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Design, Development and Characterization of PEGylated Liposomes of Gemcitabine Hydrochloride

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ABSTRACT

Gemcitabine hydrochloride (GEM) is a potential anticancer drug but it has certain limitations like short biological half life, low therapeutic index, rapid metabolism to the inactive metabolite & non-selectivity towards cancer cells resulting into common side effects of chemotherapy. The purpose of the study was formulation and evaluation of GEM loaded PEGylated liposomes to increase the residence time in systemic circulation & study its in-vivo performance. The liposomes were prepared by thin film hydration method using various phospholipids and were characterized for various parameters. The conventional & PEGylated liposomes were compared with free drug for its in-vivo performance, blood toxicity & in-vitro anticancer activity. Optimized formulations were subjected to stability studies for up to 2 months. Stable GEM loaded PEGylated liposomes having size and entrapment efficiency 400-800nm and 45-52% respectively and was obtained. In-vitro drug dissolution studies showed sustained release confirming long circulation of PEGylated liposomes. Blood toxicity studies reflected reduced toxicity of formulations than free drug. The pharmacokinetic parameters have demonstrated increased plasma half life of PEGylated formulation than conventional and free drug. In vitro anticancer activity in human lung cell lines showed many fold increase in the cyto-toxicity compared to pure drug. The study demonstrates efficient tumour targeting of GEM loaded PEGylated liposomes due to improved pharmacokinetics and residence time, reduced blood toxicity and enhanced in-vitro anticancer activity.

Keywords Gemcitabine hydrochloride, PEGylated liposomes, drug release, pharmacokinetics, cytotoxicity.

INTRODUCTION

Gemcitabine hydrochloride (GEM) is a fluorinated nucleoside analogue (2′, 2′difluorodeoxycytidine) used clinically as a very potent anti-tumor drug against different

solid tumors.[1] Unfortunately, use of effective anti-tumor doses of this drug result into haematological toxicity and other side effects. In body, it gets rapidly converted to inactive metabolite 2′-deoxy-2′, 2′-difluorouridine (dFdU) by cytidine deaminase following systemic administration. This metabolite is rapidly excreted in the urine.[2-3] Approximately, 77% of administered GEM gets excreted, either unchanged, or as the dFdU metabolite into the urine within 24 h.[4-6]

A strategic approach to overcome these problems is based on the increase in residence time and improvement in selectivity towards tumor using advanced drug delivery systems. In this context, PEGylated liposomes are suitable drug carrier systems for therapeutic applications. [7] The use of liposomes as drug carriers is mainly due to their versatility being able to encapsulate drugs with different physicochemical properties.[8] Liposome features are strictly related to chemical properties of the phospholipids used for their preparation. In fact, lipids can modify biodistribution, surface charge, permeability, release and clearance of liposomal drug delivery. They protects drug from enzymatic degradation, improves pharmacokinetics, tissue distribution and controls the release of therapeutic agents at appropriate target.[9,10] Moreover their inert biological nature, freedom from antigenic, pyrogenic reactions and similarity with composition of natural biomembrane make them popular.[11-13] Presence of polyethylene glycol moieties on the surface of liposomes provides long circulation properties, improved stability, drug defense from metabolic degradation/inactivation and increase intracellular uptake.[14-17] Liposome versatility can be of particular interest for the therapeutic treatment of various cancer diseases. Earlier studies have reported the preparation of GEM loaded liposomes using various methods of preparation, and could achieve GEM entrapment up to 46%.[18] The triblock and diblock pegylated copolymers show higher *in vitro* cytotoxicity than the others. Diblock-PEG2000 micelles possess high drug loading, low *in vitro* cytotoxicity, proper *in vitro* sustained release performance and prolonged mean residence time of drug in blood circulation.[17]

Therefore, the purpose of the study was to formulate GEM into PEGylated liposomes to improve GEM entrapment, increase the residence time of drug in systemic circulation, reduce reticuloendothelial system (RES) uptake and offer selectivity in targeting to solid tumors due to enhanced permeation and retention (EPR) effect.

The present work reports preparation of GEM-loaded PEGylated liposomes by thin film hydration method using phospholipids like DPPC, DSPC, DSPG, DPPG, MPEG-2000-DSPE & MPEG-2000-DPPE. The PEGylated liposomes were evaluated for yield, drug content and entrapment efficiency (EE). Studies like optical microscopy, transmission electron microscopy (TEM), photon correlation spectrometry (PCS), Zeta sizer, in-vitro drug release & sterility testing were also performed. Optimized formulations of PEGylated liposome were further subject to in-vivo blood toxicity and pharmacokinetic studies using Wistar albino rats, and in-vitro antitumor activity. The liposomes were subject to stability studies at 5° ± 3°C, and 25° ± 2°C (60± 5% relative humidity) over the period of three months.[19-21]

MATERIALS AND METHODS

Chemicals

Gemcitabine hydrochloride was gift sample from Cipla Pharmceuticals (Mumbai, India). 1, 2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 1, 2-Dipalmitoyl-sn-glycero-3-phosphocholine

(DPPC), Hydrogenated soya phosphatidylcholine (HSPC), N-(Carbonyl-methoxypolyethylenglycol-2000)-1, 2-distearoyl-sn-glycero-3-phosphoethanolamine, sodoium salt (MPEG-2000-DSPE) were obtained as a gift from Lipoid GmbH (Ludwigshafen, Germany). 1, 2-Distearoyl-sn-glycero-3-phosphoglycerol, sodium salt (DSPG, Na), 1, 2-Dipalmitoyl-sn-glycero-3-phosphoglycerol, sodium salt (DPPG, Na), N-(Carbonyl-methoxypolyethylenglycol-2000)-1, 2-dipalmitoyl-sn-glycero-3-phosphoetanolamine, sodium salt (MPEG-2000-DPPE) were generous gifts from Genzyme Pharmaceuticals (Liestal, Switzerland). Cholesterol was purchased from HiMedia (Mumbai, India). Chloroform & Methanol were purchased from Finar Chemicals Ltd. (Ahmedabad, India). All other solvents and chemicals used were of analytical grade and purchased from commercial sources.

Table I: Composition and particle size of liposome formulations

Formulation code	Composition		Particle size			
	Ingredients	Quantity (mg)	Before Lyophilization		After Lyophilization	
			z-Average (nm)	PDI	z-Average (nm)	PDI
GEM-1	DPPC DPPG	43 7	345.3±9	0.575±0.01	440.0±3	1.0±0.15
GEM-2	DSPC DSPG	43 7	192.5±8	0.418±0.01	208.7±5	0.690±0.01
GEM-3	HSPC	50	674.7±3	0.685±0.02	733.0±9	0.638±0.02
PGEM-1	DPPC DPPG DSPE-MPEG	36 4 10	590.1±5	0.851±0.01	605.0±8	0.694±0.02
PGEM-2	DPPC DPPG DPPE-MPEG	36 4 10	639.7±5	0.790±0.01	666.9±7	0.843±0.10
PGEM-3	DSPC DSPG DSPE-MPEG	36 4 10	447.2±4	0.800±0.02	474.4±5	0.66±0.01
PGEM-4	DSPC DSPG DPPE-MPEG	36 4 10	879.6±3	0.751±0.01	926.6±4	1.0±0.17
PGEM-5	HSPC DSPE-MPEG	40 10	408.7±8	0.693±0.01	451.1±3	0.590±0.01

PDI: Polydispersity index, All data expressed in the form of the mean ±standard deviation. (n=3)

Preparation of liposomes

Both conventional & PEGylated liposomes were prepared from various combinations of phospholipids as showed in Table I with constant amount (15mg) of GEM and cholesterol (10mg) in each formulation. The weighed quantity of phospholipids and cholesterol was dissolved in mixture of anhydrous chloroform & methanol (3:1 v/v) in a sterile round bottom flask, and subjected to evaporation at 45°C for 2 h using rotary evaporator (Evator, Medica Instruments). The thin film formed was kept in vacuum drier for 24 h to ensure complete removal of chloroform from the film. The film was allowed to hydrate using PBS (pH 7.4) containing 15 mg GEM and 15% w/v mannitol as a cryoprotectant by hand shaking for 10 minutes and further kept for 1 h at room temperature. The formed liposomes were subjected to sonication for 15 minutes for size reduction. The non-entrapped drug was removed by centrifugation (Remi- R- 8C), at 2000 rpm for 1 h at 4°C temperature; this step is called as

liposome purification.[20, 21] Final liposomal dispersion was filled in sterile glass vials covered with special stoppers for lyophilization. The liposomal dispersions were preserved by addition of sodium azide 0.05 % w/v related to total aqueous phase.

Lyophilization

Each 4ml of liposomal dispersion was filled in 10 ml glass vials, covered with special stoppers for lyophilization and placed in a freeze dryer (Martin Christ ALPHA 1-2 LD plus). For freezing the samples, the vials containing sample were cooled with 0.4°C/min from 20 to -52°C for 3 h under atmospheric pressure. After 3 h warm-up vacuum was applied for 10 minutes and then primary drying was started. In primary drying the pressure was reduced to 0.06 mbar at -20°C and under these conditions samples were dried for 30 h. Afterwards final drying was started where pressure was reduced to 0.002 mbar and sample vials were heated up to 10°C these conditions were maintained for 6 h. Finally sample vials were closed directly in the freeze dryer with rubber stoppers and sealed with aluminum crimps using inbuilt automatic sealing system. Sample vials were stored at 2-8°C.

Physicochemical characterization of liposomes

Yield

Percent yield was calculated to determine process loss during lyophilization as the weight of the lyophilized liposomes from each formulation in relation to the sum of starting material multiplied by hundred.

$$\%\text{Yield} = \frac{\text{Pr actical Yield}}{\text{Theoretical Yield}} \times 100 \qquad (1)$$

Drug content

The dried liposomal powder was dissolved in 1 ml methanol: ether (50:50, v/v) and volume was made up to mark of 10 ml volumetric flask with PBS (pH 7.4), 0.1 ml of above solution was further diluted to 10 ml and analyzed by spectrophotometrically (Shimadzu Jasco V-630) at 268 nm. The calculations were done by using eq. no.2

Shape and surface morphology

The prepared liposomes were observed under optical microscope for its appearance and shape. The diluted liposomal dispersion was taken on slide and images were captured at magnification of 10/0.25. The images of liposomal dispersion were taken under Motic Image Plus, version 2.0ML, China.

For finer details, conventional and PEGylated liposomes were observed by transmission electron microscopy (TEM). Freeze dried powder of liposomes was reconstituted with PBS (pH 7.4). 10 µl undiluted sample of liposomal dispersion was placed on copper grid which was previously coated with carbon film then the sample was dried under IR lamp for 25-30 minutes. The sample was loaded in instrument (TEM, Philips CM 200) operated at 200 kV and images were viewed & recorded with a 1k CCD camera.

Entrapment Efficiency

The entrapment efficiency (EE) is defined as the ratio of the amount of GEM encapsulated in liposomes to that total GEM added in liposomal dispersion. The freeze dried liposomal

formulation was dissolved in 1ml of methanol: ether (50:50, v/v) to disturb the vesicles in which GEM was entrapped. Final volume of formulation was made up to the mark of 10 ml volumetric flask. The solution formed was centrifuged for 15 minutes and supernatant was analyzed by UV-Visible spectrophotometer at 268 nm. The following external calibration curve was used for calculations

$$y=0.03078\ x+0.04754 \quad (2)$$

The encapsulation capacity is amount of drug that get entrapped out of total amount of drug added during liposome preparation with respect to the total concentration of lipids used in liposome preparation. The encapsulation capacity values were calculated by using the following equation

$$\text{Encapsulation capacity} = \frac{\text{Amount of encapsulated drug}}{\text{Total amount of drug added}} \times \text{Total amount of lipids added} \quad (3)$$

Vesicle size and size distribution

Size analysis was done on Malvern instrument v2.0 (Nano ZS). The average vesicle size and size distribution are important parameters because they influence the physicochemical properties and biological fate of the liposomes after administration. The vesicle mean diameter and size distribution were determined using particle size analyzer (Zetamaster, Malvern Instruments Ltd., Sparing Lane South, Worchester Shine, England). The size distribution of liposome was expressed as polydispersity index. The samples analysis was done by diluting 1 ml of liposomal dispersion up to 10 ml with double distilled water filtered with 0.1µm filter; further samples were placed in cuvett and analyzed.

Surface charge – Zeta potential

The zeta potential measurement was done on Malvern instrument v2.2 (Nano ZS). The magnitude of zeta potential gives indication of potential stability of a colloidal system hence particles in suspension has large negative or positive zeta potential tends to repel each other there by inhibiting flocculation or aggregation.

In vitro release study

The in vitro release of GEM from conventional and PEGylated liposomes was determined by dialysis method. After reconstituting the freeze dried liposomes in 10ml PBS (pH 7.4), an aliquote of each liposomal dispersion was placed in dialysis tube (Himedia Laboratories Pvt. Ltd., Mumbai) with molecular weight cutoff 14000 Da. Then, dialysis tube was immersed in a beaker containing 200 ml of release medium, i.e. PBS (pH 7.4) and stirred with magnetic stirrer at 150 rpm to maintain sink condition. The sample (5ml) were taken at predetermined time intervals from release medium and replaced by same volume of fresh medium. Concentration of GEM was determined after filtering the samples through 0.22µm syringe filter and were assayed UV spectrophotometrically at 268 nm.

Sterility test

In order to ensure the sterility of finished products, the optimized formulations were subjected to sterility test. The sterile formulations were incubated with different culture media like Fluid

thioglycolate medium for anaerobic/ aerobic bacteria, Soyabean casein digest for fungi, Nutrient agar for *bacillus subtilis*, Maconkeys agar for *E-coli*, Potato dextrose agar for *Candida albicans*, and Mannitol salt agar for *Staphelococcous aureus*. The sterility test was performed by spread plate method. Same media for positive control with specific organisms and negative control without any inoculation was incubated for 14 days and results were noted.

Blood toxicity in animals

The modification of blood biochemical indexes was evaluated to measure the blood toxicity index of free drug and drug encapsulated in conventional as well as PEGylated liposomes. Four groups each containing 3 albino rats was treated i.v. with 0.5 ml of drug, GEM-2 & PGEM-3 formulations (5 mg/kg) every three days for 30 days. Then blood samples were collected via ocular vein plexus immediately frozen on addition of anticoagulant. Different blood parameters were then measured by biochemical auto analyzer (Type 7170, Hitachi, Japan). The blood samples obtained by healthy albino rats were used as control.

Pharmacokinetic Studies

The protocol in prescribed Proforma B for animal studies was submitted to IAEC of Bharati Vidyapeeth College of Pharmacy, Kolhapur (988/C/06/CPCSEA). The Approval no. was BVCPK/ CPESEA/ IAEC/ 01/ 16. Albino rats of either sex weighing 200 to 250 gm were fasted overnight and divided in to four groups each containing three rats. The group I received conventional liposomal formulation, group II received PEGylated liposomal formulation, group III received drug solution at a dose of 5 mg/kg and equivalent weight in case of formulations, group IV received normal saline solution by injecting in tail vein of animal. The blood samples were withdrawn at an interval of 1, 6, 12 & 24 h from retro orbital plexus. The collected samples were analyzed by HPLC.

HPLC analysis

From, retro orbital plexus the blood samples were directly collected in micro cups containing 200 µl tri chlor acetic acid and 50µl of glacial acetic acid were added to decrease hydrogen bonding between nucleotide and proteins. Acetonitrile (1ml) was added to plasma samples, the mixture was vortexed and centrifuged for 15 min at 4°C. The supernant was separated in glass tube and again two washing of acetonitrile was given to extract the drug from plasma samples. The combined supernatant was filtered through 0.22 µm syringe filter and was injected into HPLC. The mobile phase was water/acetonitrile (95:5 v/v). The flow rate was 1 ml/min and UV detection was performed at 268nm. Analysis was carried out using a RP-HPLC system (Jasco PU-2080, intelligent HPLC pump) with 20 µl sample loop injector & detector consisted of UV-visible (Jasco UV-2075, intelligent). Chromatographic separation was carried out at room temperature using a HOQ SIL RP C18 column (4.6×250 mm, 5 µm particle size, KYA technology, Japan). Equipment was operated through software 'Borwin Veesion 1.5'. GEM quantification was carried out using an external standard curve in the linear concentration range between 2 and 26 μg/ml. A standard solution of GEM (1 mg/10ml) was used for the construction of the standard curve. Plasmatic amounts of GEM were determined using the standard curve according to the following equation:

$$AUC = 53325.43x - 122087.01 \quad (4)$$

Where x is the drug concentration (μg/ml), and AUC area under the curve (mAu×min).

GEM plasma concentrations were expressed as μg/ml.

In-vitro anticancer activity

The MTT assay test was used to evaluate the cellular viability, so as to determine the the cytotoxic effect of free and liposomally entrapped GEM on human lung carcinoma cells NCI-H522 (obtained from NCCS, Ganeshkhind, Pune). The cell viability was evaluated by determining the quantity of colored formazan crystals formed during the biological test. 1.6×10^3 /100 µl cancer cells were transferred aseptically in each well of 96-well plate then 100, 250, 500 & 1000 μg/ml concentrations of free drug, PEGylated formulation & conventional formulation were prepared and added to wells in triplicate. Cells were incubated for 24 h at 37°C in CO_2 incubator. After incubation, 20 µl of MTT (5mg/ml dissolved in PBS) were added in each well and incubated for 3 h. Supernatant of wells were removed after 3 h and 200 µl of dimethyl sulfoxide were added to dissolve the formazan crystals. 96-well plates were gently shaken and absorbance of various samples was measured with ELISA microplate reader (Labsystems mod. Multiskan MS Midland, ON, Canada) at 570 nm. The percentage cell viability was calculated according to following equation:

$$\text{Cell viability} = {}^{\text{AbsT}}/_{\text{AbsC}} \times 100 \qquad (5)$$

Where AbsT represented the absorbance of treated cells and AbsC the absorbance of control (untreated) cells.

Stability studies

From the all eight Formulations, GEM-2 from conventional and PGEM-3 from PEGylated liposomes were tested for stability studies. According to ICH guidelines Q1A (R2) formulation GEM-2 & PGEM-3 was divided into 2 sample sets and stored at 5° ± 3°C & 25° ± 2°C and 60% RH ± 5% RH At the interval of 15 days for 3 months, the in-vitro drug release and drug content of selected formulations (GEM-2 & PGEM-3) was determined by method discussed previously.

Statistical analysis

All data were expressed in the form of the mean ± standard deviation. For comparison of mean between the formulations, the student's t-test was used. Difference between two parameters were considered stastically significant for $P<0.05$. All the analysis of data was performed using statistical software package Graphpad Prism version 5.

RESULTS

Preparation of liposomes

The formed liposomal dispersion was homogeneous and opaque white in color.

Lyophilization

All liposome formulations evaluated in the present study could be lyophilized and redispersed without loss of overall dispersion quality. The moisture content of the lyophilized products was below 5% in all samples. Interestingly, moisture content was lower in the PEGylated formulations compared to conventional to formulations. The entire freeze dried formulations was easily redispersed, appeared macroscopically homogenous after redispersion and no precipitate

was observed in light. In preliminary experiment, macroscopically homogenous dispersion could not obtained after redispersion of lyophilized PEGylated liposomes stored at room temperature, all lyophilized samples were therefore stored at 2-8 °C in refrigerator. Reconstitution time for freeze dried liposomes were 50-60 seconds at room temperature when reconstituted after 1 month by adding 2 ml of phosphate buffer by manual shaking and vortexing.

Physicochemical characterization of liposomes

Different lipid combinations could modulate both technological and pharmacokinetic parameters of colloidal vesicles thus influencing the application of liposomes as drug delivery in chemotherapy. For this reason, different conventional and PEGylated liposomal formulations were prepared and investigated as potential colloidal carrier for GEM.

Table II: Data for entrapment efficiency, encapsulation capacity & zeta potential of conventional & PEGylated liposomes.

Formulation Code	Entrapment efficiency	Encapsulation capacity	Zeta Potential (mV)
GEM-1	43.90 ± 0.14	26.24 ± 0.21	-18.5±4.23
GEM-2	40.80 ± 1.00	24.48 ± 0.59	-24.7±3.9
GEM-3	37.86 ± 0.72	22.72 ± 0.50	-23.3±4.45
PGEM-1	51.18 ± 0.91	30.71 ± 0.54	-41.49±3.15
PGEM-2	51.74 ± 1.00	30.04 ± 0.62	-43.89±6.32
PGEM-3	47.73 ± 0.54	28.64 ± 0.33	-47.6±5.49
PGEM-4	48.35 ± 0.57	29.01 ± 0.28	-44.85±3.75
PGEM-5	44.59 ± 0.76	26.75 ± 0.45	-27.19±3.45

All data expressed in the form of the mean ± standard deviation. (n=3)

Yield

The percent yield of liposomal powder after lyophilization compared to total solid content in liposomal dispersion was in the range of 85.98 to 92.31%. as shown in Figure I. The variation in percent yield may be due to bumping effect observed during freeze drying due to reduced pressure and alteration in moisture content of liposomal powder upon storage.

Drug content

The drug content in conventional and PEGylated liposomes were in the range of 91.19 to 94.81% as shown in Figure I. The little loss of drug from the formulation was observed which may be due to bumping effect during freeze drying of liposomal dispersions.

Shape and surface morphology

Images obtained under optical microscope confirmed formation of phospholipid vesicles upon hydration of thin lipid film formed by using flash rotary evaporator. It was found that the formed vesicles were spherical in shape as shown in Figure II.

The morphology of the conventional and the PEGylated liposomes were observed by transmission electron microscopy (TEM). The image from negative-staining showed that both conventional and PEGylated liposomes were of discrete and round structure ranging size from 200 to 400 nm which were consistent with the results obtained from the particle size measurement as shown in Figure III.

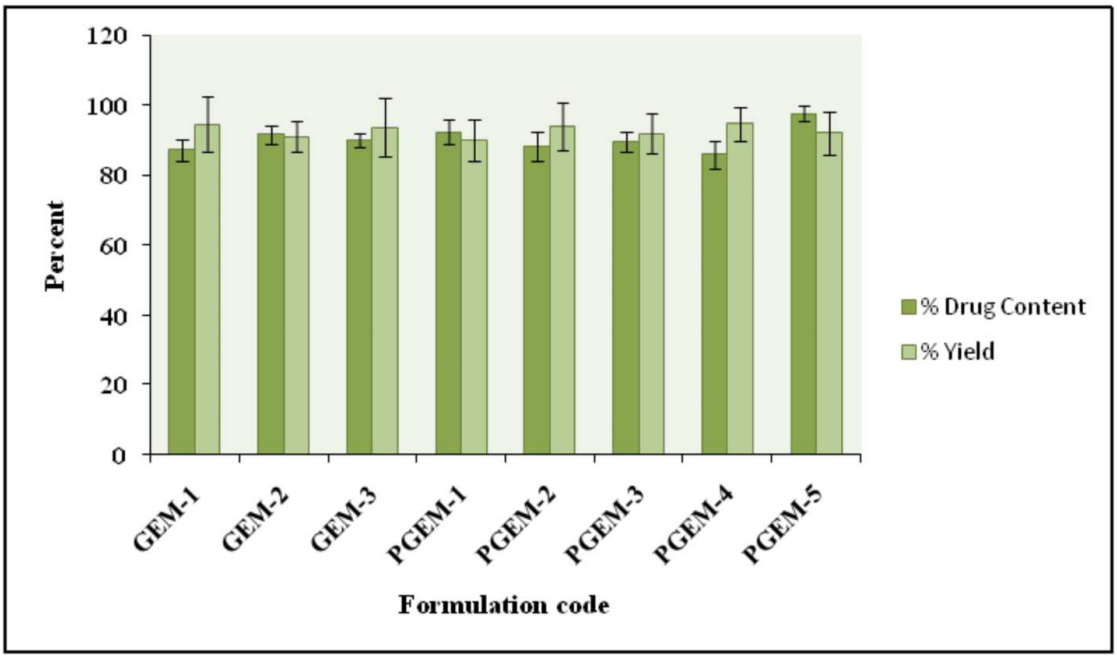

Figure I: Percent yield & Drug content of various formulations.

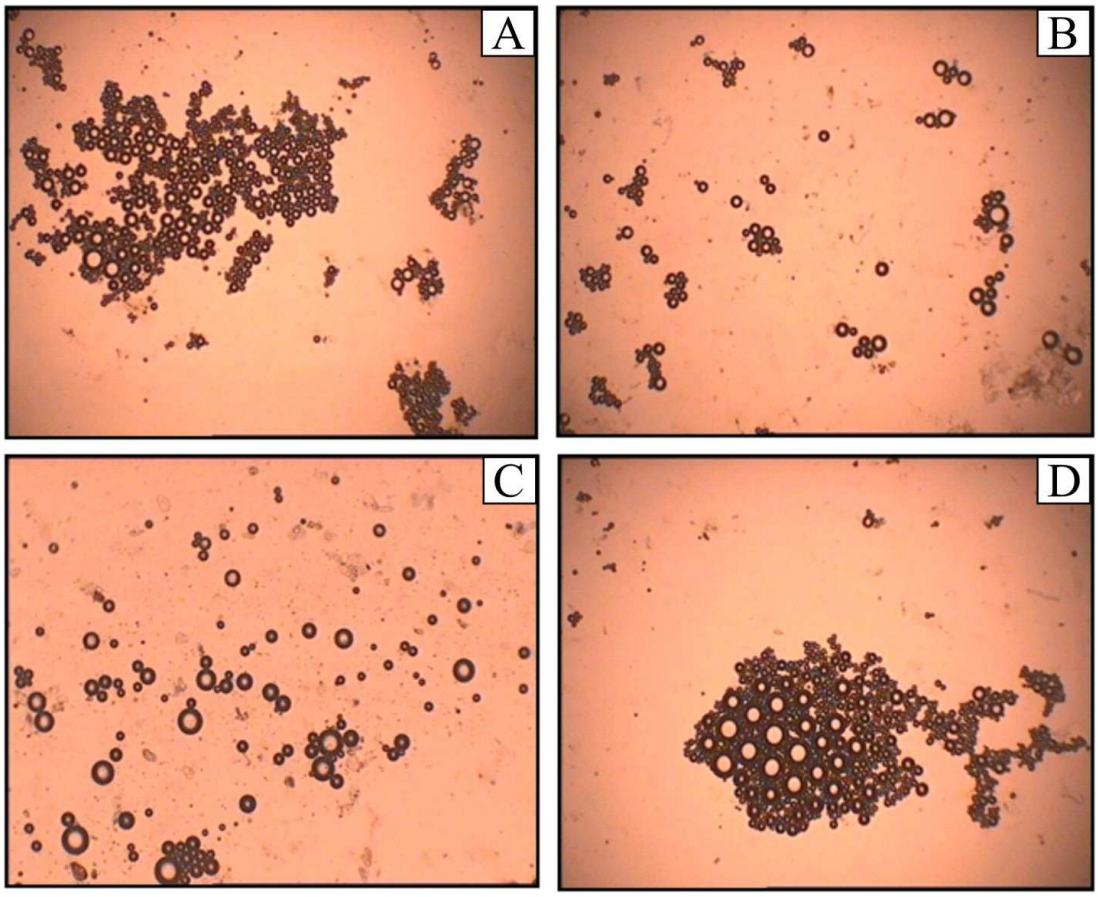

Figure II: Optical microscopic images (A) GEM-2, (B) GEM-3, PGEM-3 (C) & PGEM-4 (D) liposomal formulations.

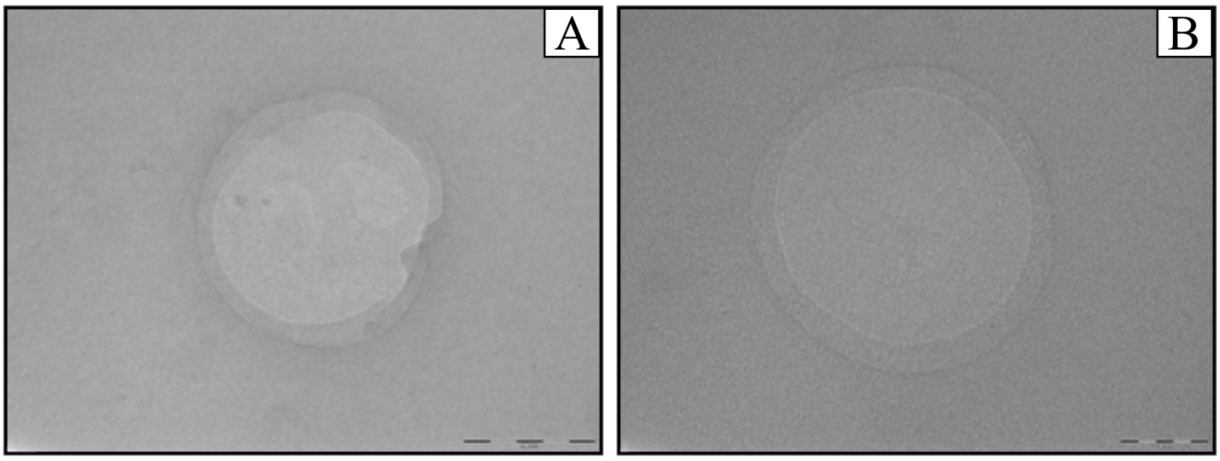

Figure III: Transmission electron micrograph of conventional (A) and PEGylated liposomes (B) of GEM.

Entrapment Efficiency

EE of different formulations of GEM-loaded conventional and PEGylated liposomes are summarized in Table II. Different lipid combinations can influence the entrapment efficiency of liposomal formulation. The PEGylated liposomes showed increased EE than the conventional liposomes. There is no significant difference in loading capacity among different liposomal formulations investigated.

Vesicle size and size distribution

Freeze drying altered the size and polydispersity of liposomal dispersion; increase in size may be due to aggregation of vesicles upon freeze drying and redispersion. The increase in size was less in case of PEGylated liposomes than conventional liposomes but the polydispersity was found to be more indicating slightly wide size distribution than conventional liposomes before and after freeze drying as shown in Table I.

Surface charge – Zeta potential

The experimental data shown in Table II reflects that zeta potential values are influenced by lipid composition. Zeta potential values of about -18.5 to -24.7 were observed in case of conventional liposomes while in case of PEGylated liposomes values were -27.18 to -47.6 which is probably related to steric effect of the MPEG-2000-DSPE & MPEG-2000-DPPE.

In vitro release study

The graphical presentation of release profile of all the conventional & PEGylated formulations is shown in Figure IV. The conventional and PEGylated liposomes released maximum 69% and 42% of GEM within 24 h at room temperature, respectively. The release of GEM showed an initial burst release phase, releasing approximately 25% and 15% of GEM during the first 2 h. Thereafter the release rate was reduced, indicating that depot effect could be achieved using liposomes, especially in the PEGylated liposomal formulations. The above results suggest that GEM would be stable in the blood circulation and would be released slowly at the tumor site. The GEM-1,2,3 & PGEM-1,5 shown Peppas model as best fit while PGEM-2,3,4 shown a matrix as a best fit model.

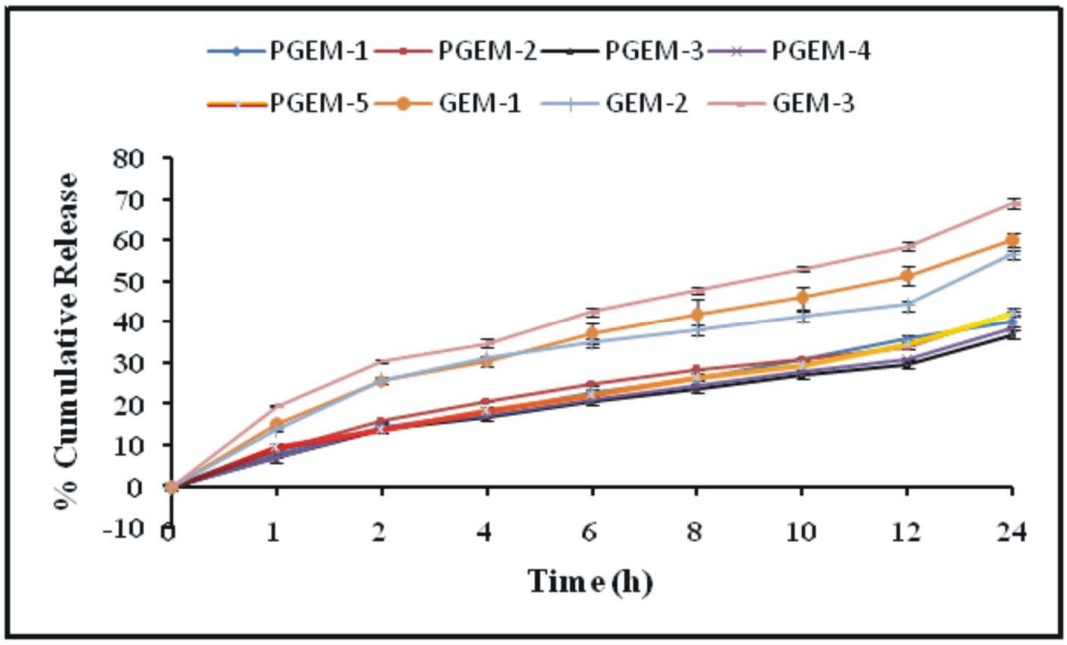

Figure IV: In vitro drug release profile for conventional liposomes & PEGylated liposomes.

Sterility Test

The results obtain showed that no growth of microorganisms on culture medium incubated with formulation while positive control showed growth on the medium. This indicates that formulation is sterile and passes the sterility test.

Table III: Haematological parameters of rat treated with the different formulations.

Parameter	Formulation		Drug	Control
	GEM-2	PGEM-3		
WBC (K/µl)	5.2 ± 0.12	6.4 ± 0.20	2.7 ± 0.33	8.1 ± 0.15
RBC (M/ µl)	3.8 ± 0.08	5.3 ± 0.09	2.3 ± 0.21	5.94 ± 0.19
Hgb (g/dl)	10.7 ± 0.39	12.9 ± 0.42	10.1 ± 0.31	13.4 ± 0.24
Hct (%)	28.0 ± 1.23	35.2 ± 1.99	25.8 ± 2.01	40.9 ± 1.54
MCV(fl)	50.5 ± 2.55	49.9 ± 1.66	47.2 ± 3.07	56.0 ± 1.98
MCH (pg)	18.0 ± 1.5	18.9 ± 0.8	15.9 ± 1.2	21.9 ± 2.1
MCHC (g/dl)	38.0 ± 0.55	38.2 ± 1.2	31.5 ± 1.54	39.1 ± 0.98
MPV (fl)	8.5 ± 0.44	9.4 ± 0.31	7.7 ± 0.19	11.0 ± 0.21
PCT (%)	0.324 ± 0.02	0.399 ± 0.03	0.247 ± 0.01	0.584 ± 0.02
PDW (%)	15.9 ± 1.39	16.5 ± 1.51	15.0 ± 1.25	17.4 ± 0.95
Plt (K/µl)	382 ± 22	425 ± 24.8	321 ± 45.8	531 ± 31.4
RDW (%)	14.0 ± 0.34	16.3 ± 0.98	20.4 ± 0.54	14.7 ± 0.84

All data expressed in the form of the mean ± standard deviation. (n=3)
White blood cells (WBC), Red blood cells, Hemoglobin (Hgb), Hematocrit (Hct), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), Mean platelet volume (MPV), Plateletcrit (PCT), Platelet distribution width (PDW), Platelet (Plt), red cell distribution width (RDW)

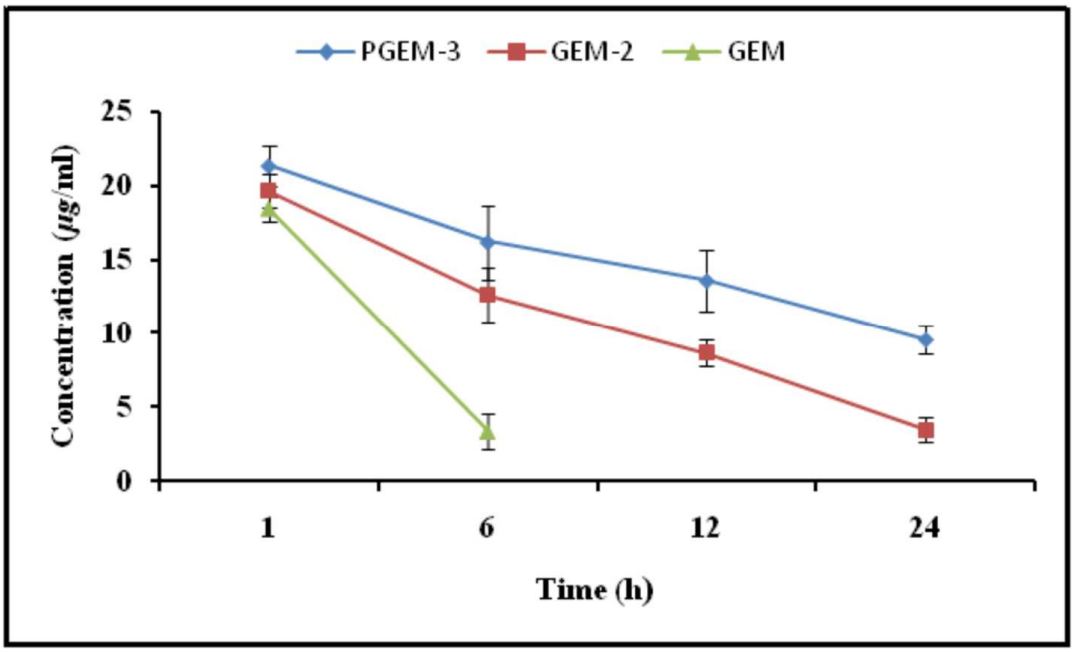

Figure V: Plasma concentration profile of GEM after intravenous administration.

Blood toxicity in animals

The haematological data animals treated with conventional, PEGylated, pure drug and control is given in Table III. The result shows that pure drug treatment to animal shows maximum toxicity i.e. reduction in blood indices than normal but in case of conventional liposomal formulation treatment the toxicity is less compared to pure drug this may be due to less exposure of drug to blood because of encapsulation. In case of PEGylated formulation the toxicity is least this may be due to localizing and long circulation effect of PEGylated formulation.

Table IV: Plasmatic pharmacokinetic parameters of GEM after a single intravenous administration in wistar albino rats.

Sr. No.	Pharmacokinetic parameters	Free Drug	GEM-2	PGEM-3
1	**AUC (µg/ml × h)[a]**	11.37 ± 0.049	18.87 ± 0.0368[*]	21.37 ± 0.098[#,*]
2	**$t_{1/2}$ (h)**	1.57 ± 0.12	7.29 ± 0.26[*]	13.86 ± 0.39[#,*]
3	**k_e (h^{-1})**	0.44 ± 0.001	0.095 ± 0.002[*]	0.05 ± 0.001[#,*]
4	**V_d (ml)**	8.69 ± 0.19	14.28 ± 0.39[*]	14.81 ± 0.25[#,*]
5	**C_{max} (µg/ml)**	27.0 ± 1.25	29.0 ± 2.1[*]	46.0 ± 1.87[#,*]

All data expressed in the form of the mean ± standard deviation. (n=3)
[*] p< 0.05 compared with free drug
[#] p< 0.05 compared with conventional liposomes GEM-2
[a] The areas under the plasma concentration-time curve (AUC) (starting from the first to the last sampling time) was calculated using the trapezoidal rule.

Pharmacokinetic studies

GEM showed good linearity (r = 0.997) over the concentration range of 2-26 µg/ml in plasma. Hence, GEM was found to obey Beer- Lambert's law over this range. No interference coming from plasma components was observed for GEM and its metabolite (dFdU). The chromatographic method provided a suitable separation of the peaks of GEM and dFdU, which

showed a retention time of 4.0 and 6.0 min, respectively. The GEM given to animal was very less in quantity at 6 h and further it was not found in blood samples of the same group of animals. While the GEM-2 & PGEM-3 formulation injected animals shown presence of GEM till 24 h. Further evidence of metabolic protective role of liposomes on the encapsulated GEM was obtained by evaluation of pharmacokinetic parameters of the drug with respect to free drug administered intravenously shown in Table IV and plasma concentration profile of GEM, conventional & PEGylated formulations is shown in Figure V.

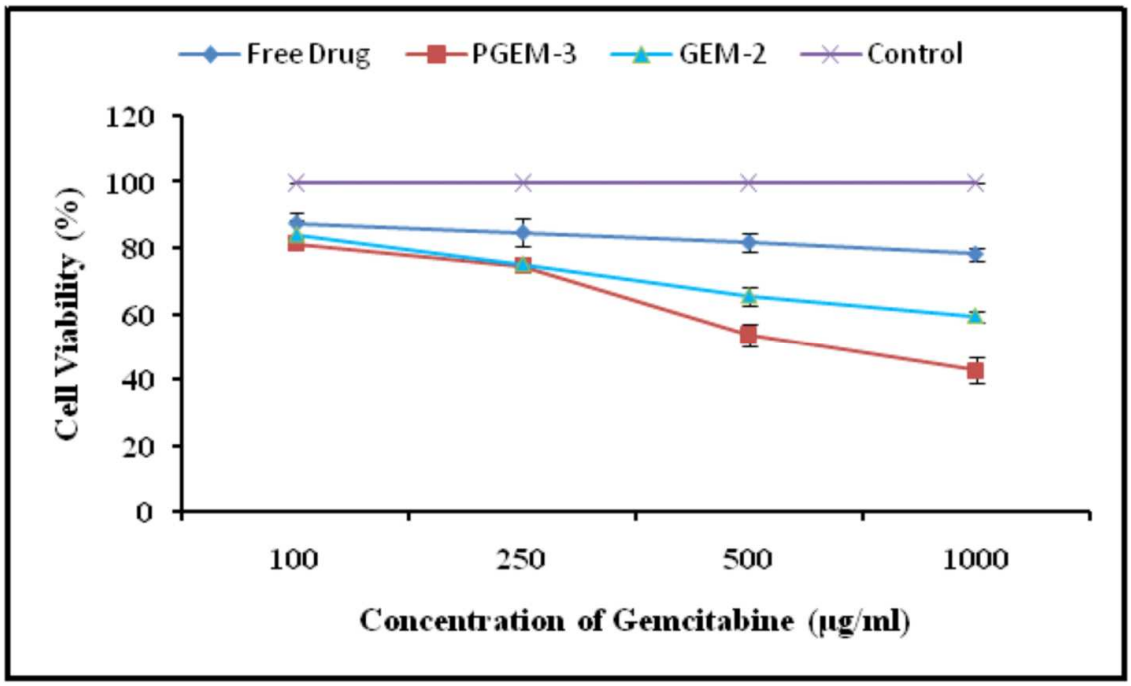

Figure VI: Dose-dependent anticancer activity of free drug, conventional & PEGylated formulations on human lung carcinoma cells by MTT assay.

In-vitro anticancer activity

Biological efficacy of GEM entrapped in PEGylated & conventional liposomes was tested on human lung carcinoma cells (NCI-H522) by using MTT assay. Empty liposomes were used to evaluate possible toxic effect of the carrier on human lung carcinoma cells. After incubation free GEM elicited little cytotoxic effect at the investigated concentrations on lung carcinoma cells, which presents a vitality of about 78 to 87 %. A significant improvement of drug anticancer activity with respect to the free drug was obtained by using PEGylated & conventional GEM-loaded liposomes. Both conventional as well as PEGylated formulations showed a dose-dependent anticancer activity on human lung carcinoma cells. A significant difference was observed between conventional and PEGylated formulation at a concentration of 1000 µg/ml i.e. cell viability is 59.61 % & 43.07 % respectively after 24 h incubation. Lung carcinoma cells showed an improvement of efficacy of PEGylated formulation over the conventional formulation and free drug. The improvement of anticancer efficiency of GEM on lung carcinoma cells provided by PEGylated formulation suggests the protective and long circulation properties of it as shown in Figure VI.

Stability studies

Stability studies of optimized formulations, GEM-2 and PGEM3, at 25° ± 2°C and 60% RH ± 5% showed insignificant change in the drug release profile ($P<0.05$), suggesting developed formulations to be stable. The alteration in drug release profile of optimized formulations stored at 5° ± 3°C was negligible. Drug content of optimized formulations stored at different temperature and humidity conditions was not changed significantly ($P<0.05$).

DISCUSSION

Earlier findings have suggested that type and various lipid combinations along with physicochemical state of the lipid bilayer influences the release rate of the drug. Thus the effect of different lipid combinations and physicochemical state of GEM-loaded liposomes on the in-vivo fate of both conventional and PEGylated liposomes and has been established in the present work.

All formulations evaluated in the present study could be lyophilized and redispersed without overall loss of colloidal quality. The slight increase was observed in the size and polydispersity of vesicles redispersed after lyophilization. The presence of PEGylated phospholipids resulted in both a more or less distinct increase in size and polydispersity as well as in an alteration of morphology was observed by optical microscopy and TEM. The findings of EE can be supported by an interaction of GEM with the negatively charged polar head group of phospholipids along with simple drug entrapment in aqueous compartment of liposomes due comparatively bigger size.

The results of in-vitro release studies suggests that GEM takes time to release from liposomes because of lipid bilayers are stabilized by cholesterol and depot and long circulating effect could be achieved by PEGylated liposomal formulations.

Reduction in blood toxicity of PEGylated liposomes than conventional liposomes and free drug ensures the reduced exposure of drug to the blood due to encapsulation of drug in the vesicles. To prolong the circulation time of vesicles, ‘stealth’ liposomes are frequently used by addition of PEGylated phospholipids providing surface modification of the vesicles by the polyethylene glycol residues.[22-25] A further evidence of the metabolic protective role of liposomes on the encapsulated GEM was obtained by the evaluation of the pharmacokinetic profile of the drug with respect to the free form after i.v. administration. All pharmacokinetic parameters confirmed that the encapsulation of GEM in liposomes confined the drug in the systemic circulation thus decreasing the amount of this antitumoral agent that was removed from blood stream.

The findings regarding in-vitro anticancer activity of free or liposomally entrapped GEM could be correlated with improvement of antitumoral efficiency as well as their long circulating properties, suggesting that PEGylated liposomal formulation could be used as possible carrier for GEM delivery and treatment of solid cancers. The increased charge on PEGylated liposomes further improved stability may be due to steric hindrance of MPEG-2000-DSPE & MPEG-2000-DPPE. The storage of liposomal formulations at 25° ± 2°C and 60% RH ± 5% RH altered release profile insignificantly, this may be due to less transition temperature of lipids.

Overall, results are in agreement with earlier studies where an increase in size and polydispersity as well as changes in the morphology has been in the literature.[26-28]

CONCLUSION

Long circulating liposomes of GEM were successfully developed by use of PEGylated phospholipids. Similar to in vitro release profile, in vivo performance of the PEGylated liposomes have demonstrated extended drug release (depot effect), increased biological half life of gemcitabine hydrochloride and reduction in elimination rate constant. Moreover, blood toxicity has been reduced due to drug encapsulation. Many fold increase in the anticancer activity (on cancer cell lines) is an indicator of improved therapeutic efficacy of GEM. Stability studies revealed no significant change in the release profile confirming storage stability of liposomes. Hence, it can be concluded that, PEGylated GEM liposomes can be considered suitable for systemic administration of gemcitabine hydrochloride for treatment of solid tumors.

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