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R E S E A R C H A R T I C L E

Formulation and Characterization of Phospholipid Based Micelles: A Potential Carrier for Cancer

Mayank Chaudhari, Deepa H. Patel*, Drashti G. Pathak, Sneha K. Patel,

Associate Professor, Parul Institute of Pharmacy and Research, P.O. Limda, Ta. Waghodia, Dist. Vadodara-391760

A B S T R A C T

Cancer is a broad group of diseases involving unregulated cell growth. In cancer, cells divide and grow uncontrollably; forming malignant tumors. Mitoxantroneis an anthracenedione antineoplastic agent. It is a potent inhibitor of topoisomerase II that intercalates into deoxy-ribonucleic acid (DNA) through hydrogen bonding, causes cross links and strand breaks. However, it has a cytocidal effect on both proliferating and non proliferating cultured human cells, suggesting lack of cell cycle phase specificity, which prompt for development of novel carrier that could effectively target tumor site without producing undesirable side effects. The aim of the present investigation was to formulate and evaluate phospholipids based micelles: A potential carrier for anticancer drug, mitoxantrone. Fourier transform infrared spectroscopy (FTIR) was employed to study drug-excipient incompatibility. Analytical method was performed by UV spectrophometer. Phospholipids Micelles were prepared by co-precipitation and reconstitution of drug and phospholipids. Phospholipids micelles were successfully prepared by taking the ratio of Mitoxantrone:Phospoipids (1:3) and N-(carbonyl-methoxy polyethylene glycol-2000)- 1,2-distearoyl-*sn*-glycero-3 phosphoethanolamin (DSPE MPEG 2000) : Phosphatidylcholine (PC) (7:3) by co-precipitation method and evaluated for micelles size and zeta potential, percent drug entrapment, micelles morphology using transmission electron microscopy (TEM), *in vitro* drug release, sterility testing and stability studies. Drug and excipients were found to be compatible to each other which were confirmed by FTIR study. The micelles size, zeta potential and percent entrapment was found to be 23.3±3.12 nm, -40.6±0.21mV and 91±0.06 respectively. *In-vitro* drug release study of the optimized batch was found to be 97.73 ± 0.89 % for optimized batch up to 24 hrs. Sterility test suggested that the developed phospholipid micelles were successfully sterile using membrane filtration method. Stability study shows phospholipids micelles were stable at refrigerated condition. The present study demonstrated that, phospholipids micelles are a promising nanocarrier for mitoxantrone delivery to cancer cell and should be used as a novel targeted nanomedicine.

Keywords: Mitoxantrone, N-(carbonyl-methoxy polyethylene glycol-2000)- 1,2-distearoyl-*sn*-glycero-3phosphoethanolamin (DSPE MPEG 2000), Phosphatidylcholine (PC) , Phospholipids Micelles.

A R T I C L E I N F O

Corresponding Author
Deepa H. Patel
Associate professor
Parul Institute of Pharmacy and Research,
P.O. Limda, Ta. Waghodia, Dist. Vadodara-391760
MS-ID: IJCPS3657

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C O N T E N T S

1. Introduction

Cancer is a broad group of diseases involving unregulated cell growth. In cancer, cells divide and grow uncontrollably, forming malignant tumors. The resultant unnatural cell behavior leads to expansive masses of abnormal cells that destroy surrounding normal tissue and can spread to vital organs and invading nearby parts of the body resulting in disseminated disease . The cancer may also spread to more distant parts of the body through the lymphatic system or bloodstream resulting a precursor of inevitable patient death.[1]

Mitoxantrone is an antibacterial, antiprotozoal, immunomodulating, and antineoplastic cytostatic anthraxquinone derivative. It is used in the treatment of certain types of cancer, mostly multiple sclerosis, metastatic breast cancer, acute myeloid leukemia, and non-Hodgkin's lymphoma.[2] Currently multi dose vials of this drug are available in the market.[2, 3] Disadvantage of this formulation is that drug molecule can extravasculate in any vasculature in the body which causes high toxicity to healthy tissue. The formation of drug aggregates can also result in high-localized concentrations at the sites of deposition associated with local toxicity and/or lowered systemic bioavailability. Intravenous administration of aggregates formed by insoluble drug can cause embolization of blood vessels.[4, 5, 6]

Phospholipids micelles are particles with diameters typically smaller than 100 nm formed by amphiphilic phospholipids dispersed in aqueous media.It solubilize poorly water-soluble drugs by incorporating them into their hydrophobic core thus increased its bioavailability.[7,8] These phospholipid micellar systems are safe, biocompatible, and nontoxic. The (Poly ethylene glycol) PEG on the surface of the micelles renders them sterically stabilized, preventing opsonization and reticular endothelial system uptake. Because of their low critical micellar concentration (CMC) values and also most likely because of the strong interaction between the acyl chains in the core region, are also relatively stable on dilution. In addition, because of their small size, these carrier systems can provide targeted delivery to cancer tissue or other injured tissues by selective extravasation through leaky vasculature.[9]

Pegylated phospholipid such as N-(carbonyl-methoxy polyethylene glycol-2000)-1, 2 distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE-MPEG), phosphatidylcholine (PC) used in this formulation that incorporate the drug in its hydrophobic core and thus improve the solubility of hydrophobic compound, decreased the local toxicity and improve the systemic bioavailability. So, aim of the present work was to formulate and characterize phospholipids based micelles a potential carrier for anticancer drug, mitoxantrone. [10]

2. Materials and Methods

Table 1: List of materials

Material	Use	Supplier
Mitoxantrone	Anticancer drug (API)	Sun Pharma Ltd, Vadodara, India
N-(carbonyl-methoxy polyethylene glycol-2000)-1,2-distearoyl-*sn*- glycerol phosphoethanolamine (DSPE-MPEG-2000)	PEGylated Phospho lipid	Lipoid ltd. Germany
Phoshatidylcholine (PC)	Phospho lipid	Chemdyes Corporation, Vadodara, India
Methanol	Solvent	SRL, Mumbai, India

Table 2: List of equipments / machines

Equipment	Purpose	Make
Digital pH Meter	pH check	Systronics
Weighing balance	Weighing of ingredients	Shimadzu AUW220D
Fourier transform Infrared spectrometer	Identification and compatibility studies	Bruker, Mumbai, India
Rota Evaporator	Micelle preparation, Solvent Evaporator	Local supplier, India
Cooling Centrifuge	Separation	Remi Electrotechnic LTD.
Probe sonicator	Size reduction	Sonics, Vibra cell
UV spectrophotometer	Drug analysis	Shimadzu UV-1800, Japan
Malvern zeta sizer	Size and Zeta potential measurement	Malvern instrument, UK

Preformulation studies

Preformulation is an exploratory activity that begins early in drug development. Preformulation studies are designed to determine the compatibility of initial excipients with the active substance for a biopharmaceutical, physicochemical, and analytical investigation in support of promising experimental formulations. Data from preformulation studies provide the necessary groundwork for formulation attempts.

Solubility study:

Solubility study of drug was performed in different solvents like water, methanol, chloroform and ethanol. Results of solubility study in different solvents are shown in table 5.1.

Fourier transforms infrared spectroscopy (FTIR):

Fourier transform Infrared Spectroscopy (FTIR) was carried out using KBr press by pressed pellet technique in which dried KBr was used for preparation of pellets. Pellets were prepared by mixing KBr and samples in mortar and pestle and then pressedin KBr press. The prepared pellet was placed in the sample holder in the instrument torecord the FTIR peaks. The results of the infrared studies for the selected batch are recorded. Compatibility of the drug (mitoxantrone) with excipients (DSPE-MPEG 2000, PC) used to formulate micelles were established by infrared absorption spectral analysis. [11] FTIR spectral analyses of following samples were carried out to investigate any changes in chemical composition of the drug after combining it with the excipients. FTIR spectra of mitoxantrone and mitoxantrone with physical mixture of all excipients are shown in figure 5.1 and spectral interpretations are shown in table 5.2.

Analytical method development:

Analytical method was developed by UV visible spectrophotometer. Method for determination of mitoxantrone gave absorption peaks at 660 nm in methanol and phosphate buffer saline pH 7.4 (PBS pH 7.4).

Preparation of standard stock solution in methanol:

The stock solution was prepared by accurately weighing 5 mg of mitoxantrone reference substance, which was transferred to a 50 ml volumetric flask, dissolved and diluted up to 50 ml with methanol to obtain a concentration of 100µg/ml. [12]

Preparation of calibration curve

Aliquots of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 ml of reference stock solution were transferred to 10 ml volumetric flasks and the mixture was diluted up to 10 ml with methanol to obtain 2-14 µg/ml concentration range. The absorbance was measured at 660 nm against methanol as blank. The solutions were stabilized for 10 minutes. Calibration curve and data of mitoxantrone in methanol are shown in figure 5.2 and table 5.3 respectively.

Preparation of standard stock solution in PBS pH 7.4:

The stock solution was prepared by accurately weighing 5 mg of mitoxantrone reference substance, which was transferred to a 50 ml volumetric flask, dissolved and diluted up to 50 ml with PBS pH 7.4 to obtain a concentration of 100µg/ml.

Preparation of calibration curve:

Aliquots of1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 ml of reference stock solution were transferred to 10 ml volumetric flasks and the mixture was diluted up to 10 ml with methanol to obtain 10-50 µg/ml concentration range. The absorbance was measured at 660 nm against methanol as blank. The solutions were stabilized for 10 minutes. Calibration curve and data of mitoxantrone in PBS pH 7.4 are shown in figure 5.3 and table 5.4 respectively.

Preparation of phospholipid based micelles of Mitoxantrone[13,14,15]: Micelles containing Mitoxantrone was prepared by co-precipitation method. Mitoxantrone, N-(carbonyl-methoxy polyethylene glycol-2000)-1, 2-distearoyl-*sn*-glycero phosphoethanolamine (DSPE-MPEG-2000) and phosphatidylcholine (PC), was dissolved in methanol. The solvent was then removed by vacuum rotary evaporation at 45ºC and 100rpm for 30 minutes. The dried film was rehydrated with phosphate buffer pH 7.4 (PBS pH 7.4) at 50ºC and 80 rpm for 1.5 hours, The solution was sealed, and equilibrated for 12 hours at room temperature which will approximately 25°C. The excess of free mitoxantrone was removed by centrifugation at 13,000 rpm for 5 minutes to obtain a clear dispersion. The prepared dispersions was then characterized for their particle size, zeta potential and percent drug entrapment.

Formulation Development:

Optimization of formulation parameter:

The various formulation parameters were studied for optimization of the method by keeping process parameter constant. Thin Film was prepared at 100 rpm, for time duration of 30 min, under 600 mm Hg pressure at 45 °C temperature. The film was hydrated using 10 ml hydration media (PBS pH 7.4) for a period of 1.5 hr at 50˚C and 80 rpm. The dispersion was sonicated for 10 min.

- Drug: Phospholipid molar ratio
- Phospholipid (DSPE-MPEG 2000): phospholipid molar ratio (PC)

Drug: phospholipid molar ratio should be such that precipitation of drug does not occur and also optimal entrapment should achieved. Two different phospholipids were used in the micellar formulation. The proportion of phospholipids should be such that optimal micelles size and entrapment of drug should achieve. For the formulation optimization different drug: phospholipid (mitoxantrone: DSPE-MPEG 2000 and PC) ratio such as 1:1, 1:3, 1:5, 1:10 were tried. Different ratio (9:1, 8:2, 7:3) of two phospholipids (DSPE-MPEG 2000: PC) were tried. [16, 17]

Hydration Media:

Phosphate buffer saline pH 7.4 (PBS pH 7.4) 10ml was used as a hydration media.

Optimization of Process Parameters

The various process parameters include Solvent evaporation time, speed of rotation, film formation, hydration of film, hydration time, sonication time were performed to achieve optimum process by considering the following formulation parameters of optimized batch (table 2.4).

Solvent evaporation time: Solvent evaporation is required for the formation of thin dried film of lipid. The thin film was prepared at 600mm Hg, 100 rpm and 45ºC the different time periods were tried to evaporate the organic solvent. Results are shown in table 5.6.

Speed of rotation

The speed of rotation of round bottom flask is important for uniformity of film as well as thickness and hydration of

film. The film was prepared at 600 mm Hg, 45ºC for 30 minutes to optimize the film formation and 1.5 hours for hydration of the film. [18, 19] Different speeds of rotation were tried for film formation and hydration of film. Results are shown in table 5.6.

Hydration time: An optimal hydration time is required for complete hydration of thin lipid film. The film was prepared at 600 mm Hg, at 50ºC for 1.5 hrs for hydration of the film. Different time period were tried for the complete hydration of the film. Results are shown in table 5.6.

Sonication time: Sonication is required for uniform size reduction. Prepared micelles were sonicated from 5 to 10 minutes. Results are shown in table 5.6.

Characterization of Phospholipids Micelles:

Particle size and zeta potential:

The size of micelles and zeta potential were measured by dynamic light scattering with a Malvern Zetasizer 3000 HS (Malvern Instruments, Malvern, UK). Diluted micelles were added to the sample cuvette and then cuvette was place in zetasizer. Sample was stabilized for two minutes and reading was measured. Results of micelle size and zeta potential are shown in figure 5.4, figure 5.5 and table 5.5

Percent drug entrapment (PDE) and Percent drug loading (PDL): [20-24]

Percentage drug entrapment:

The percentage of drug encapsulated was determined by UV visible spectrophotometric method. Formulation containing phospholipid micelles was centrifugated at 18000 rpm & at 4°c for 30min for removal of free drug. Sediment was collected and dissolved in methanol and estimated spectrophotometrically using UV visible spectrophotometer at 660 nm for entrapped drug. Percent drug entrapment and Percent drug loading was calculated from Eq 1 and Eq 2. Results of Percent drug entrapment and Percent drug loading are shown in table no 5.5.

Percent drug entrapment (PDE) =

$$\frac{\text{Actual drug loading}}{\text{Theoretical drug loading}} \times 100 \quad (1)$$

Percent drug Loading (PDL =

$$\frac{\text{Amount of drug present in PMs}}{\text{Weight of PMs sample analyzed}} \times 100 \quad (2)$$

In vitro drug release: [33]

One milliliter of micelles solution with known mitoxantrone content was placed into a dialysis bag. The dialysis bag was immersed into a flask containing 50 mL of release medium (phosphate buffer saline (PBS), pH 7.4) which was kept in a constant temperature at 37°C and 50 rpm on magnetic stirrer. At predetermined time intervals, aliquots (1 mL) of the release medium was taken and replaced by fresh medium. The content of mitoxantrone in the medium was measured by UV visible spectroscopy method as described above. The cumulative release percentage of mitoxantrone was calculated. The results of *in vitro* drug release are shown in figure 5.6 and table 5.9.

Transmission Electron Microscopy:

The morphology of phospholipid micelles was examined using transmission electron microscopy. A drop of phospholipids micelles was visualized after staining with phosphotungstic acid on a copper grid under TEM. The TEM image of phospholipids micelles of optimized batch is shown in figure 5.7.

Stability studies: [25-27] Comparative stability study was performed on the formulation at two different condition, i.e, 4-8 ± 2°C/45±5% RH (Refrigerator, RF) and 25± 2°C/65± 5% RH (Room temperature; RT) for a period of 30 days. The results of stability study are shown in table 5.10.

Sterility test: [28-30]

Sterilization was performed by membrane filtration method and sterility test was performed as per USP for large and small volume of parenterals. The test compound was filtered through 0.45µ Polytetrafluroethylene (PTFE) membranes which were incubated in a fluid thioglycolate medium in lieu of incubating the compound itself. Autoclaved the freshly prepared fluid thioglycolate medium at 121°c and 15psi for 15min.Three test tubes were placed,

1) Negative control which has only fluid thioglycolate medium.

2) Positive control which has fluid thioglycolate medium and Staphylococcus aureus.

3) Sample and fluid thioglycolate medium.

These three test tubes were incubated for 14days and assessed for bacterial growth. The presence of turbidity in the medium indicates a failed sterility test. The results of sterility testing are shown in table 5.11 and figure 5.8 and 5.9 respectively.

3. Results and Discussion

Preformulation Studies

Solubility Study:

Solubility data of drug and excipients in different solvents are shown in table 5.1.

Discussion:

Based on solubility study in different solvents, methanol shows maximum solubility for drug, DSPE-MPEG 2000 and PC. So, methanol was selected as a solvent for the preparation of thin film.

Fourier Transform Infrared Spectroscopy (FTIR):

FTIR spectroscopy was used for identification of compounds. The combination of fundamental stretching or bending of various functional groups and the interactions of these functional groups with other atoms of the molecule result in unique FTIR spectrum for each individual compound. Thus FTIR represents potentially highly useful and sensitive technique of determining the identities of materials in multicomponent formulation. The FTIR spectra of mitoxantrone and mixture of drug with excipients are shown in figure 5.1.

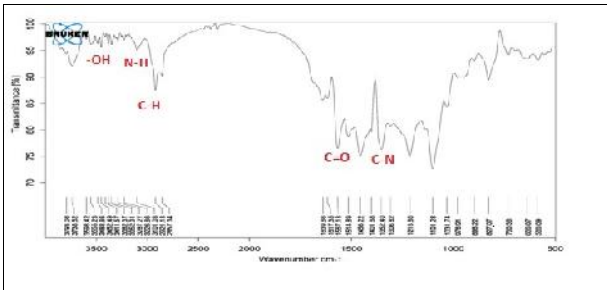

Fig 1: FT-IR Spectra of mitoxantrone

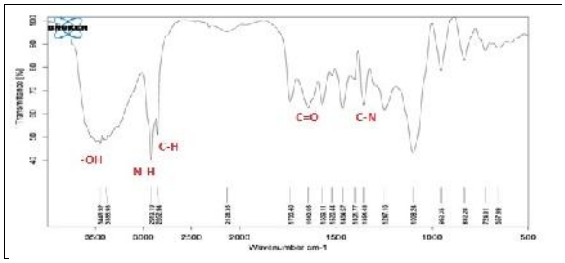

Fig 2: FT-IR Spectra of Mixture of Mitoxantrone with excipients

Discussion:

The FTIR spectral interpretation is shown in table 5.2. Mitoxantrone showed the typical infrared absorption character at 3418.27 cm^{-1} , 2921.13 $cm^{-1,}$ 1322.40 cm^{-1} , 1639.66 cm^{-1} , 3101.2 cm^{-1} corresponding to –OH, C-H, C-N, C=O, N-H respectively. Also in case of mixture of drug with excipients, no new peak was observed indicating no strong interaction and thus no incompatibility was observed.

Analytical method delovelopement: [31, 32]

Calibration curve of mitoxantrone was performed in methanol and phosphate buffer saline pH 7.4(PBS pH 7.4) using UV spectrophotometric method. The absorption spectrum showed highest absorbance peaks at 660 nm, which was selected for the analytical studies. Calibration curve of mitoxantrone and absorbance data of mitoxantrone in methanol are shown in figure 5.2 and table 5.3 respectively. Calibration curve of mitoxantrone and Absorbance data of mitoxantrone in PBS pH 7.4 are shown in figure 5.3 and table 5.4 respectively.

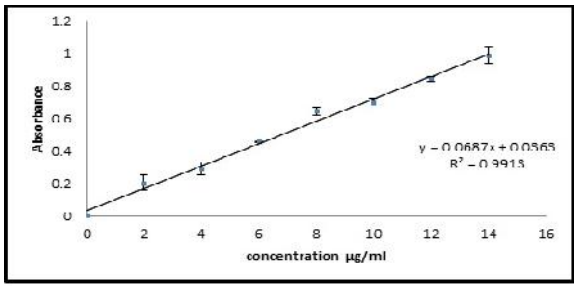

Fig 3: Calibration curve of mitoxantrone in methanol

Discussion:

From the results shown in table 5.3 and figure 5.2, the calibration curves was found to be linear in the concentration range of 2-14 µg/ml and the R^2 was found to be 0.991 in methanol. This standard concentration method obeys beers law and found to be suitable for the determination of drug entrapment study.

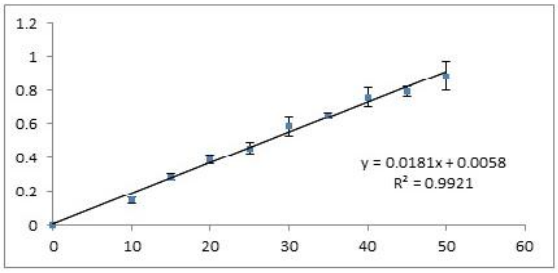

Fig 4: Calibration curve of Mitoxantrone in PBS pH 7.4

Discussion:

From the results shown in table 5.4 and figure 5.3, the calibration curves was found to be linear in the concentration range of 10-50 µg/ml and the R^2 was found to be 0.992 in PBS pH 7.4. This standard concentration method obeys beers law and found to be suitable for the determination of *in vitro* drug release study.

Formulation Development

Optimizations of the method have been achieved by optimizing various formulations as well as process variables. **Optimization of Formulation Parameters**

The various Formulation parameters were studied for optimization of the method by keeping process parameter constant. Thin Film prepared at 100 rpm, for a t ime duration of 30 min, under 600 mm Hg at 45°C temperature. The film was hydrated using 10 ml phosphate buffer saline pH 7.4 as hydration media for a period of 1.5 hr at 50°C and 80 rpm. The formulation was sonicated for 10 min.

Drug: Phosphoipid Molar ratio: [33-35]

Discussion:

Drug: phospholipid molar ratio 1:3 shows good micelles with higher PDE so it was found to be optimum. Comparative lower PDE was found in 1:1 ratio of drug: Phospholipid. Drug entrapment was found to be decrease at the 1:10 ratio of drug to phospholipid. Result showed that upon increasing the total lipid concentration the fraction of lipid taking part in encapsulation was reduced.

DSPE-MPEG2000: PC ratio

Discussion:

Increase in PC content up to 7:3, it was found to be increased in drug entrapment. The PDE was increased non-significantly with increasing PC concentration from 1 to 2% (i.e from 9:1 to 8:2 and 7:3). Phosphatidyl choline is a hydrophobic phospholipid. Addition of a small amount of it into a micellar system increases the volume of the hydrophobic region of the micelle. Therefore it would provide a larger space for drug.

Different molar ratio of drug: phospholipid and DSPE-MPEG 2000: PC was taken to get minimum vesicle size and maximum PDE. Results of batches of optimization of micelles are shown in table 5.5.

Discussion:

The result shown in table 5.5 indicates the effect of drug: phospholipid molar ratio and DSPE-MPEG 2000: PC molar ratio on particle size and PDE. PDE was increased and particle size was decreased with increase in drug: lipid molar ratio from 1:1 to 1:5 but further increased in drug: phospholipid ratio (1:10) shows decreased in PDE. Different phospholipid ratios shows that increase in phosphatidyl choline (PC) content leads to increased in PDE due to larger hydrophobic space. So, 7:3 was found to be optimum DSPE-MPEG 2000: PC molar ratio. Upon comparing the ratio of maximum PDE (7:3), F6 showed highest PDE (91±0.06%) with smallest particle size (23.3±3.12 nm). So, batch F6 was selected as optimized batch and taken for to optimize process parameters.

Optimization of Process Parameters

Solvent evaporation time:

Discussion: The maximum drying of film was found at 30 min. Retention of solvent was found at 10 min and at 45

min the film was over dried and becomes rigid so there was a problem in hydration of film.

Speed of rotation:

Discussion: A speed of 100 rpm was found to be adequate to give thin, uniform and completely dry film. During hydration period rotation at higher speed causes improper hydration. Hence, 80 rpm speed of rotation during hydration of film was selected as optimum speed.

Hydration time:

Discussion: The lipid film was hydrated from 45 minutes to 90 minutes. Lower hydration time led to a non-uniform shape and size of the micelles and also the un-hydrated part posed difficulty in size reduction.90 minute hydration time was found to be optimum.

Sonication time:

Discussion: Sonication for 5 minute produce micron and submicron sized micelles and was not able to convert it into nano range. And sonication for 10 minute gave nanosized micelles. Thus optimum time was found to be 10 min

Characterization of Phospholipid micelles:

Particle size and zeta potential:

Particle size was measured by zeta potential analyzer. Results of particle size and zeta potential of optimized batch F6 are shown in table 5.8, figure 5.4 and figure 5.5 respectively.

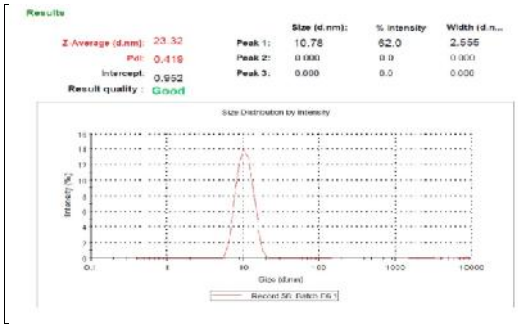

Fig 5: Particle size of optimized batch F6

Zeta Potential analysis is a technique for determining the surface charge of colloidal dispersion. The electric potential at the boundary of the double layer is known as the Zeta potential of the particles and has values that typically range from +100 mV to -100 mV. The magnitude of the zeta potential is predictive of the colloidal stability. Dispersions with a low zeta potential value will eventually aggregate due to Van Der Waal inter-particle attractions. Zeta Potential is an important tool for understanding the state of the nano size surface and predicting the long term stability of the nano size carrier.

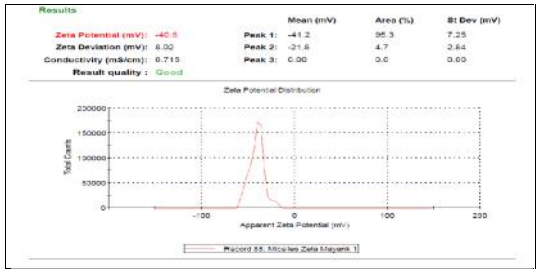

Fig 6: Zeta potential of optimized batch F6

Discussion:

Figure 5.4 demonstrate the particle size of the optimized batch was found to be 23.32±3.12 nm. Size of the PMs was found below 100nm which is usual size for this type of formulation. This small size would help micelles to transfect from biological membranes and their tendency to evade scavenging by reticulo-endothelial system (RES). Selective accumulation of drug- loaded PMs at the targeted sites is desired and achieved by enhanced permeation and retention effect (EPR) for such small sized particles.

The zeta potential is indicative of the stability of the colloidal system. Figure 5.5 demonstrate zeta potential of the optimized batch was found to be -40.6±3.6 mV, which indicate high negative surface charge on drugs loaded phospholipid micelles. This confirms higher stability because of the anticipated surface repulsion between similar charged particles and avoiding aggregation of the micellar particles.

In vitro drug release: [36]

One important characteristic of the drug delivery system is its ability to preserve an encapsulated drug under sink condition. To establish that the incorporated drug is associated firmly with micelles, phospholpid micelles loaded with mitoxantrone was dialyzed against phosphate buffer saline pH 7.4. When developing intravenous delivery systems for hydrophobic drugs such as mitoxantrone, it is important to adequately control the release rate in order to avoid precipitation upon dilution in blood. The *in vitro* release of mitoxantrone from phospholipid micelles was investigated. The sink condition was respected by addition of 30% (v/v) ethanol in the release medium. Results of in vitro drug release are shown in table 5.9.

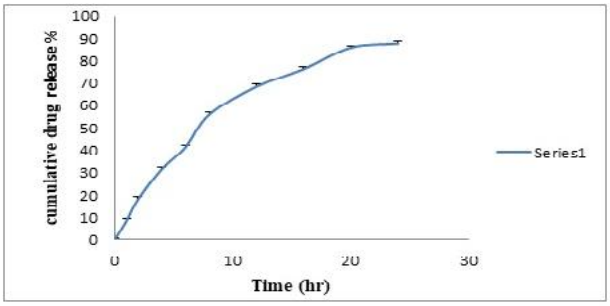

Fig 7: Cumulative drug release of phospholipid micelles

Discussion:

The drug release was found to be 87.7309±1.29 for phospholipid micelles after 24 hrs, which shows slow and sustain release of drug from the dosage form. Drug release from the formulation was found to be slow and sustained over 24 hrs. As phospholipid micelles contains in outer shell structure it would help micelles to retain in systemic circulation for prolonged period of time by preventing phagocytic clearance of the reticuloendothelial system (RES).

Transmission Electron Microscopy:

Transmission Electron Microscopy (TEM) is a vital characterization tool for directly imaging nanomaterials to obtain quantitative measures of particle and/or grain size, size distribution, and morphology.TEM images the

transmission of a focused beam of electrons through a sample, forming an image in an analogous way to a light microscope. The morphology of the mecelles were studied using a Transmission electron microscope (TEM). It was performed for batch F6 (with 1 % phototungusting acid). TEM images of batches F6 are shown in figure 5.7.

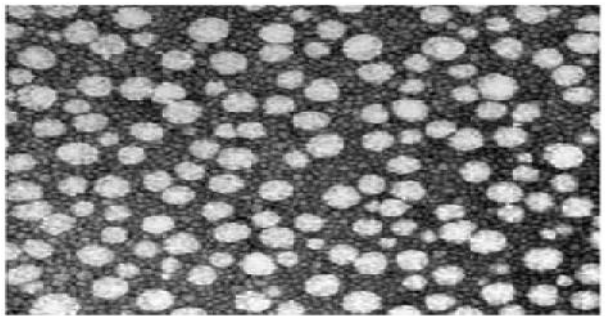

Fig 8: Transmission electron microscopy of Mitoxantrone loaded phospholipid micelles

Discussion:

TEM image of the optimized PMs formulation showed the distinct smooth surfaced micelles with spherical in shape. No aggregations of phospholipid crystals were observed.

Stability study:

The stability study was carried out for the optimized batch F6 at two different temperature condition i.e. 4-8 ± 2˚C/45±5% RH (Refrigerator, RF) and 25± 2˚C/65± 5% RH (Room temperature; RT) for a period of 30 days. The formulation shows good stability with no significant change in visual appearance, percent drug entrapment, percent drug loading and particle size. Result of the stability study are shown in table 5.10.

Discussion: Stability study was performed for final optimized F6 batch. The phospholipid micelles stored at refrigeration condition remained stable with no significant change in micelle size without any significant decrease in PDE values during the study period of 30 days.A gradual increase in micelle size and decrease in PDE values was observed with increasing temperature at 25^{o}C±2^{o}C/65±5%RH storage conditions suggesting the micelle size growth to be a function of storage temperature.On storage at 25^{o}C±2^{o}C/65±5%RH, the vesicle size increased from 23.3±3.54 to 28.45±4.87 nm and the PDE changed from 90.54±0.72 to 86.54±0.87% at the end of 30 days.

Sterility test:

Sterilization study was carried out by membrane filtration method. The test compound was filtered through 0.45 µ Polytetrafluroethylene (PTFE) membrane. Sterility study was performed as per USP method using Fluid thioglycollate medium.

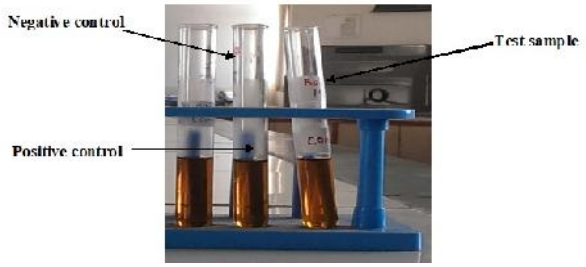

Fig 9: Sterility test of phospholipids micelles using fluid thioglycolate medium (Initial)

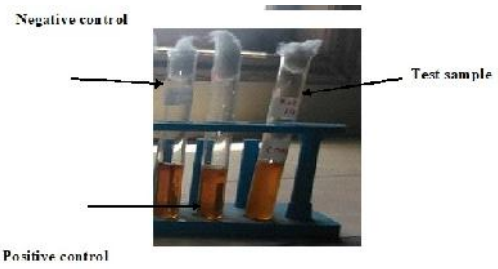

Fig 10: Sterility test of phospholipids micelles using fluid thioglycolate medium(After 14 days)

Discussions:

Sterility study was performed as per USP method using fluid thioglycolate medium and staphylococcus aureus bacteria. Figure 5.8 shown three test tubes which have negative control containing fluid thioglycolate medium, positive control containing fluid thioglycolate medium and staphylococcus aureus bacteria and test sample (PMs) and fluid thioglycolate medium. Figure 5.9 shows results after incubation for 14 days. no growth of micro organism in negative control, positive control has growth of microorganism which describes as fluid thioglycolate medium provide nutrition to the bacteria moreover test sample has no growth of micro-organism which conclude that sample was sterilized.

Table 3: Formulation optimization batches for selection of drug: Phospholipids and DSPE-MPEG 2000: PC molar ratio

Batch No	Drug: Lipid (DSPE-MPEG 2000:PC) molar ratio	DSPE-MPEG 2000:PC ratio
F1	1:1	9:1
F2		8:2
F3		7:3
F4	**1:3**	9:1
F5		8:2
F6		**7:3**
F7	1:5	9:1
F8		8:2
F9		7:3
F10	1:10	9:1

F11		8:2
F12		7:3

Table 4: Formulation parameters of optimized batch selected to optimize the process parameters

Sr. no	Materials	Amount
1	Mitoxantrone	2mg
2	DSPE-MPEG 2000	13.14mg
3	Phosphatidyl choline	1.37mg
4	PBS pH 7.4	10ml

Drug: Lipid (DSPE-MPEG 2000: PC) molar ratio was selected - 1:3;
DSPE-MPEG 2000: PC molar ratio was selected- 7:3

Table 5: Solubility study of Mitoxantrone and excipients

Solvents	Solubility		
	Drug	DSPE-MPEG 2000	Phopsphatidy choline (PC)
Water	Insoluble	Insoluble	Insoluble
Methanol	Soluble	Soluble	Soluble
Chloroform	Insoluble	Soluble	Soluble
Acetone	Insoluble	Soluble	Soluble

Table 6: FTIR spectral interpretation

Functional Group	Principle Peaks(cm^{-1})				
	-OH Bonded	C-H Stretching	C-N	C=O	N-H
Mitoxantrone	3418.27	2921.13	1322.40	1639.66	3101.2
Mitoxantrone + excipients	3428.23	2919.19	1324.48	1643.02	2991.19

Table 7: Absorbance data of Mitoxantrone in methanol

Concentration (µg/ml)	Absorbance (Mean ± SD)
2	0.202±0.041
4	0.293±0.032
6	0.427±0.033
8	0.644±0.021
10	0.701±0.022
12	0.844±0.017
14	0.991±0.029
λmax	660nm
R^2	0.991
Linearity range	2-14 µg/ml
Solvent	Methanol

Table 8: Absorbance data of Mitoxantrone in PBS pH 7.4

Concentration (µg/ml)	Absorbance
10	0.147±0.019
15	0.284±0.021
20	0.39±0.029
25	0.422±0.038
30	0.282±0.067
35	0.621±0.012
40	0.728±0.02
45	0.799±0.04
50	0.887±0.01
λ max	660nm
R^2	0.992
Linearity range	10-50 µg/ml
Solvent	PBS pH 7.4

Table 9: Result of batches of optimization for selection of drug: phospholipid and DSPE-MPEG 2000: PC molar ratio

Batch No.	Drug: phospholipid molar ratio	DSPE-MPEG 2000:PC ratio	Percent drug entrapped (PDE)	Percent drug loading (PDL)	Particle size (nm)	Zeta potential (mv)
F1	1:1	9:1	66±0.02	13.44±0.22	27.2±1.53	-32.2±1.2
F2		8:2	72±0.21	15.15±0.18	29.4±1.85	-27.7±3.4
F3		7:3	69±0.10	16.32±0.90	90.3±1.06	-28.9±4.2
F4	**1:3**	9:1	80.2±0.81	4.95±1.2	26.4±0.90	-32.3±1.2
F5		8:2	89±0.18	5.55±0.46	36.4±2.42	-13.4±2.3
F6		**7:3**	**91±0.06**	**6.27±0.55**	**23.3±3.12**	**-40.6±3.6**
F7	1:5	9:1	92±0.17	3.15±0.82	61.2±3.50	-16.8±1.1
F8		8:2	93±0.19	3.61±1.26	83.6±1.32	-31.7±2.4
F9		7:3	82±0.33	3.60±1.11	84.1±4.56	-35.9±1.5
F10	1:10	9:1	83±0.37	1.52±0.75	67.6±3.88	-41.9±2.8
F11		8:2	81±0.07	1.62±0.41	66.7±3.25	-44.6±0.6
F12		7:3	82±0.19	1.90±0.51	61.7±1.45	-38.7±3.8

Table 10: Optimized Process Condition

Sr. no	Process	Condition
1	Temperature for film formation	45ºC
2	Solvent evaporation time	30 min
3	Speed of rotation for film formation	100 rpm
4	Temperature for hydration	50ºC
5	Speed of rotation for hydration	80 rpm
6	Hydration time	90 min
7	Sonication time	10 min

Table 11: Optimized formula of Mitoxantrone loaded phospholipid micelles

Sr.no	Materials	Amount
1	Mitoxantrone	2mg
2	DSPE-MPEG 2000	13.14mg
3	Phosphatidyl choline	1.37mg
4	PBS pH 7.4	10ml

*****Ratio of drug: phospholipid (DSPE-MPEG 2000: PC)- 1:3
Ratio of DSPE-MPEG 2000: PC -7:3

Table 12: Particle size, zeta potential and PDE of optimizedbatch

Batch	Drug:Lipid Molar Ratio	DSPE MPEG-2000: PC Ratio	Particle size (nm)	Zeta potential (mV)	PDE
F6	1:3	7:3	23.32±3.12	-40.6±3.6	91±0.06

Table 13: In-vitro Release Study of optimized batch F6

Time (hr)	%CDR of PMs
1	8.4081±1.58
2	18.2854±1.34
4	31.6467±0.96
6	41.3928±1.25
8	55.9661±1.48
12	68.4215±1.65
16	76.2905±1.68
20	85.8972±1.47
24	87.7309±1.29

Table 14: Stability study for optimize batch

Sr. No	Parameter	Storage period (Days) 4-8±2˚C/ 45±5% RH (Refrigerator, RF)	Storage period (Days)25± 2˚C/65± 5% RH (Room temperature; RT)

		Initial	**15 days**	**30 days**	**Initial**	**15 days**	**30 days**
1	Visual appearance	Clear dark blue	Clear dark blue	Clear dark blue	Clear dark blue	Clear dark blue	Clear dark blue
2	Percent Drug Entrapment (%)	91.30 ±0.06	90.81 ± 0.8	92.95 ± 0.26	90.54 ± 0.72	89.95 ± 0.65	86.54 ± 0.87
3	Percent Drug loading (%)	6.25 ±0.55	6.42 ± 0.61	6.27 ± 0.75	6.52 ± 0.44	5.89 ± 0.21	5.96 ± 0.61
4	Particle size	23.3 ± 3.12	24.8 ± 4.15	25.1 ± 5.21	23.3 ± 3.54	26.6 ± 4.44	28.45 ± 4.87

(Where n=3, Mean ±SD)

Table 15: Results of sterility study data for Optimized Batch F6

Days	**Negative control**	**Positive control**	**Test sample**
1	-	+	-
2	-	+	-
3	-	+	-
4	-	+	-
5	-	+	-
6	-	+	-
7	-	+	-
8	-	+	-
9	-	+	-
10	-	+	-
11	-	+	-
12	-	+	-
13	-	+	-
14	-	+	-

Table 16: Result Summary for Optimized Formulation

Sr.no	**Parameters**	**Results**
1	Particle size	23.3±3.12 nm
2	Zeta potential	-40.6±3.6 mV
3	PDE	91±0.6 %
4	*In vitro* drug release	87.7309±1.29 % after 24 hrs
5	Transmission electron microscopy	Smooth surfaced particle
6	Sterility study	Pass in sterility test
7	Stability study	Stable at refrigeration condition

4. Conclusion

The micellar formulations of mitoxantrone with DSPE-MPEG 2000 and Phosphatidylcholine was optimized and prepared successfully by co-precipitation technique with good PDE (Percent drug entrapment) and particle size. From FTIR studies it was concluded that there were no incompatibility between drug and excipients. Regression co-efficient (R^2) was found to be near to one and which showed linear relationship between absorbance and concentration. Particle size of phospholipid micelles was found to be 23.32±3.12 nm, which is suitable for higher percent drug entrapment (91±0.06%). Zeta potential of PMs was found to be -40.6±3.6 mV, which indicates the stability of the formulation.Result of drug release shows slow and sustains release of drug through phospholipid micelles. Sterility test shows no growth of microorganisms. Results of stability study show that the phospholipid micelles were found to be remains stable after 1 month at refrigerator condition. Mitoxantrone loaded phospholipid micelles were successfully optimized and developed using co precipitation reconstitution method with good percent drug entrapment, particle size and stability. From FTIR studies it was concluded that there were no incompatibility between drug and excipients. Nanosize of the micelles significantly reduce drug distribution and toxicity to normal tissue further the drug entrapped in the micelles will not be free to interact with blood component and normal tissue thus phospholipid micelles reduce systemic toxicity and give better safety to patient, longer and sustained release of the micellar formulation would be more appealing with better patient compliance by minimizing dose related toxicity as

compared to the simple injection solution. The result obtained in the present study suggested that mitoxantrone incorporated in to micellar carrier can be effectively use in the treatment of cancer. Extensive preclinical and clinical trials on suitable animal and human model may justify the potential roll of phospholipids micelles containing anticancer drug in future development for cancer treatment.

Conflict of Interest: We declare no conflict of interest.

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