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Development and Chbaracterization of Liposomal Formulation of Carfilzomib with Hydrogenated Palm Oil

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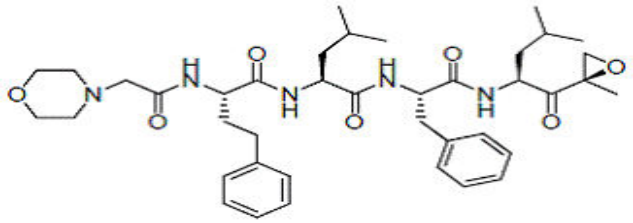
Abstract: Carfilzomib is a peptide epoxy ketone derivative, chemically; it is a tetra peptide epoxy ketone and an analog of epoxomicin. Carfilzomib is commercially available Carfilzomib for Injection, which is a lyophilized formulation available as 30 mg / vial and 60 mg / vial a sterile, white to off - white lyophilized powder and is available as a single - use vial. The current investigation was designed to alternative stable liposomal formulations.The inventors of the present invention have surprisingly found that it is possible to prepare a stable lipid Nano composition of Carfilzomib. Liposomal formulations are less toxic than drugs alone and have better pharmacological parameters. Although they seem to be the first choice for drug delivery systems for various diseases.The usage of natural products in pharmaceuticals has steadily seen improvements over the last decade, and this study focuses on the utilization of palm oil in formulating liposomal Carfilzomib. The liposomal form of Carfilzomib generally minimizes toxicity and enhances target delivery actions. Taking into account the antiproliferative and antioxidant properties of palm oil, the aim of this study is to design and characterize a new liposomal Carfilzomib by replacing phosphatidylcholine with 5% and 10% palm oil content. Liposomes were formed using the freeze thaw method, and Carfilzomib was loaded through pH gradient technique and characterized through in vitro and ex vivo terms. Based on TEM images, large lamellar vesicles (LUV) were formed, with sizes of 438 and 453 nm, having polydispersity index of 0.21 ± 0.8 and 0.22 ± 1.3 and zeta potentials of about −31 and −32 mV, respectively. In both formulations, the entrapment efficiency was about 99%, and whole Carfilzomib was released through 96 hours in PBS (pH = 7.4) at 37°C. Comparing cytotoxicity and cellular uptake of LUV with on MCF7 and MDA-MBA 231 breast cancer cell lines indicated suitable uptake and lower IC50 of the prepared liposomes[4].

Keywords:1 Carfilzomib,2 Liposomal,3 Hydrogenated palm oil:,4polyethylene glycol,5L-alpha-phosphatidylcholine,6Malvern Zetasizer,7 Zeta Potential, 8Polydispersity Index

Introduction[6]:

Carfilzomib is a modified tetra peptidyl epoxide, isolated as the crystalline free base. The chemical name for carfilzomib is (2S)-N-((S)-1-((S)-4-methyl-1-((R)-2-methyloxiran- 2-yl)-1-oxopentan-2-ylcarbamoyl)-2-phenylethyl)-2-((S)-2-(2-morpholinoacetamido)-4-phenylbutanamido)-4-

methylpentanamide. Carfilzomib is a peptide epoxy ketone derivative, chemically; it is a tetra peptide epoxy ketone and an analog of epoxomicin. Carfilzomib is white to off white amorphous non hygroscopic powder. It is soluble in dichloromethane and practically insoluble in water.
Carfilzomib has the following structure:



Mechanism of action[3]:
Carfilzomib is a tetrapeptideepoxyketone proteasome inhibitor that irreversibly binds to the N-terminal threonine-containing active sites of the 20S proteasome, the proteolytic core particle within the 26S proteasome. Carfilzomib had antiproliferative and proapoptotic activities in vitro in solid and hematologic tumor cells. In animals, carfilzomib inhibited proteasome activity in blood and tissue and delayed tumor growth in models of multiple myeloma, hematologic, and solid tumors.
Carfilzomib is an antitumor antibiotic derived from anthracyclines. While the usage of anthracyclines is limited due to their dose-related cardiotoxicity and myelosuppression, applying liposomal Carfilzomib in ovary, lung, and breast cancer therapies has been approved by WHO due to its superior efficacy and minimum cardiotoxicity. Furthermore, the liposomal forms allow Carfilzomib to remain in the circulation system for longer periods of time, which will allow for the delivery of a greater amount of the drug to cancerous cells or tumors. Both the prolonged exposure of tumor cells to liposome and the capability to distinguish the differential between tumors via tissue cells are valuable reasons to develop liposomes. On the other hand, as nanoparticles are regarded as a valuable carrier, nanoliposome is also one of the well-known and established developments in drug delivery systems.
Since palm oil has antiproliferative and antioxidant properties due to presence of components such as carotenes, tocopherol, tocotrienols, terpenoids, and flavonoids, it is viable for use in pharmaceutical products, on top of its nutritional advantages. In addition, its antioxidants help resist rancidity and improve the stability of palm oil.
Considering the anticancer properties of palm oil and great advantages of liposome, the aim of this study was to prepare liposomal Carfilzomib by applying palm oil fractions[2].

Materials[1]:
Carfilzomib – A gift sample from MSN labs, Hyderabad. Hydrogenated palm oil (palm oil), cholesterol (CH), L-alpha-phosphatidylcholine (PC), polyethylene glycol (PEG), methanol, and chloroform were purchased from Sigma-Aldrich. Sodium hydroxide and potassium dihydrogen phosphate were purchased from Merck.

Methods[8,9]:
Liposomes were prepared using the freeze-thaw method and pH gradient technique, carried out in order to maximize the loading of Carfilzomib, withinliposome. Two formulations were designed; both consisted of 45 mg CH and 5 mg PEG with different percentages of PC and palm oil. The first formula (Fa) contains 5% palm oil and 45% of PC, while the second formula (Fb) contains 10% palm oil and 40% PC in their respective formulations. Then, all of the lipid components and PEG were dissolved in a chloroform: methanol mixture of (2 : 1, v/v) in a round-bottom flask. The solvent was removed under vacuum using a rotary evaporator (Rotavapour R-124, BÜCHI) at 40°C and 50 rpm. After a thin lipid film was formed in the interior of the flask, the system was purged with nitrogen to remove organic solvent entirely. The lipid film layer was hydrated with 10 mL Citrate buffered solution (pH = 4) and then sonicated for 30 minutes in a bath type sonicator (Sonicor). The freeze-thaw cycle was carried out five times via freezing under −80°C and then heated mixture in water bath at 65°C with the intention of decreasing the size, further entrapping the acidic buffer inside the liposome. Bicarbonate buffer (pH = 0.5) was added dropwise to the mixture (for the reason of adjusting outer liposomes space into a physiological pH) until its pH reaches 7. Afterwards, 10 mL of Carfilzomib

medium in distilled water (2000 µg/mL) was added to the mixture and shaken at room temperature for 30 minutes at 60 rmp.

Formation develeopment[7]:

The formation of liposomes was observed with a transmission electron microscope (TEM). Samples were prepared by applying a drop of the mixture to a carbon-coated copper grid and left for a minute to allow some of the particles to adhere onto the carbon substrate. After removing the excess dispersion with a piece of filter paper, a drop of 1% phosphotungstic acid solution was applied for one minute and then left to be air-dried. The samples were viewed with a TEM .

Particle Size Distribution, Polydispersity Index (PDI), and Zeta Potential (ZP) Measurement

To evaluate the size distribution, PDI, and value ZP of each sample, 50 mg of liposome was weighted and dispersed in 20 mL distilled water and then those parameters were measured by the zetasizer (ZetasizerNanoseries, Malbern Instrument). This test was repeated thrice.

Liposome Particle size distribution (PSD)

Dynamic Light Scattering (also known as Photon Correlation Spectroscopy or Quasi-Elastic Light Scattering) is a technique for measuring the size of particles typically in the sub-micron region.Dynamic Light Scattering (DLS) measures Brownian motion and relates this to the size (Hydrodynamic diameter) of the particles. The relationship between the size of a particle and its speed due to Brownian motion is defined by Stokes-Einstein equation.

Hydrodynamic Diameter: The size of a particle is calculated from the translational diffusion coefficient by using the Stokes-Einstein equation;

$D(H) = kT / 3\pi\eta D$

Where

D (H) = hydrodynamic diameter, D = translational diffusion coefficient

k = Boltzmann's constant, T = absolute temperature, η = viscosity

It does this by illuminating the particles with a laser and analysing the intensity fluctuations in the scattered light. Brownian motion is the random movement of particles due to the bombardment by the solvent molecules that surround them.

The particle size distribution of Carfilzomib liposome injection lies between approx. 30 nm to 300 nm. So the formulation can be analyzed using Zeta-Sizer instrument with detector positioned either at 173° (Nano S & ZS range: 0.6nm to 6µm) or 90° (Nano S90 &ZS90 range: 2nm to 3µm).

Procedure:

Approx 1mL Sample was put into disposable Polystyrene cuvette and particle size distribution was analysed using Malvern ZetasizerNano ZS using Particle absorbance 0.010 and Refractive index 1.35.

The following parameters are considered important during the Particle size analysis:

- Average Particle Size $Z_{(avg)}$
- Polydispersity Index (PDI)
- D10, D50 and D90 values
- SPAN (D90-D10)/D50

Zeta Potential/Electrical Surface Potential:

Surface charge/zeta potential on liposomes can affect the clearance, tissue distribution, and cellular uptake.

The magnitude of the zeta potential gives an indication of the potential stability of the colloidal system. If all the particles in suspension have a large negative or positive zeta potential then they will tend to repel each other and there is no tendency to flocculate. If the particles have low zeta potential values then there is no force to prevent the particles coming together and flocculating.

The general dividing line between stable and unstable suspensions is generally taken at either +30mV or -30mV. Particles with zeta potentials more positive than +30mV or more negative than -30mV are normally considered stable.

The Zeta potential analyser calculates the zeta potential by determining the Electrophoretic Mobility and then applying the Henry equation.

The development of a net charge at the particle surface affects the distribution of ions in the surrounding interfacial region, resulting in an increased concentration of counter ions (ions of opposite charge to that of the particle) close to the surface. Thus an electrical double layer exists around each particle.
The liquid layer surrounding the particle exists as two parts; an inner region, called the Stern layer, where the ions are strongly bound and an outer, diffuse, region where they are less firmly attached. Within the diffuse layer there is a notional boundary inside which the ions and particles form a stable entity. When a particle moves (e.g. due to gravity), ions within the boundary move with it, but any ions beyond the boundary do not travel with the particle. This boundary is called the surface of hydrodynamic shear or slipping plane.

The potential that exists at this boundary is known as the Zeta potential.
Concept of Zeta potential and electric double layer were derived from The Henry equation:
Z: Zeta potential.
$\mathbf{U_E}$: Electrophoretic mobility.
ε: Dielectric constant.
η: Viscosity.
***f*(Ka):Henrys** function.

$$U_E = \frac{2\varepsilon z f(ka)}{3\eta}$$

Two values are generally used as approximations for the *f*(Ka) determination either 1.5 or 1.0. Electrophoretic determinations of zeta potential are most commonly made in aqueous media and moderate electrolyte concentration. *f*(Ka) in this case is 1.5, and is referred to as the Smoluchowskiapproximation.For small particles in low dielectric constant media *f*(Ka) becomes 1.0 and allows an equally simple calculation. This is referred to as the Huckelapproximation. Non-aqueous measurements generally use the Hackleapproximation.
Construction of Standard Curve:
Dilutions of Carfilzomib were in the range of 400, 200, 100, 50, 25, and 12.5 ng/mL, prepared and detected by HPLC with a fluorescence detector Mixture of Acetonitrile. Heptanesulfonic acid (0.2%, pH 4) by a ratio of 25: 75 was applied as mobile phase with the flow rate of 1 mL/min.
Carfilzomib has an excitation wavelength of 480 nm and an emission wavelength of 560 nm.

Evaluation of Entrapment Efficiency and In Vitro Release:
The mixture was centrifuged (Universal 32) for 70 minutes at 14000 rpm, the supernatant containing free Carfilzomib was obtained, and the absorbance was measured using HPLC [15]. The entrapment efficiency of liposomes was determined by the following formula:where EE is the concentration of entrapped drug (ng/mL), is the initial concentration of drug used in formulating the liposomes (ng/mL), is the concentration of drug in the supernatant (ng/mL), and EE (%) is the percentage of the drug's entrapment.
To estimate the in vitro drug release of liposomal Carfilzomib, a dialysis bag was used. After separating free drug, 100 mg of liposome was weighted and then placed directly into the dialysis bag (Mw12000). The dialysis bag was sealed at both ends and located in a 1000 mL fresh PBS buffer medium (pH 7.4) at 37°C, at 90 rpm under perfect sink conditions. At predetermined time intervals, 1 mL of the medium was sampled for further analysis by HPLC. The concentrations of Carfilzomib

throughout the releasing time were calibrated using the calibration equation. The results recorded are the mean value of the three runs carried out for each liposome concentration. The percentage of released Carfilzomib at certain time was plotted using Microsoft Excel and was defined by the following formula: where is the concentration of drug released (ng/mL) at time and is the initial drug concentration (ng/mL).

Statistical Analysis

All of the results were remarked with the mean ± SD, and the one-way analysis of variance (ANOVA) was employed for statistical analysis of the data,

RESULTS:

TEM images in Figure -1 & Figure -2 demonstrate the formation of vesicles. Considering the TEM images, one layer liposome with large inside capacity confirms the fine formation and well shape of the LUV in both formulations.

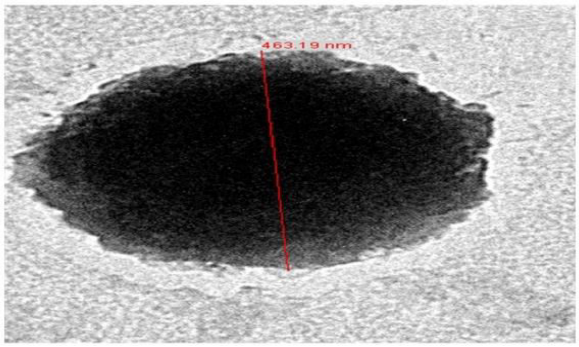


Figure -1

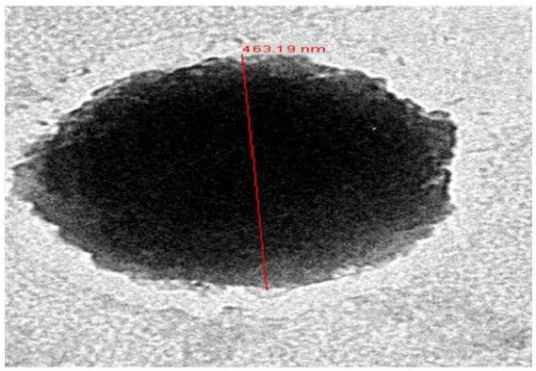


Figure - 2

TEM images of Carfilzomib liposome with magnification 8000x,

Particle Size Distribution and Zeta Potential Measurement

Particle size determinations are mostly performed to confirm that the desired liposome size range has been obtained during preparation because suitable size of particles is important for their interaction with the biological situation; for instance, through intravenous administration of loaded particles, their ability to pass or leave the vascular capillaries effectively is dependent on the sizing. Referring to Table 1, F (A) has a size of 438 nm, while F(B) has a size of 453 nm; the nanosize of LUVs would result in advance drug delivery.

The polydispersity index value is a measure of the heterogeneity of particle sizes in a compound. Liposomes with PDI value between 0.1 and 0.25 display more uniformity and physical stability. Further PDI value more than 0.5 indicates the poor uniformity of mixture. Looking at Table 1, the PDI values of liposomes are 0.22 and 0.21 which confirm the uniformity and homogeneity of LUVs in the mixture as well.

Table 1: Particle size, zeta potential, and entrapment efficiency of the liposomes.

Formulation	Mean particle size (nm, ±SD)	Mean zeta potential (mV, ±SD)	Mean polydispersity index (PDI)	Mean entrapment efficiency (%, ±SD)
F(A)	438.74 ± 1.9	-31.1 ± 2.6	0.22 ± 1.3	99.98 ±3.18
F(B)	453.71 ± 1.1	-32.2 ± 4.1	0.21 ± 0.8	99.99 ± 5.22

The value of zeta potential (ZP) proves the stability of the particulate systems. It is a measurement of the repulsive forces between the particles. Particles having a ZP of less than −30 mV or more than +30 mV are usually regarded as stable. Considering the ZP values were higher than −30 mV (Table 2), which confirms the acceptable stability of LUVs as well as their uniformity and size homogeneity suspension.

Table 2: IC50 Results of F (A), F(B).

Formulation	IC50 MCF7 (µg/mL,n =3)	IC50 MDA-MBA 231 (µg/mL, n=3)
F(A)	376.45 ± 9.20	726.40 ± 7.58
F(B)	387.22 ± 6.93	755.73 ± 6.81

Physics chemical Characterization and In Vitro Drug Release

From Table 3, Results of Carfilzomib Liposomal Injection with both formulations were described.

Table 3: Study results of Carfilzomib Injection 10 mg/mL

Sr. No.	Tests	Specification Stage	Carfilzomib injection 10 mg/mL F(A)	F(B)
1	Assay (%)	Between 90-110	98.50	94.2
2	Lipid content (%)			
	palm oil content	Between 80-120	96.90	92.73
	L-alpha-phosphatidylcholine	Between 80-120	95.30	92.31
	Cholesterol	Between 80-120	97.20	93.79
3	Free Drug (%)	NMT 5% of assay	2.50	1.59
4	Entrapped Drug (%)	NLT 92% of assay	98.50	98.77
5	Zeta Potential (mV)	-5mV to -15mV	-9.38	-9.81
6	Osmolality (mOsm/kg)	250-400mOsm/kg	324	327
7	pH	Between 6.0 to 7.0	6.49	6.4
8	Particle Size Distribution			
	Zavg (nm)	95 ± 15	91.7	91.2
	D10 (nm)	45 to 80	64	63.1
	D50 (nm)	80 to 120	95.4	94.97
	D90 (nm)	120 to 190	143	143
9	In-Vitro Release (%) {Dissolution media pH 6.4; Temperature 52±0.5°C}			

Sr. No.	Tests	Specification Stage	Carfilzomib injection 10 mg/mL	
			F(A)	F(B)
	After 0.5 Hrs.	1.5 Hrs - NLT 10%	14	2
	After 1.5 Hrs.	1.5 Hrs - NLT 15%	32	24
	After 3.0 Hrs.	3.0 Hrs - NLT 35%	57	54
	After 5.0 Hrs.	5.0 Hrs - NLT 60%	81	78
	After 7.0 Hrs.	7.0 Hrs - NLT 80%	98	94

Conclusion:

In order to take advantage of the therapeutic effects of palm oil, liposomal Carfilzomib formulations were prepared by replacing PC with different ratios of palm oil. Liposomal formulations containing 5% - F(A) and 10% - F(B)of palm oil were made through the freeze-thaw method, and then the TEM images revealed satisfactory morphology and formation of LUVs, respectively. Liposomal size distribution, zeta potential, and stability remain in the acceptable range. The HPLC results confirm the optimal drug loading through pH gradient technique and sophisticated in vitro release profile as well.

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