrimack Pharmaceuticals Inc.
8.	Abelcet®(1995)	i.v.	AmphotericinB	Invasive severe fungal infections	Sigma-Tau Pharmaceuticals
9.	Ambisome®(1997)	i.v.	Amphotericin B	Presumedfungalinfections	Astellas Pharma
10.	Amphotec®(1996)	i.v.	Amphotericin B	Severe fungal infections	Ben Venue Laboratories Inc.
11.	Visudyne®(2000)	i.v.	Verteporphin	Choroidal neovascularisation	Novartis
12.	DepoDur™(2004)	Epidural	Morphinesulfate	Pain management	SkyPharmaInc.
13.	Exparel®(2011)	i.v.	Bupivacaine	Pain management	Pacira Pharmaceuticals, Inc.
14.	Epaxal®(1993)	i.m.	Inactivated hepatitis A virus (Strain RGSB)	Hepatitis A	Crucell, Berna Biotech
15.	lnflexal®V (1997)	i.m.	Inactivated hemaglutinine of Influenza virus strains A and B	Influenza	Crucell, Berna Biotech

i.v. (intravenous); i.m. (intramuscular); HSPC (hydrogenated soy phosphatidylcholine); PEG (polyethylene glycol);DSPE (distearoyl-sn-glycero-phosphoethanolamine); DSPC (distearoylphosphatidylcholine);DOPC (dioleoylphosphatidylcholine); DPPG (dipalmitoylphosphatidylglycerol); EPC (egg phosphatidylcholine);DOPS (dioleoylphosphatidylserine); POPC(palmitoyloleoylphosphatidylcholine); SM (sphingomyelin);MPEG (methoxy polyethylene glycol); DMPC (dimyristoyl phosphatidylcholine);DMPG (dimyristoylphosphatidylglycerol); DSPG (distearoylphosphatidylglycerol);DEPC (dierucoylphosphatidylcholine);DOPE(dioleoly-sn-glycero-phophoethanolamine).

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Table 2: Name of Drug Product and Phospholipids used for Liposome preparation

PRODUCT	PHOSPHOLIPIDS	DRUG
Ambisome®	HSPC and DSPG	Amphotericin B
Doxil®	HSPC and DSPE-PEG$_{2000}$	Doxorubicin
DaunoXome®	DSPC	Daunorubicin
Myocet™	EPC	Doxorubicin
DepoDur™	DOPC and DPPG	Morphine sulfate
DepoCyt®	DOPC and DPPG	Cytosine
Lipo-Dox®	DSPC and DSPE-PEG$_{2000}$	Doxorubicin
Marqibo®	ESM	Vincristine

Table 3: Observations from the FDM analysis

S. No.	Description of Events	Temperature
1	Freezing temperature	-10.9°C
2	Initiation of collapse temperature	-11.0°C
3	Complete collapse temperature	-0.7°C

Different Compositions of Liposomes formulationswere given in the table-4.

Table 4: Different Compositions Liposome formulations

Formulation #	HSPC (mg/mL)	DSPG (mg/mL)	Cholesterol (mg/mL)	Alpha Tocopherol (mg/mL)	Sucrose (mg/mL)	Disodium Succinate Hexahydrate (mg/mL)	NaOH / HCl [q.s to pH 6.5(5.5 to 7.5)]
Formulation 1	18	4	3	0.02	25	2	6.5
Formulation 2	18	5	4	0.02	50	2	6.5
Formulation 3	18	6	5	0.02	25	2	6.5
Formulation 4	18	4	3	0.01	50	2	6.5
Formulation 5	18	5	4	0.02	25	2	6.5
Formulation 6	9	6	2	0.01	50	2	6.5
Formulation 7	9	4	3	0.02	25	2	6.5
Formulation 8	9	5	4	0.01	50	2	6.5
Formulation 9	9	6	2	0.02	25	2	6.5
Formulation 10	9	4	3	0.01	50	2	6.5

Table 5: Physicochemical properties of Liposomal formulations

Formulation #	Drug Content (%)	D_{90} (nm)	pdi	%EE	Zeta Potential (mV)	Cumulative % *in vitro* drug release*
Formulation 1	98.1	189	0.102	88.1	-16.4	78.2±2.2
Formulation 2	98.9	191	0.081	94.7	-23.6	86.9±1.2
Formulation 3	99.3	178	0.090	82.9	-19.2	71.2±0.8
Formulation 4	97.8	175	0.057	89.4	-15.1	82.5±0.8
Formulation 5	99.8	198	0.086	98.5	-25.9	95.1±0.8
Formulation 6	101.2	189	0.068	45.2	-26.1	78.9±0.8
Formulation 7	96.8	172	0.081	39.5	-26.5	76.9±1.2
Formulation 8	99.5	175	0.083	54.1	-22.3	75.6±1.9
Formulation 9	98.4	168	0.115	68.2	-26.7	74.5±0.9
Formulation 10	98.1	179	0.095	55.1	-21.2	58.9±0.8

*Each value represents mean ±s.d (n=3)

Determination of percentage entrapment efficiency (%EE): The percentage of drug entrapped in the Lposomal formulation was determined by measuring the concentration of the drug in the aqueous phase by ultra-filtration method using centrisart devices (Sartorious)which is equipped with a filter membrane (molecular wt cut off 20,000 daltons) at the base of the sample recovery chamber. About 1 mL of undiluted sample

is placed in the outer chamber on the top of the sample holder and kept in the centrifugator. The unit is centrifuged at 20000 rpm for 1hr. The Liposomes along with the encapsulated drug remain in the outer chamber and the aqueous phase is moved into the sample recovery chamber through the membrane. The amount of drug in the aqueous phase is estimated by HPLC at 250 nm by using the below equation.

$$EE(\%)=\left[\frac{C_d - C}{C_d}\right] X\ 100\ (1)$$

Where, C_d is the concentration of total drug and C is the concentration of un entrapped drug.

***In vitro* drug release studies:** The USP-4 apparatus (Make:Sotax) IVR assay was established for evaluation of liposomal formulations. While the conditions of the IVR assay temperature of 55 °C and 5% w/v of γ-CD acceptor in release media. They were optimized to facilitatedrug release within 24 h without disrupting liposome structure. The principles applied during IVR assay development could be applied by others to set up USP-4 based IVR assays for other complex products such as liposomes, nanoparticles, microspheres, gels, or suspensions.The released Cyclophosphamide amount was monitored by UV absorption hourly. During the method development stage, an equal amount of free Cyclophosphamide solution (10 μg/mL) was placed in the release media directly as a control to mimic the complete release of Cyclophosphamide from formulations and monitor any changes in UV absorption of released Cyclophosphamide over the release period. The in-vitrodrug release profiles of all the Cyclophosphamide formulations loaded Liposomes are shown in **Fig.1.** A range of Liposomal formulations were prepared to evaluate the effect of various formulation parameters on the in-vitrodrug release profile. As per theory, drugs Loaded in the Liposomal systems are released possibly by the following mechanisms: a) passive diffusion b) vesicle erosion. The Cyclophosphamide drug release from Liposomal formulations was found to be slow, gradual and extended over 24 hours. After 24 hours, there was no further rise in the values of the cumulative percent drug release. The *in vitro* drug release profiles of all the Cyclophosphamide loaded Liposomes are shown in **Fig.1.** The Liposomal drug release mechanism is a complex process; it depends on many factors like nature of drug, Lipid ratio, drug-lipid matrix interactions, lamellarity, dispersion medium and the method of preparation of Liposomal. *In vitro* drug release studies were conducted in order to determine the effect of surfactant: cholesterol ratio on drug release. The stability and drug release properties of formulations of Cyclophosphamide HCl loaded Liposomes were determined by their surfactant: cholesterol ratio. The release pattern from Liposomes extended over 24 hours, depending upon the proportion of surfactant and cholesterol. Formulations having higher concentration of cholesterol gave decreased rate of drug release. Cholesterol makes the lipid bilayers more rigid and retards the release of the drug. When the combined effect of surfactant and cholesterol was studied, it was observed that, at the medium level of these components, the percent drug release was the maximum. It indicated that the lipid composition in Liposome determines its membrane fluidity, which in turn influences the rate of drug release.

Liposome Morphology and number of Lamellae:

Liposome Injection is Small Unilamellar Liposome (SUV) prepared. It is having only on bilayer of lipids. Liposome morphology and lamellarityinfluences drug retention, the rate of drug release from the liposomes, Transmission Electron Microscope (TEM) of Indian Institute of Science (IISc) Bangalore supported for study.

Phase transitions of Lipid bilayer:

Lipid bilayer phase transitions important to know at which temperature liposomal bilayer soften and for hydration temperature selection. Nano-Differential scanning

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calorimetry (Nano DSC) TA Instrument used, Indian Institute of Technology Mumbai supported for study. Phase transition temperature (Tm) of formulation 5 sample products is 53 deg.

Technique: The Nano DSC differential scanning calorimeter is designed to characterize the molecular stability of dilute in-solution biomolecules. The Nano DSC obtains data using less sample than competitive designs. Solid-state thermoelectric elements are used to precisely control temperature and a built-in precision linear actuator maintains constant or controlled variable pressure in the cell. Automated, unattended continuous operation with increased sample throughput is achieved with the optional Nano DSC Autosampler.The Nano DSC utilizes capillary cells to achieve highest sensitivity and maximum sample flexibility.

Zeta potential /Electrical surface potential or charge

Surface charge on liposomes can affect the clearance, tissue distribution, and cellular uptake. Further stability of product in liquid phase. The zeta potential of a particle is the overall charge that a particle acquires in a particular medium; it has been defined as the potential at the hydrodynamic shear boundary. Larger zeta potentials predict a more stable dispersion, which means that all the particles in suspension will tend to repel each other thus preventing aggregation. Normally, particle suspensions with zeta potentials > +20 mV or < −20 mV are considered stable. Measurement of liposomes zeta potentials can provide insight about their stability, circulation times, protein interactions, particle cell permeability, and biocompatibility. In drug delivery system, specific zeta potential has the possibility to improve biological performance by circumventing surface charge related toxicities. Liposomes are vesicles in which an aqueous volume is entirely enclosed by a membrane composed of lipid molecules, usually phospholipid. They can be prepared so that they entrap materials both within their aqueous compartment (water-soluble materials) and within the membrane (oil-soluble materials). They are extensively used as vehicles for the targeted delivery of drugs. The fate of intravenously injected liposomes is determined by a number of properties. Two of the most important are particle size and zeta potential. Knowledge of the zeta potential of a liposome preparation can help to predict the fate of the liposomes in vivo. Any subsequent modification of the liposome surface can also be monitored by measurement of the zeta potential. Malvern Zetasizer Nano ZS used for measuring of Surface potential of liposome samples ,obserzetapotential of formulation – 15.1 is -26.7 mV. Analysis was carried out at 25°C temperature keeping the angle of detection at 90°. The prepared niosomal solution and ROP-HCl solutions were diluted (1:100) with 0.1M sodium chloride solution. All measurements were carried out in triplicate. The zeta potential is expressed in terms of surface charge of the system (mV).

Liposome Size Distribution:

Liposomes are bilayer vesicles made of phospholipids derived from natural or man-made materials. They are mainly used in the pharmaceutical field for treatment of cancers as carriers of chemotherapeutic drugs to the tumor area. The amount of drug loaded into the liposomes and the size of the liposomes play pivotal roles in the pharmacokinetic and pharmacodynamic parameters of the drug. Particle size of Doxorubicin liposome is highly critical for drug targeting to specific cancer cell. Malvern zetasizer Nano ZS was used for measurement of Particle size distributions (PSD) of liposome samples. Analysis was carried out at 25°C temperature keeping the angle of detection at 90°. The prepared niosomal solution and ROP-HCl solutions were diluted (1:100) with 0.1M sodium chloride solution. All measurements were carried out in triplicate. The mean vesicle size is expressed in terms of diameter in nanometers (average of the vesicle size). The size distribution of vesicles is expressed in terms of poly dispersity index (PDI). The zeta potential is expressed in

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terms of surface charge of the system (mV).

Determination of pH: The pH of the Liosomal dispersions was measured by a pH meter of model Orion Star A211 Benchtop pH Meter (Thermofisher scientific) by following calibration standards.

Stability Studies: Stability studies of formulations are the critical factor for product integrity during their shelf life. A series of guidelines on the design and conduct of stability testing of pharmaceutical products have been published by International Conference on Harmonization (ICH) and World Health Organization (WHO) in the recent past.

The required vials of test products i.e. Formulation number 5 were stored long term at both refrigerator condition (2-8°C) and accelerated stability conditions (25± 2°C/60 ± 5% RH) in stability chamber. The samples were withdrawn at different time intervals of 3 and 6 months. The test products were analyzed for characteristics such as Description, percentage drug content, particle size (Z average), zeta potential, Osmolality, Water content and pH.

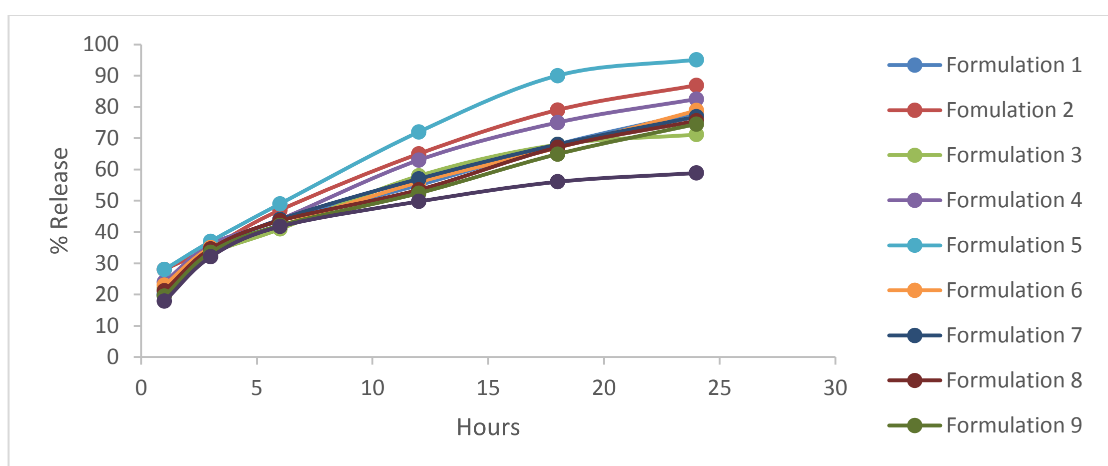

Fig 1: *In vitro* Cumulative % drug release vs time

Fig 1represents the cumulative percent drug release of Cyclophosphamide from Formulation1 to Formulation10 Liposomal formulations. Among these ten batches of Liposomes, F5 formulation showed maximum cumulative percent drug release(i.e. 98.5).

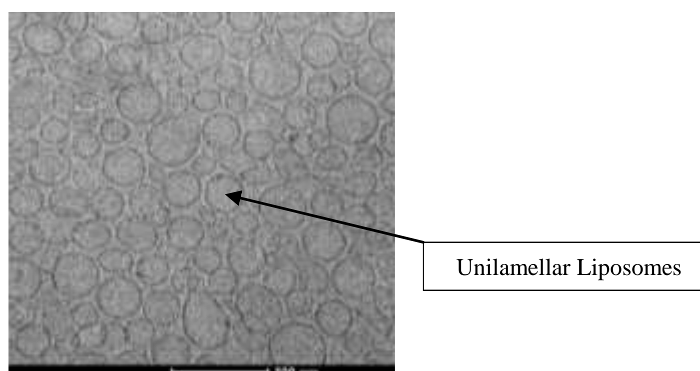

Fig 2: Transmission Electron Microscope image of formulation 5

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Table 6: Transmission Electron Microscope results of formulation 5

Batch No	Size (nm)	Sphericity [Count]	Wall Thickness (mm)	Lamellarity
Formulation 5	85.02	0.93[250]	6.53	~ 99% Unilamellar

Based on above data formulation 5 is unilamellar with bilayer thickness of 6.53nm

Table 7: Zeta Potential Results Liposomal formulations.

S. No	Formulation #	Zeta Potential (mV)
1.	Formulation 1	-16.4
2.	Formulation 2	-23.6
3.	Formulation 3	-19.2
4.	Formulation 4	-15.1
5.	Formulation 5	-25.9
6.	Formulation 6	-26.1
7.	Formulation 7	-26.5
8.	Formulation 8	-22.3
9.	Formulation 9	-26.7
10.	Formulation 10	-21.2

Table 8: Particle size distributions (PSD) of liposome samples

Formulation #	D_{10}	D_{50}	D_{90}	Z-Average	pdi
Formulation 1	61.9	98.8	189	93.34	0.102
Formulation 2	60.9	92.8	191	88.72	0.081
Formulation 3	61.1	94.3	178	89.81	0.090
Formulation 4	72.0	105	175	101.7	0.057
Formulation 5	65.8	102	198	96.93	0.086
Formulation 6	66.6	99.4	189	95.35	0.068
Formulation 7	64.9	98.7	172	94.39	0.081
Formulation 8	65.1	100.2	175	95.30	0.083
Formulation 9	59.3	89.5	168	88.12	0.061
Formulation 10	62.7	96.3	179	91.8	0.092

Table 9: Stability data of batch prepared with final optimized Formulation 5

S. No	Tests		Specifications (Tentative)	Initial	2-8 °C (Real Time)		25°C±2°C/60%±5 %RH (Accelerated)	
					3M	6M	3M	6M
1	Description		*	*	*	*	*	*
2	Percentage drug content		90.0-110.0%	95.9	96.1	96.1	95.8	95.7
3	Reconstitution Time (seconds)		NMT 300	156	230	242	240	241
4	Particle Size	Z average (nm)	120 ± 40	95.8	111.5	115.4	120.2	125.7
5	Zeta potential		Not less than -15	-25.9	- 27.8	-28.8	-25.3	-24.6
6	pH		5.0 to 7.0	5.89	5.91	5.86	5.42	5.34
7	Osmolality(mOsmol/kg)		250 to 350	273	291	271	263	280
8	Water content (%)		NMT 5.0%	0.38	0.68	0.59	0.86	0.79
* Yellow color Lyophilized mass filled in 25 mL clear, colorless glass vial with grey rubber stopper and sealed with violet flip off seal.								

OBSERVATION AND CONCLUSION

Analytical results of Liposomal formulation 5 batch shows that all Critical quality attributes of formulation are well within proposed specification. Hence, this formulation 5 liposomal formation is stable and it can be used for further evaluation.

DISCUSSION

Short-term genotoxicity study for free drug and liposome encapsulated in mice[1]

In the present experiment, mice were injected with 50 mg/kg free Cyclophosphamide or encapsulated in liposomes to compare their ability to induce mutagenic damages including chromosomal aberrations, changes in Sister Chromatid Exchange (SCEs) frequencies, and in Mitotic Index (MI), as well as in cell cycle kinetics. Both forms of Cyclophosphamide induced an increase in chromosomal aberrations and SCEs at the different sampling time. On the contrary, a decrease in mitotic index and delay in cell cycle kinetics was observed at all stages of the experiment. Encapsulation of Cyclophosphamide increased its mutagenicity, especially at a longer sampling time. This may due to interaction of liposomes with cells which is mainly through endocytosis or fusion resulting in accumulation of drug inside the cell causing chromosomal damage. Further evaluation of possible toxicity of encapsulation drugs in healthy tissue is needed. Cyclophosphamide as one of the widely used anti-tumor agents creates cross-links and strand breaks in DNA of many cells like germ cells. Such commonly used anticancer agents fail to distinguish normal cells from cancerous cells, so it kills normal proliferating cells as well. In fact use of most available anticancer drugs including Cyclophosphamide for killing cancer cells is a compromise between necessity and undesirable toxicity to normal cells.

Some studies have shown intraperitoneal administration of Cyclophosphamide can cause an increase in chromosomal aberrations and Sister Chromatid Exchanges (SCEs) as well as decrease in mitotic index. It has been reported that Cyclophosphamide and its metabolites induce oxidative stress and react with electron rich areas of the susceptible molecules such as nucleic acids and proteins. Therefore Cyclophosphamide targets rapidly dividing cells causing disruption of cell growth, mitotic activity and functions via alkylation of DNA at the N7 position of guanine. Liposomes-encapsulated anticancer drugs appear to represent an increasingly useful method for delivery of chemotherapeutic agents reducing their nonspecific toxicity and enhance their anticancer effect. The above mentioned results of our study indicated that animals treated with single dose of freeCyclophosphamideat 24, 48 and 72 hours sampling times showed several times increase in frequency of aberrant cells, SCEs and decrease in the mitotic index. This is in compliance with previous investigations which reported the ability of Cyclophosphamide to produce chromosome aberrations and SCEs. The most serious and frequent complication of Cyclophosphamide chemotherapy is suppression of the immune system, immunological dysregulation, and increasing intracellular amount of reactive oxygen species and glutathione depletion; such compounds can exert clastogenic effects, especially by acting as spindle inhibitors, thereby causing c-anaphasis (abnormal mitosis) and consequently aneuploidy and/or polyploidy. So it is reasonable to assume that liposome encapsulation of cancer chemotherapy agents aim to down-regulate the mutagenic effect of such anticancer alkylating agents.

CONCLUSION & FUTURE PROSPECTUS

In passive loading technique, it is possible to prepare stable Nano Liposomal formulation of Cyclophosphamide with high entrapment efficiency. Analytical results of Liposomal formulation 5 batch shows that all Critical quality attributes of formulation are well within proposed specification. Hence, this formulation 5 liposomal formation is stable, further procedure be optimized for free drug elimination and evaluation of feasibility for in commercial manufacturing equipment with higher batch sizes

It is possible to conclude that the higher effect of Cyclophosphamide encapsulated in liposomes may be attributed to the accumulation of high concentrations of the

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released drug inside cells, not in tissues as a whole, where it can directly affect cell content. Also development of nontoxic biodegradable sustained release systems for Cyclophosphamide represents a significant advance in cancer chemotherapy. However, further evaluation of possible toxicity in healthy tissues is needed.

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