Synopsis of the Thesis titled

DESIGN, DEVELOPMENT AND EVALUATION OF NANOCARRIERS LOADED WITH DRUG COMBINATIONS FOR THE MANAGEMENT OF CANCERS PREDOMINANT IN WOMEN

By

Vankani Ankit Krushnachandra

(Registration No.: FOPH/18)

M. Pharm (Pharmaceutics)

Under the Guidance of

Dr. (Mrs.) Krutika K. Sawant

Professor and Dean, Faculty of Pharmacy

Submitted for the Degree of

Ph.D. in Pharmacy

Submitted to

Faculty of Pharmacy,

THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA

Vadodara – 390001, Gujarat (INDIA)

October 2023

TABLE OF CONTENTS

1. INTRODUCTION

The most prevalent cancer and the leading cause of cancer-related death among women globally is breast cancer. There were 685 000 deaths and 2.3 million new cases of breast cancer in women worldwide in 2020 [1]. Breast cancer is the most common cancer in the world, affecting 7.8 million women who were diagnosed with breast cancer in past 5 years and are alive as of the end of 2020, making it the world's most widespread cancer as per World Health Organization (WHO).In 2040, it is anticipated that breast cancer will account for over 3 million new cases and 1 million fatalities [2]. Breast cancer survival rates vary greatly around the globe, with high-income nations having an estimated 5-year survival rate of 80% and low-income nations having a survival rate of about 40%. The incidence of breast cancer has increased by more than 20% globally since 2008. The mortality rate has risen by 14% [3, 4].

Increased incidence of cancer in recent years and its impact on different physical, mental, and social dimensions of human life have turned it to a major problem of the century. The incidence of this disease in developed countries varies from 1 to 2 percent, with almost 5% yearly increase in less developed countries [5, 6]. According to estimates, more than 7 million people globally die from cancer. Meanwhile, breast cancer is the most prevalent type of malignant neoplasms among women with more than one million new cases per year [7, 8].

Effective drug delivery has been a major contributor to the improvement of cancer treatment. Ineffective drug delivery has resulted in inadequate tumour response, severe adverse effects, and the emergence of infamous cancer drug resistance. Due to the fact that anticancer drugs are typically toxic to healthy proliferating cells, dosage must be restricted to avoid potentially fatal adverse effects. Such factors as limited systemic circulation lifetime, undesirable biodistribution, non-specific cellular uptake, and inadequate tumour vascularisation can further reduce the therapeutic efficacy of such a low drug dose. Consequently, each course of chemotherapy has typically resulted in partial treatment, which has led to a selective pressure that favours mutations and drug resistance among the surviving cancer cells. Drugs with a favourable initial response are frequently rendered ineffective after repeated administrations, making the treatment of recurrent tumours more challenging [8-10].

The rise of chemotherapeutic drug resistance has been linked to the emergence of mutations. Compromised apoptotic signalling, enhanced damage repair mechanisms, increased drug metabolism, altered drug targets, and up-regulation of drug efflux pumps are among the identified

mutation types. Therefore, an effective method for enhancing the treatment of cancer cells would involve activating multiple pathways to prevent tumour cells from acquiring mutations. In addition, the majority of malignancies have been linked to multiple genetic alterations or abnormalities that result in tumour heterogeneity [11-13].

Utilizing a singular chemotherapeutic agent to treat cancer has resulted in the development of drug resistance, which has been a significant barrier to the success of cancer therapy [12]. In order to decrease the development of drug-resistance phenotypes, an efficient method for enhancing therapeutic efficacy would be to employ treatment strategies with agents that act via multiple mechanisms. Multiple agent delivery via efficient nanocarrier platforms would be a promising strategy for overcoming all of the aforementioned obstacles [14]. Taxanes (Paclitaxel) and alkylating agents (Cyclophosphamide) are the first line of treatment in metastatic breast cancer [15, 16].

Paclitaxel and Cyclophosphamide is a widely used combination for treatment of Metastatic breast cancer. [17, 18] Paclitaxel is hydrophobic in nature and consists of Cremophor EL and Ethanol in the marketed formulation. A high level of Cremophor EL is associated with severe anaphylactic hypersensitivity reactions, hyperlipidaemia, abnormal lipoprotein patterns, aggregation of erythrocytes and peripheral neuropathy [17, 18, 20]. Cyclophosphamide has aqueous solubility but undergoes spontaneous hydrolysis when comes in contact with water [18, 19]. Basic or alkaline degradation and hydrolytic degradation is common problem for both the active ingredients. Nanostructured lipid carriers (NLCs) and Microemulsion with these drug combinations will lead to effective targeted therapy and reduced risk of resistance which is common in monotherapy [13].Therefore, the proposed combination is incorporated in Nanostructured Lipid Carriers (NLC) and Microemulsion considering the various advantages.

Nanostructured Lipid Carriers are second generation of lipid-based nanocarriers formed from mixture of solid and liquid lipids and have unstructured-matrix due to the different moieties of the constituents of NLCs. NLCs are composed of biocompatible solid lipid matrices and liquid lipid which have different chemical structure from the solid lipid. Besides, NLCs have the usual particle diameter ranging 10–1000nm.Nanostructured lipid carriers (NLC) are composed of solid lipid matrix which are incorporated with liquid lipids. The presence of liquid lipids with different fatty acid C-chains produces NLC with less organized crystalline structure and therefore provides better loading capacity for drug accommodation. Liquid lipids are better solubilizers of drugs than solid lipids.

These carriers are composed of physiological and biodegradable lipids exhibiting low systemic toxicity and low cytotoxicity [21-23].

NLCs were selected as carriers for the parenteral delivery of chemotherapeutic drugs because NLCs are an advanced and efficient carrier system in particular for lipophilic substances with various advantages of better physical stability, ease of preparation and scale-up, increased dispersability in an aqueous medium, high entrapment of lipophilic drugs, controlled particle size, extended release of the drug, improve benefit/risk ratio, limited side effects, longer drug circulation time, lower cytotoxicity, improving drug bioavailability, enhancing drug permeability and retention in tumour tissues. These nanocarriers possess the utility in delivery of hydrophilic as well as lipophilic drugs. NLCs have emerged as a promising carrier system for the delivery of pharmaceuticals via oral, parenteral, ocular, pulmonary, topical, and transdermal route [21, 24, 25].

Microemulsions are isotropic, thermodynamically stable transparent (or translucent) systems of oil, water, and surfactant,frequently in combination with a cosurfactant with a droplet size usually in the range of 20-200 nm [26, 27]. These homogeneous systems, which can be prepared over a wide range of surfactant concentration and oil to water ratio, are all fluids of low viscosity. Microemulsions as drug delivery tool show favourable properties like thermodynamic stability (long shelf-life),easy formation (zero interfacial tension and almost spontaneous formation), optical isotropy, ability to be sterilized by filtration, high surface area (high solubilisation capacity) and very small droplet size [28-30].

The formulation of lipophilic and hydrophobic drugs into parenteral dosage forms has proven to be difficult. O/W Microemulsions are beneficial in the parenteral delivery of sparingly soluble drugs where the administration of suspension is not desirable. They provide a means of obtaining relatively high concentration of these drugs which usually requires frequent administration. Other advantages are that they exhibit a higher physical stability in plasma than liposomes or other vesicles and the internal oil phase is more resistant against drug leaching [26, 27].

2. AIM

The present research focuses on the development of nanostructured lipid carriers and microemulsion for the simultaneous delivery of Paclitaxel and Cyclophosphamide for the treatment of Breast Cancer. Thus, development of Paclitaxel-Cyclophosphamide dual drug NLCs and microemulsion may provide a new attractive treatment option with following advantages: (i) Enhanced efficacy (ii) Improved target selectivity (iii) Superior solubility (iv) Improved stability.

3. OBJECTIVES

The objectives of present work were:

- To formulate novel nanocarriers (NLCs and Microemulsion) for delivery of selected anti-cancer drugs.
- To optimize composition and process parameters of the nanocarriers formulation and to characterize the physico-chemical properties.
- To carry out in vitro studies and in vivo studies of drug loaded nanocarriers.
- To assess the stability of drug loaded nanocarriers.

4. PLAN OF WORK

a) Literature review
b) Procurement of excipients and therapeutic moieties
c) Preformulation studies
d) Formulation, optimization and characterization of drug-loaded carriers
e) In-vitro and In-vivo evaluation of nano-carriers
f) Stability studies of formulated nano-carriers

5. GENERAL METHODS & ANALYTICAL TECHNIQUES

5.1. Description

NLC and Microemulsion sample was observed in white light at visual inspection booth against black and white background [31].

5.2. Melting Point determination

Melting point of Paclitaxel and Cyclophosphamide was determined by capillary glass method [32].

5.3. FTIR Spectroscopy

IR-spectrum of drug was measured in the solid state by preparing a Potassium Bromide (KBr) pellet. The pure drug was previously ground individually and mixed thoroughly with KBr separately, an infrared transparent matrix at 1:100 (sample KBr) ratio. The pellets were then scanned over a wavelength range of 4000-400 cm-1 and a spectrum was obtained by using a FTIR spectrometer-430 (Shimadzu, Japan) [33].

5.4. Determination of Wavelength Maxima

Fixed amount of API was dissolved in methanol and UV scan of solution was executed against the methanol as blank in UV visible spectrophotometer in between the wavelengths of 200 to 400nm [34].

5.5. Particle size distribution

Malvern Zetasizer (Nano ZS) was used to determine the particle size distribution of NLCs at 25°C with equilibrium time of 60 seconds. [35-38]

5.6. Globule size distribution

Malvern Zetasizer (Nano ZS) was used to determine the globule size distribution of microemulsion at 25°C with equilibrium time of 60 seconds. [35-38]

5.7. HPLC Method for quantification of Paclitaxel

Aliquots were taken from working standard solution of Paclitaxel and diluted up to 10mL with methanol to give a final concentration of 0.1µg/mL, 0.5µg/mL, 1.0µg/mL, 10.0µg/mL, 20.0µg/mL, 50.0µg/mL, and 100.0µg/mL. 100 µL of each sample was injected in sampler loop of HPLC for each concentration, and chromatogram was taken under the condition mentioned in Table 1. The

calibration graph was constructed by plotting peak area against concentration of Paclitaxel and the regression equation was calculated [39].

Table 1: HPLC Parameters for Paclitaxel

Sr. No.	Parameter	Value
1	HPLC	Shimadzu, Prominence-I LC-2030 plus
2	Column	250 mm × 4.6 mm × 5 µm
3	Wavelength	227nm
4	Flow rate	1.5mL/minute
5	Run time	15 minutes
6	Injection volume	100µL
7	Mobile Phase	Water: Acetonitrile (11:9)

A calibration plot in the range of 0.1µg/mL to 100.0µg/mL was obtained. It was observed that the correlation coefficient for the system was >0.95, suggesting a linear relationship between peak area and concentration of Paclitaxel. The retention period was in between 9.5 and 10.5 minutes. The method was adopted from the monograph of Paclitaxel Injection given in USP 43 NF 38.

5.8. HPLC Method for quantification of Cyclophosphamide

Aliquots were taken from working standard solution of Cyclophosphamide and diluted up to 10mL with methanol to give a final concentration of 0.5µg/mL, 1.0µg/mL, 2.5µg/mL, 5.0µg/mL, 10.0µg/mL, 20.0µg/mL, 50.0µg/mL, and 100.0µg/mL. 100 µL of each sample was injected in sampler loop of HPLC for each concentration, and chromatogram was taken under the condition mentioned in Table 2. The calibration graph was constructed by plotting peak area against concentration of Cyclophosphamide and the regression equation was calculated [40].

Table 2: HPLC Parameters for Cyclophosphamide

Sr. No.	Parameter	Value
1	HPLC	Shimadzu, Prominence-I LC-2030 plus
2	Column	250 mm × 4.6 mm × 5 µm
3	Wavelength	195nm
4	Flow rate	1.5mL/minute
5	Run time	15 minutes
6	Injection volume	100µL
7	Mobile Phase	Water: Acetonitrile (30:70)

A Cyclophosphamide calibration plot in the range of 0.5ppm to 100.0ppm was obtained. It was observed that the correlation coefficient for the system was > 0.95, suggesting the presence of a linear relationship between peak area and concentration of Cyclophosphamide. The retention period was in between 6.0 and 7.0 minutes.

5.9. % Entrapment efficiency

The desired amount of nanostructured lipid carriers/ microemulsion was diluted with 1.5mL of phosphate buffer solution (PBS) with pH 7.4 containing 2 % Tween 80. They were then vortexed for 5 minutes to dissolve the free drug. The dispersion was centrifuged for 30 min at 15 000 rpm (Centrifuge model 2-16P, Sigma, Germany). The supernatant samples were suitably diluted and the amount of the free drug in the dispersion medium was estimated by HPLC. The entrapment efficiency was calculated using following equation:

$$\% \text{ Entrapment efficiency} = \frac{W_{total} - W_{free}}{W_{total}} \times 100$$

Here, the W_{total} is the weight of total drug added and W_{free} is the free drug. [41, 42]

5.10. % Filterability

% filterability test is to monitor flow decay and gradual pore plugging caused by the product and to determine the maximum volume of product that can be filtered through a 0.2µ filter. Fixed volume of NLCs/ microemulsion was filtered through 0.2µm PES filter and the % volume filtered was recorded. [43]

5.11. Morphology by Transmission Electron Microscopy

The shape of the optimized NLCs and Microemulsion was examined by JEM-2100 TEM at Sophisticated Analytical Instrument Facility, North Eastern Hill University, Shillong. One drop of each sample was applied to a carbon film covered copper grid (200-mesh). Five minutes later, a filter paper placed at the edge of the copper grid was used to remove any excess liquid present. Samples were air-dried in room temperature and then examined at 200 kV power. [45]

5.12. Viscosity

Viscosity of Microemulsion was measured using Anton Paar Rheometer, Model: MCR 102 in rotational mode with spindle plate of Parallel plate (PP50), rotational speed of 50 RPM and gap of

0.1mm. 0.5 to 1.0mL of Microemulsion sample was placed on cleaned and dry plate of Rheometer, spindle was lowered down and excess sample was trimmed down and measurement was started. [46]

5.13. Zeta Potential

Malvern Zetasizer (Nano ZS) was used to determine the particle zeta potential of Paclitaxel NLCs at 25°C with equilibrium time of 60 seconds. Purified water was used as medium for dispersion. [35-38]

5.14. Dilution test

The dilution test was performed for formulated microemulsion to confirm the stability of microemulsion upon dilution and type of microemulsion. Microemulsion was diluted with water at 1:10, 1: 100, and 1: 250 ratios. Clarity of solution upon dilution was evaluated. If water was easily dispersed in the continuous phase, the microemulsion was defined as oil-in-water microemulsion and if microemulsion becomes turbid upon dilution, it was defined as bicontinuous. [46, 47]

5.15. Redispersibility of NLCs

To evaluate the Redispersibility of lyophilized NLCs, fixed volume of water for injection was added to lyophilized NLCs and further shaken vigorously to redisperse the lyophilized NLCs. Redispersibility was evaluated in Sterile Water for Injection and phosphate buffer pH 7.4. [48]

5.16. Differential Scanning Calorimetry

Paclitaxel, Cyclophosphamide, Physical mixture of Paclitaxel and Cyclophosphamide and Lyophilized NLCs were weighed directly into DSC aluminium pan and scanned in the temperature range of 25–300 °C under an atmosphere of dry nitrogen. Heating rate of 10°C/min was used and thermograms obtained were observed. [49-51]

5.17. In-Vitro release studies

The in vitro release of Paclitaxel & Cyclophosphamide loaded nanocarriers was evaluated by the dialysis method. The dialysis membrane was soaked for 24 hours before the experiment in the release medium (phosphate buffer, pH 7.4, and containing Tween-80). Accurately measured nanocarriers were placed in the dialysis membrane, which was thoroughly tied to prevent leakage of the drug. The dialysis membrane was put in a beaker containing fixed volume of the release medium under continuous stirring at 200 RPM and the temperature of release medium was maintained as 37.5±2.0°C. At predetermined time intervals, a fixed volume of release medium samples were

withdrawn and replaced by the same volume of fresh release media. The samples were analyzed immediately using HPLC method for quantification of Paclitaxel and Cyclophosphamide. [52, 53]

5.18. Sterility

Membrane Filtration Method was used to determine the sterility of optimized NLCs and Microemulsion as it is a regulatory method of choice for filterable pharmaceutical products. The microemulsion/ reconstituted NLCs were passed through the membrane filter of pore size 0.45µm under aseptic conditions. Once the filtration is complete, filter was added to the culture media and kept for 14 days incubation. Two types of growth media i.e. Fluid Thioglycolate Medium (FTM) and Soybean casein digest medium (SCDM) were used in tests. Sterile water for injection was used as negative controlled whereas growth media exposed to atmospheric conditions was used as positive control.

6. DETERMINATION OF SYNERGISM

Paclitaxel and Cyclophosphamide are widely used for treatment of Metastatic breast cancer. However no literature exists which proves the synergism between Paclitaxel and Cyclophosphamide. Therefore, there arises a need to confirm the synergism between the two drugs.Literature review suggests that200mg/m^2dose of Paclitaxel and 1750mg/m^2dose of Cyclophosphamide is suitable for the treatment of breast cancer. Therefore, ratio of Paclitaxel to Cyclophosphamide as1: 8.75 was considered for the determination synergism [57].

The Response Additivity approach was adapted for the determination of synergism between Paclitaxel and Cyclophosphamide. The MTT assay was performed for Paclitaxel, Cyclophosphamide alone and Paclitaxel + Cyclophosphamide combination (1: 8.75) on MCF-7 cell line to determine the % viability and IC_{50} for all the three components. Based on the results obtained, the Response Additivity graph of Paclitaxel and Cyclophosphamide alone versus Paclitaxel and Cyclophosphamide combination was derived and is presented in Figure 1. The presented graph clearly depicts that Paclitaxel and Cyclophosphamide combination exhibits synergism and therefore the combination is suitable for being loaded in the novel carriers [58-60].

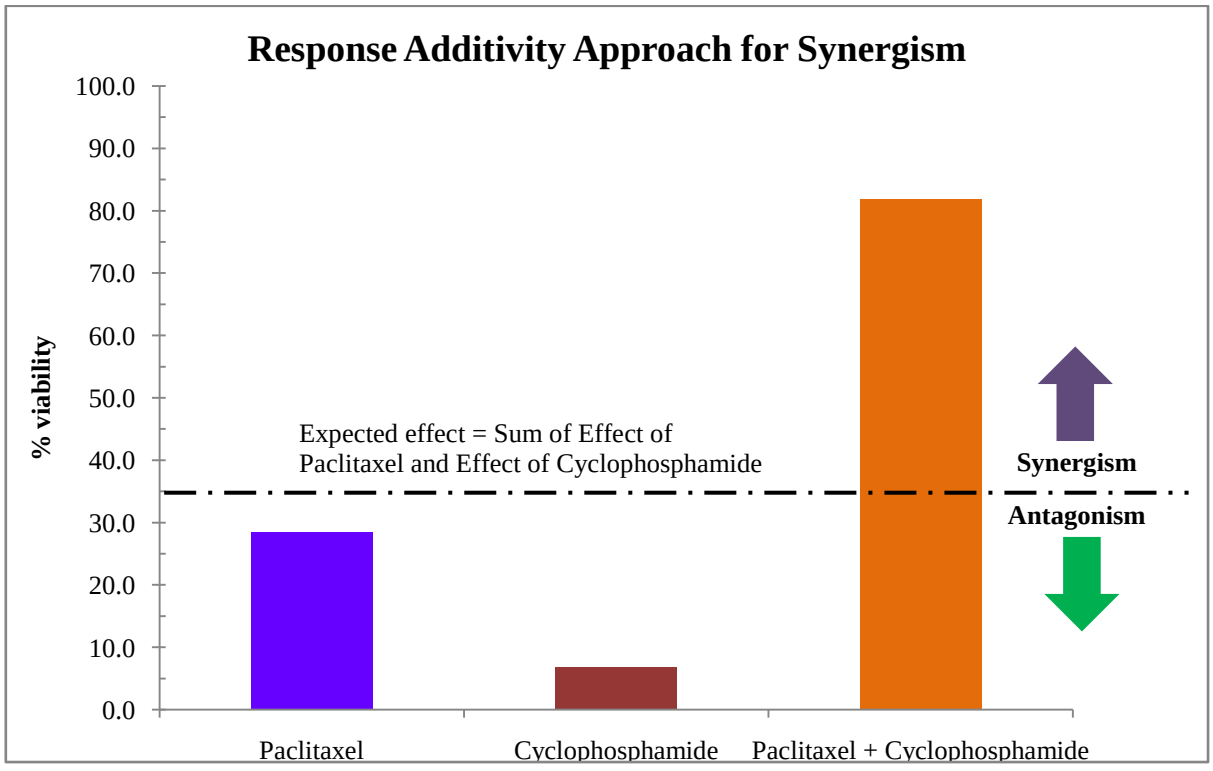


Figure 1: Response Additivity graph of Paclitaxel and Cyclophosphamide alone versus Paclitaxel and Cyclophosphamide combination (1: 8.75)

7. PREFORMULATION STUDIES

7.1. Authentication of Paclitaxel

Authentication of Paclitaxel was done by Melting Point determination, FTIR and by determining maximum wavelength by UV visible spectrophotometer.

7.1.1. Melting Point Determination of Paclitaxel

The melting point of Paclitaxel was found as 215-217°C with decomposition which is in the reported range of 213-220°C with decomposition.

7.1.2. FTIR Spectroscopy of Paclitaxel

The FTIR scan of Paclitaxel API was found to match with the IR scan of reference standard with reference peaks identical with the Paclitaxel standard. The FTIR spectrum of Paclitaxel API is presented in Figure 2.

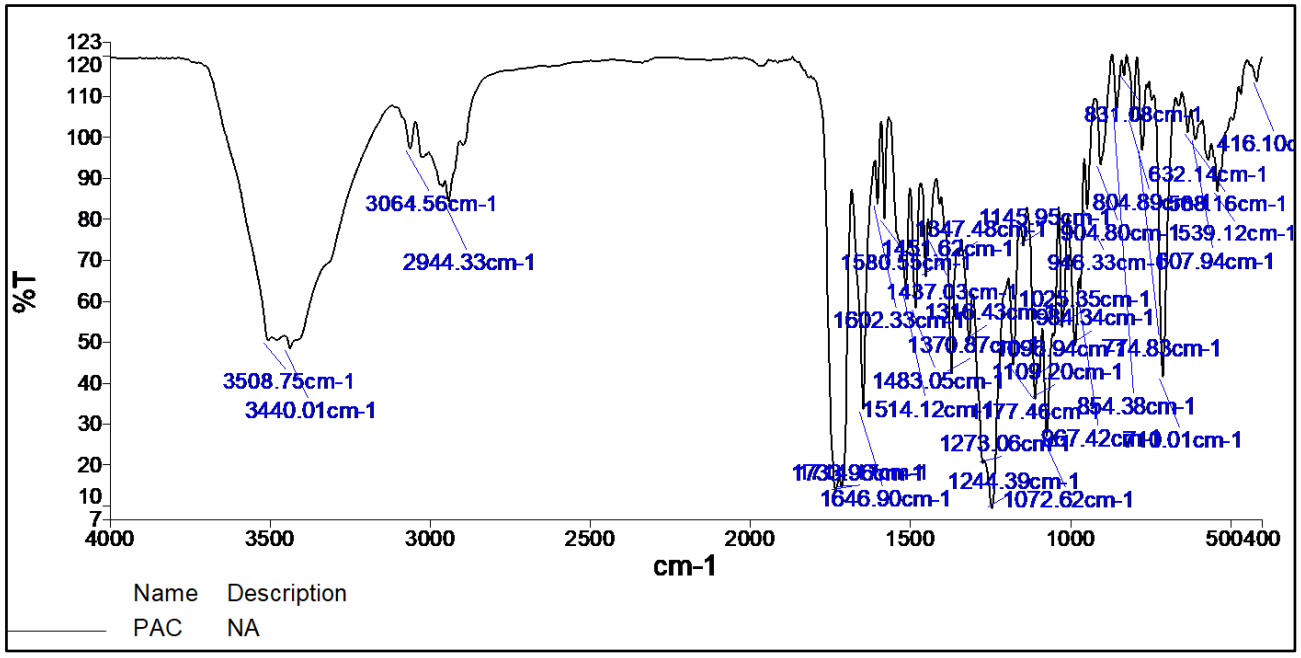


Figure 2: FTIR Spectrum of Paclitaxel

7.1.3. Determination of wavelength maxima of Paclitaxel

The wavelength maximum of Paclitaxel was obtained as 227nm which is in line with the reported value.

7.2. Authentication of Cyclophosphamide

Authentication of Cyclophosphamide was done by Melting Point determination, FTIR and by determining maximum wavelength by UV visible spectrophotometer.

7.2.1. Melting Point Determination

The melting point of Cyclophosphamide was found as 45°C which is in the reported range of 43-45°C with decomposition.

7.2.2. FTIR Spectroscopy of Cyclophosphamide

The FTIR scan of Cyclophosphamide API was found to match with the IR scan of reference standard with reference peaks identical with the Cyclophosphamide standard. The FTIR spectrum of Cyclophosphamide API is presented in Figure 3.

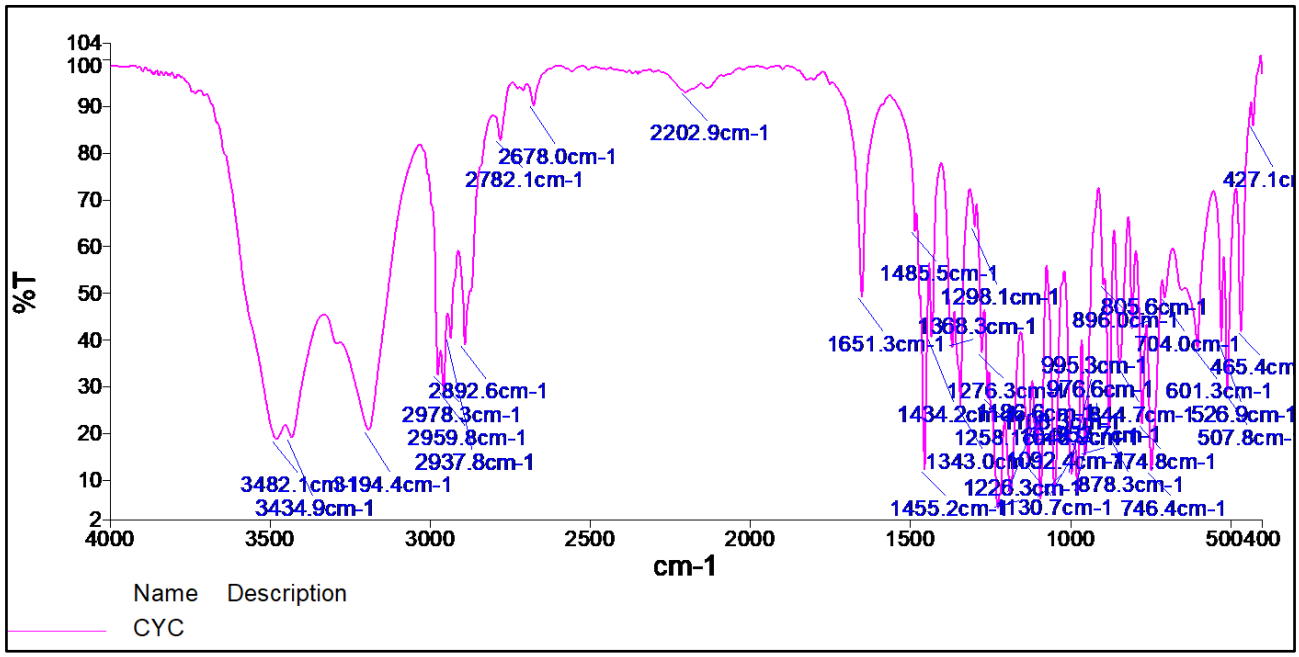


Figure 3: FTIR Spectrum of Cyclophosphamide

7.2.3. Determination of wavelength maxima of Cyclophosphamide

The wavelength maximum of Cyclophosphamide was obtained as 218nm which is in line with the reported value.

7.3. Screening of Solid Lipids

In a 10 ml clear glass vial 2.0 g solid lipid was taken and melted at temperature 10 °C above its melting point on a hot plate cum magnetic stirrer. To this melted lipid, drug was added in small increments (5 mg) with constant stirring using a magnetic bead. After addition of each increment of drug, the mixture was stirred for 15 minutes to allow complete solubilisation of drug and equilibration. Loss of transparency indicated saturation solubility of drug in the lipid.[61-65]

Results &Discussion: The solubility of Paclitaxel was tested in various solid lipids and Paclitaxel was found to have highest solubility in Stearic acid and PEG-100 Stearate. Further the solubility of Cyclophosphamide was evaluated in Stearic acid and PEG-100 Stearate. Cyclophosphamide was found to have higher solubility in PEG-100 Stearate and therefore, PEG-100 Stearate was selected as solid lipid for the preparation of nanostructured lipid carriers.

7.4. Screening of Liquid Lipids/ Oils

2 ml of liquid lipid was taken in a clear glass vial and heated to 60°C. To this drug was added in small increments (5 mg). This was then subjected to stirring to ensure thorough mixing of drug in the liquid lipid. Loss of transparency indicated saturation solubility of drug in the lipid. The precipitation of drug indicated liquid lipid is not compatible with drug.[61-65]

Results & Discussion: The solubility of Paclitaxel was tested in various liquid lipids/ oils and Paclitaxel was found to have highest solubility in PEG-8 Caprylic/Capric Glycerides. As Paclitaxel was found to have highest solubility in PEG-8 Caprylic/Capric Glycerides,same liquid lipids/ oils were further evaluated to check the solubility of Cyclophosphamide. Cyclophosphamide was also found to have very good solubility in clove oil and PEG-8 Caprylic/Capric Glycerides. PEG-8 Caprylic/Capric Glycerides was selected as liquid lipid for the manufacturing of nanostructured lipid carriers and microemulsion.

7.5. Screening of Co-surfactants

2 ml of co-surfactant was taken in a clear glass vial and heated to 60°C. To this, drug was added in small increments (5 mg). This was then subjected to stirring to ensure thorough mixing of drug in the co-surfactant. Loss of transparency indicated saturation solubility of drug in the co-surfactant. The precipitation of drug indicated co-surfactant is not compatible with drug.[61-65]

Results & Discussion: Paclitaxel was found to have highest solubility in Propylene Glycol and PEG-400. Hence, same co-surfactants were further evaluated to check the solubility of Cyclophosphamide. Cyclophosphamide was also found to have very good solubility in Propylene Glycol and PEG-400.

7.6. Solid Lipid-Liquid Lipid Compatibility Studies

Solid Lipid-Liquid Lipid compatibility was evaluated by solidification test. 1.0 g of solid lipid and 1.0 g of liquid lipid was taken in clear glass vial and heated at temperature 10°C above the melting

point of solid lipid on a hot plate cum magnetic stirrer and mixed using magnetic bead. After sufficient mixing, the magnetic bead was removed and the solid lipid-liquid lipid mixture was allowed to cool to room temperature (25±5°C). After cooling, mixture was observed for any droplets of oil on the surface as well as a small piece of mixture was smeared on a filter paper and observed for any oil spots on the filter paper. Similarly solidification test was performed for the mixture of 1.0 g of solid lipid and 3.0 g of liquid lipid. [61]

Results & Discussion: PEG-100 Stearate and clove oil mixture was found to be compatible at 1:1 ratio with no droplets on surface as well as the filter paper.However, the compatibility was not established at ratio of 1:3 where droplets of olive oil were observed on the surface of solidified mixture as well as on the filter paper.PEG-100 Stearate and PEG-8 Caprylic/Capric Glycerides mixtures were observed to be compatible with each other at 1:1 as well as1:3 ratio with no droplets on the surface of solidified mixture and as well as on the filter paper. Hence, mixture of PEG-100 Stearate and PEG-8 Caprylic/Capric Glycerides was selected for formulation of nanostructured lipid carriers.

7.7. Selection of co-solvent

Acetone is well accepted solvent due to its lower boiling point (56°C) leading to faster evaporation during compounding for NLCs and Microemulsion. Paclitaxel and Cyclophosphamide have very good solubility in acetone making it a preferred co-solvent for the formulation of NLCs and microemulsion. [66, 67]

7.8. Drug Excipient Compatibility Studies

Isothermal stress testing method was used to assess the compatibility of drug-drug/drug-excipient. Briefly, a fixed amount of pure drugs and excipients were weighed separately and in combination. Individual drugs and drug-drug/drug-excipient combinations were transferred in to an appropriately labelled glass vial. Each vial was sealed properly and placed in stability chamber at 40±2°C/75±5%RH for 4 weeks. To identify the physical instability, organoleptic parameters of samples such as colour and texture were observed initially and at the end of 4^{th} week. To identify the chemical instability, samples were divided into two parts at the end of 4^{th} week. First part of samples was used to record the Fourier-Transform Infrared (FT-IR) spectrum using FT-IR Spectrometer. Disappearance of absorption bands or reduction of the band intensity combined with the appearance of new bands give a clear evidence for interactions. The second part of samples were separately mixed with fixed quantity of methanol and sonicated for 5 minutes followed by filtration through

0.22 µm membrane and analysed using the developed High Performance Liquid Chromatography (HPLC) methods. [68, 69]

Results & Discussion: All the mixtures were found to be physically stable with no change in their description. Similarly, FTIR scan of drug alone versus the drug-excipient mixtures showed no change in the spectral peaks. The assay of drug alone versus the drug-excipient mixtures was found to be within the range of 98.0 to 102.0% as analyzed by HPLC. Thus it was concluded that all the selected excipients were compatible with Paclitaxel and Cyclophosphamide.

8. FORMULATION DEVELOPMENT, OPTIMIZATION, & EVALUATION

8.1. NANOSTRUCTURED LIPID CARRIERS

8.1.1. Selection of Method for NLCs manufacturing

Lipid phase and aqueous phase were prepared separately at optimized elevated temperature, Lipids and API were dissolved in solvent at elevated temperature, and further aqueous phase was added to lipid phase at elevated temperature under continuous stirring to evaporate the solvent and further cooled at 5 ± 3°C under continuous stirring to form NLCs [70-72].

As a part of formulation development studies, process parameters and formulation parameters were optimized using Monothetic analysis technique.

8.1.2. Optimization of Formulation Parameters

[A] Selection of Surfactants

Various surfactants which can be used for i.v. route viz. Polysorbate 20, Polysorbate 80, Poloxamer 188, Soluplus, and Cremophor EL were tried for preparation of NLCs as per Table 3.

Table 3: Surfactants, type, HLB value, and maximum allowable concentration

Sr. No.	Surfactant	Type of Surfactant	HLB Value	Maximum allowable Concentration
1	Polysorbate 20	Hydrophilic	16.7	1.00 %
2	Polysorbate 80	Hydrophilic	15	69.33 %
3	Poloxamer 188	Hydrophilic	29	2.0 %
4	Soluplus	Amphiphilic	14	75.0 %
5	Cremophor ELP	Amphiphilic	13	65.0 %

Results and Conclusion: The results of impact of surfactants are presented in Table 4.

Table 4: Results of optimization of surfactant concentration

Sr. No.	Surfactant Concentration (%)					Z-Average (nm)	PDI	% Entrapment efficiency		% Filterability
	Polysorbate 20	Polysorbate 80	Poloxamer 188	Soluplus	Cremophor ELP			PAC	CYC	
1	1					427.5 ± 66.1	0.400 ± 0.020	76.5 ± 1.2	60.0 ± 2.1	70.0 ± 5.0
2		1				509.7 ± 19.4	0.511 ± 0.082	72.4 ± 2.0	63.1 ± 1.5	65.0 ± 3.0
3			1			468.1 ± 25.7	0.445 ± 0.036	49.2 ± 0.6	65.5 ± 0.9	50.0 ± 6.0
4				1		177.7 ± 36.2	0.187 ± 0.010	97.0 ± 0.2	97.5 ± 0.3	95.0 ± 4.0
5					1	250.6 ± 11.8	0.200 ± 0.015	95.5 ± 0.3	95.4 ± 0.4	95.0 ± 3.0
6				1	1	158.2 ± 10.1	0.116 ± 0.010	98.9 ± 0.2	96.5 ± 0.3	100.0 ± 0.0
7				1	2	142.7 ± 14.5	0.123 ± 0.010	99.0 ± 0.5	99.6 ± 0.3	100.0 ± 0.0
8				1	4	120.2 ± 4.2	0.138 ± 0.009	100.1 ± 0.7	99.7 ± 0.7	100.0 ± 0.0

Conclusion: Polysorbate 20 has the lowest maximum allowable concentration of 1% and therefore experiments to manufacture NLCs for the selection of surfactants were performed with 1.0 % concentration for all the surfactants. Based on the experiments conducted using single surfactant, NLCs manufactured using Soluplus as surfactant was observed to have lowest Z-average, highest % entrapment efficiency, and % filterability followed by NLCs manufactured using Cremophor ELP. Soluplus turns sticky when added in lipid phase; it turns sticky whereas it has a good solubility in aqueous medium. Cremophor ELP is in liquid state and enhances the solubility of Paclitaxel and Cyclophosphamide in lipid phase. Both surfactants are amphiphilic in nature with HLB value 13 and 14 which is ideal for the production of stable lipid based formulations. Therefore Soluplus and Cremophor ELP were used in combination for the development of NLCs.

[B] Total Lipid Concentration

Effect of total lipid content on Particle Size, PDI, %Entrapment Efficiency, and rate of filtration was investigated by varying the total lipid concentration and the results are presented in Table 5.

[C] Solid Lipid: Liquid Lipid Concentration

Effect of ratio of solid: liquid lipid was investigated by preparing batches of varying amount of Solid Lipid and Liquid Lipid. Their effect on Particle Size, PDI, % entrapment efficiency and rate of filtration was evaluated and the results are presented in Table 5.

[D] Drug Substance Concentration

Effect of drug amount was investigated by preparing batches of varying amount of drug. Effect of amount of drug on Particle Size, PDI, Entrapment Efficiency, stability, and filtration efficiency of NLCs was evaluated and the results are presented in Table 5.

Results and Discussion: The results of optimization of total lipid concentration, solid lipid: liquid lipid concentration, drug substance concentration is presented in table 5:

Table 5: Results of optimization of total lipid concentration, solid lipid: liquid lipid concentration, drug substance concentration

Sr. No.	Total Lipid Conc. (%)	Solid lipid: liquid lipid Ratio	Drug substance concentration (mg/mL)		Z-Average	PDI	% Entrapment efficiency		% Filterability
			PAC	CYC			PAC	CYC	
1	**2**	1:1	1	8.75	114.2 ± 3.3	0.147 ± 0.010	99.2 ± 0.6	99.9 ± 0.7	100.00 ± 0.00
2	**4**	1:1	1	8.75	147.7 ± 12.0	0.175 ± 0.013	98.9 ± 0.6	97.9 ± 0.7	83.33 ± 3.06
3	**6**	1:1	1	8.75	267.4 ± 35.2	0.318 ± 0.019	99.1 ± 0.5	98.2 ± 0.6	76.67 ± 6.11
4	2	**1:2**	1	8.75	164.2 ± 11.9	0.208 ± 0.022	100.1 ± 0.4	99.1 ± 0.7	82.50 ± 1.90
5	2	**1:3**	1	8.75	197.8 ± 9.1	0.212 ± 0.057	100.0 ± 0.2	98.5 ± 0.4	60.14 ± 6.65
6	2	1:1	**2**	**17.5**	153.5 ± 8.2	0.179 ± 0.018	88.3 ± 0.6	89.0 ± 1.1	100.00 ± 0.00
7	2	1:1	**3**	**26.25**	279.0 ± 25.0	0.328 ± 0.017	67.5 ± 2.3	63.5 ± 2.6	88.67 ± 2.31
8	2	1:1	1	8.75	120.2 ± 4.2	0.138 ± 0.009	99.1 ± 0.7	98.7 ± 0.7	100.00 ± 0.00
9	2	1:1	1	8.75	200.4 ± 3.6	0.305 ± 0.069	95.7 ± 1.2	90.6 ± 0.5	78.10 ± 4.00
10	2	1:1	1	8.75	258.2 ± 40.6	0.351 ± 0.046	91.3 ± 1.5	84.8 ± 1.0	50.17 ± 6.44

Based on the above results, total lipid concentration was finalized as 2%, solid lipid: liquid lipid ratio as 1:1, drug concentration for Paclitaxel as 1mg/mL and Cyclophosphamide as 8.75mg/mL.

8.1.3. Optimization of Process Parameters

[A]Mixing Speed

Different mixing speeds between 250, 500 and 1000 RPM been evaluated.

[B]Mixing Time

Different mixing time between 5 minutes, 15 minutes and 30 minutes were evaluated.

[C]Temperature for Lipid Phase

Different temperatures for heating of Lipid Phase between 50 ± 2°C, 70 ± 2°C and 90 ± 2°C were evaluated.

Results & Discussion: The results of mixing speed, mixing time, and temperature of liquid phase is presented in Table 6:

Table 6: Results of mixing speed, mixing time, and temperature of phases

Mixing Speed (RPM)	Mixing time (Minutes)	Phase Temperature	Z-Average (nm)	PDI	% Entrapment efficiency	
					PAC	CYC
250	15	70 ± 2°C	461.4 ± 17.0	0.243 ± 0.05	85.5 ± 0.2	92.8 ± 0.7
500	15	70 ± 2°C	152.1 ± 3.8	0.200 ± 0.01	99.6 ± 0.4	100.0 ± 0.2
1000	15	70 ± 2°C	176.2 ± 11.6	0.194 ± 0.02	94.1 ± 0.4	99.2 ± 0.8
500	**5**	70 ± 2°C	495.2 ± 15.9	0.336 ± 0.05	96.2 ± 0.1	98.1 ±
500	**30**	70 ± 2°C	173.1 ± 7.1	0.204 ± 0.02	98.4 ± 0.6	99.3 ± 0.1
500	15	**50 ± 2°C**	427.6 ± 22.7	0.390 ± 0.023	62.8 ± 1.3	75.1 ± 1.0
500	15	**90 ± 2°C**	217.1 ± 11.0	0.232 ± 0.010	100.1 ± 0.5	89.6 ± 1.1

The optimum mixing speed of 500 RPM for mixing time of 15 minutes and phase temperature of 70±2°C was observed to manufacture NLCs with lowest particle size distribution and highest % entrapment efficiency.

8.1.4. LYOPHILIZATION OF NLCs

Mannitol, Lactose Monohydrate and Dextrose Monohydrate were evaluated as cryoprotectants. The final Cryoprotectant was selected from a freeze thaw study where NLCs containing 4%w/v of each cryoprotectant were frozen at freezing rate of 0.1°C/minute and again brought to room temperature (25°C) at heating rate of 0.1°C/minute.

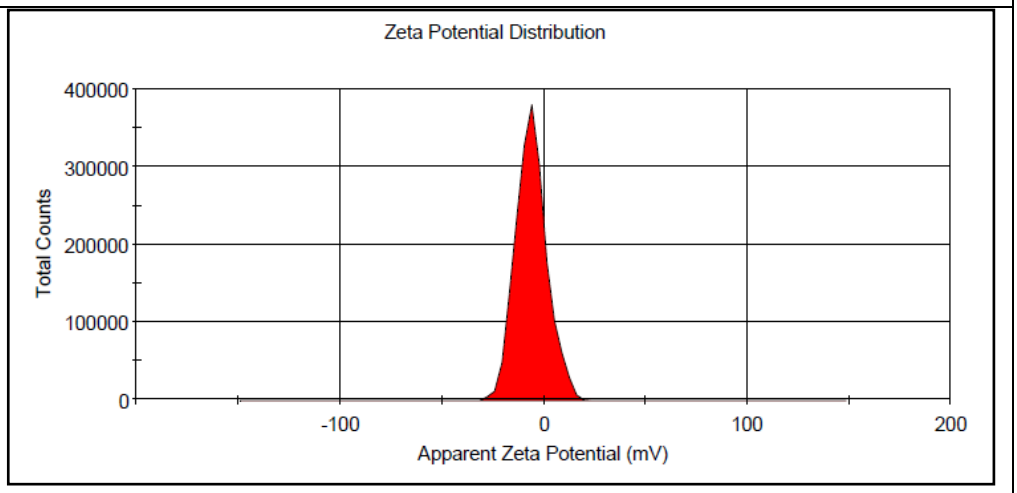
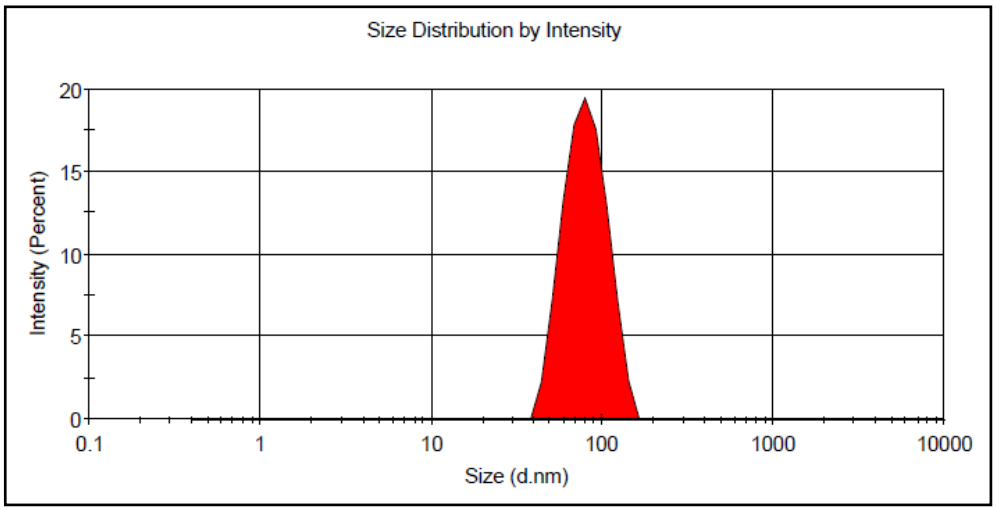
The selected cryoprotectant was studied at 3 different concentrations of 2%w/v, 4%w/v, and 6%w/v to optimize the concentration of selected cryoprotectant. SMART**™** cycle was executed using Lyostar III Lyophilizer of SP Scientific, USA. SMART™ Freeze-Dryer technology eliminates the trial-and-error approach normally involved in developing new lyophilisation cycles. Based on manometric temperature measurement equation and heat and mass transfer theory, pressure rise data is analyzed by an algorithm and converted to a number of critical process parameters. Lyophilization parameters were selected by using SMART™ Programme of Lyostar-3 Lyophilizer (Make: SP Scientific) [73-79].

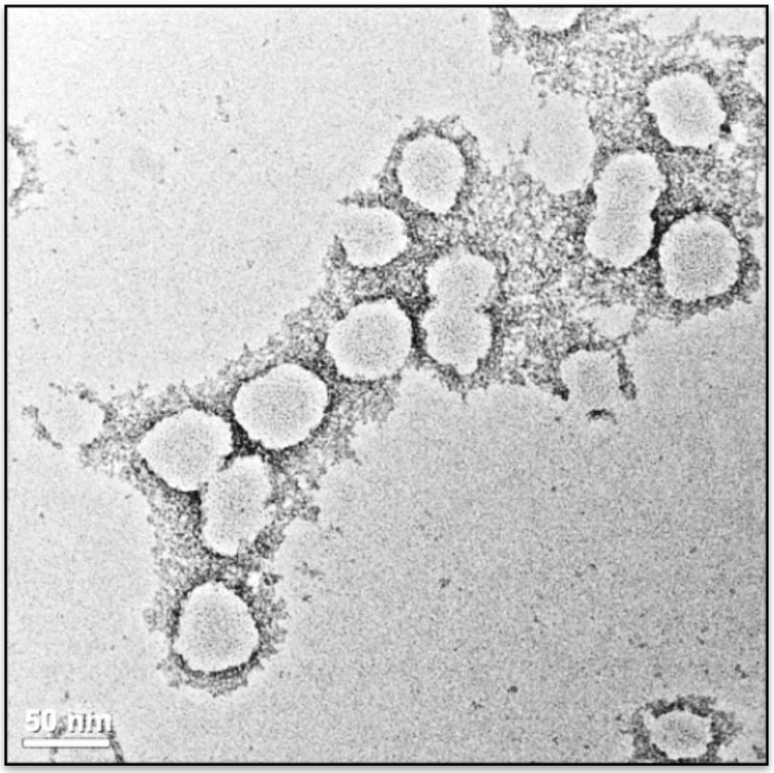
8.1.5. CHARACTERIZATION OF NLCS

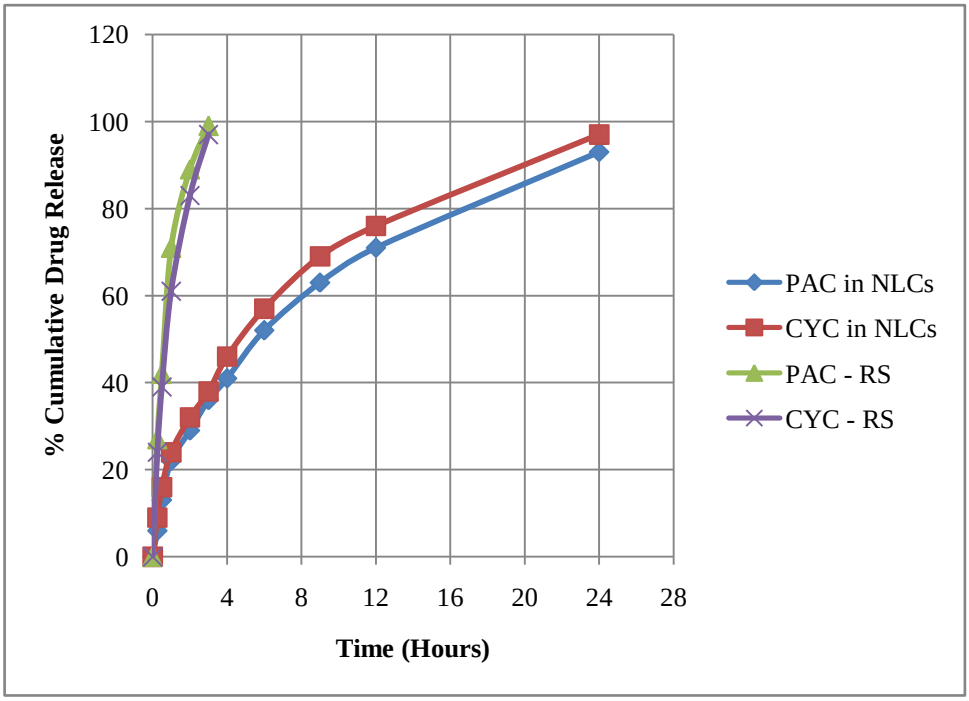
Paclitaxel and Cyclophosphamide loaded NLCs was characterized for morphology by TEM, Particle size distribution, Zeta potential, Redispersibility of NLCs, Differential Scanning Calorimetry, X-ray diffraction, and FTIR spectroscopy. The results and discussion are as presented in Table 7:

Table 7: Results and Discussion of characterization of NLCs

Sr. No.	Test Name	Results and Discussion
1	Redispersibility	NLCs were found to be readily redispersible in sterile water for injection and phosphate buffer pH 7.4. The time required to redisperse in sterile water for injection was 69 seconds and for phosphate buffer pH 7.4 was 74 seconds.
2	Zeta Potential	The zeta potential of the NLCs was observed to be -5.3 ± 2.7 mV. The graph of Zeta potential is show in Figure 4 revealed that there is no significant charge on the NLCs as the surfactants used are non-ionic in nature and therefore they do not impart any charge on the NLCs.

Sr. No.	Test Name	Results and Discussion
		 **Figure 4: Graph of Zeta Potential of NLCs**
3	Particle size distribution	The particle size distribution of the optimized NLCs revealed that the average Z-average was 74.3 ± 3.7nm with PDI of 0.072 ± 0.004. The graph of particle size distribution is presented in Figure 5 below:  **Figure 5: Graph of Particle size distribution of NLCs**
4	Morphology by TEM	The image obtained by TEM as shown in (Figure 7) revealed that the optimized NLCs were discrete and spherical with particles size (<100 nm), which was close to the results obtained by dynamic light scattering (DLS) method.

Sr. No.	Test Name	Results and Discussion
		 **Figure 6: TEM Image of Optimized NLCs**
5	Differential Scanning Calorimetry	Paclitaxel, Cyclophosphamide and Paclitaxel & Cyclophosphamide loaded NLCs were analyzed by DSC to investigate the crystalline nature of Paclitaxel and Cyclophosphamide. Thermogram of NLCs did not show the melting peak of Paclitaxel and Cyclophosphamide suggesting that paclitaxel and Cyclophosphamide were present in the NLCs either in amorphous form or molecularly dispersed.
6	FTIR	The paclitaxel-loaded SLNs were characterized by FTIR spectra for their surface chemistry. FTIR spectra of Paclitaxel, Cyclophosphamide, and Paclitaxel & Cyclophosphamide loaded NLCs were obtained. The IR-spectrum of NLCs showed absence of significant drug peaks which indicates efficient and complete drug encapsulation within lipid matrix.
7	In-vitro drug release study	In-vitro release studies were performed for Reference Standard of Paclitaxel, Reference Standard of Cyclophosphamide and Paclitaxel & Cyclophosphamide loaded NLCs. The graph of % cumulative release versus time in hours is as follows:

Sr. No.	Test Name	Results and Discussion
		 **Figure 7: Graph of % cumulative drug release vs. time in hours for Paclitaxel & Cyclophosphamide loaded NLCs, Paclitaxel Reference standard and Cyclophosphamide Reference standard**
8	Sterility	At the end of incubation period of 14 days, the Petri dish containing negative control, positive control and test sample (NLCs) were observed for any signs of microbial growth. No growth was observed in Negative control and test sample, whereas growth was observed for Positive control. Therefore the optimized NLCs passed the test for sterility.

8.2. MICROEMULSION

8.2.1. Selection of Method for Microemulsion

The Microemulsions containing drug combination of Paclitaxel and Cyclophosphamide were prepared by mixing of Oil Phase and Aqueous phase. Paclitaxel API was dissolved in oil phase containing liquid lipid, surfactant and co-surfactant at elevated temperature. Further the oil phase was cooled to room temperature and Cyclophosphamide API was dissolved under continuous stirring. Aqueous phase was added to oil phase under continuous stirring to form a microemulsion. This microemulsion was cooled at 5 ± 3°C under continuous stirring. Pseudo ternary phase diagrams were constructed to identify the oil-in-water microemulsion forming compositions. [35, 36, 80-82].

Based on the compatibility studies, Paclitaxel and Cyclophosphamide were compatible with Caprylic/Capric Glycerides, PEG-400, Cremophor ELP and Soluplus. Ternary phase diagram was constructed with levels of Caprylic/Capric Glycerides in the range of 4.0 to 8.0%, PEG-400 in the range of 1.0 to 4.0%, Cremophor ELP in the range of 1.0 to 8.0 % and Soluplus at 1.0%. The generated phase diagram is presented in Figure 8.

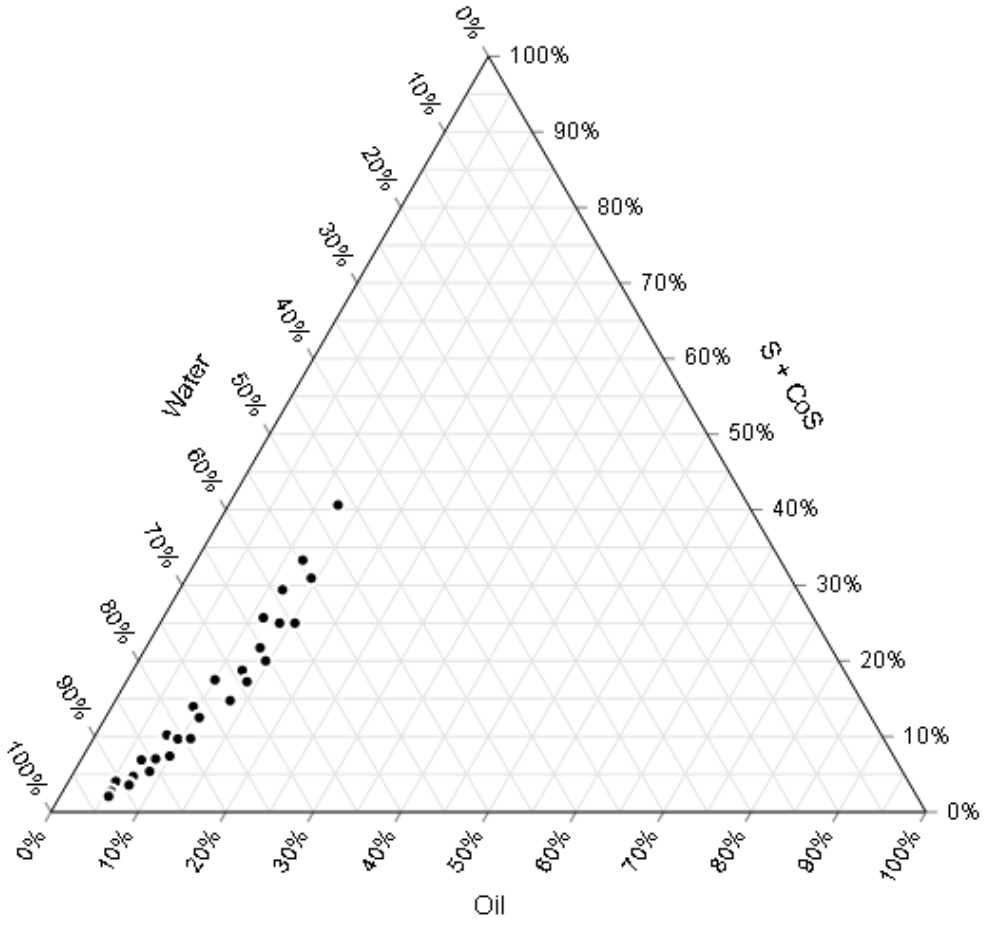


Figure 8: Pseudo ternary phase diagram for Microemulsion

8.2.2. Optimization of Drug Substance Concentration

The effect of amount of drug on Particle Size, PDI, Entrapment Efficiency, stability, and filtration efficiency of Microemulsions was evaluated. The results are presented in table 7.

Results & Discussion: The results of optimization of drug substance concentration are presented in table below:

Table 8: Results of optimization of drug substance concentration

Sr. No.	Drug substance Conc. (mg/mL)		Z-Average	PDI	% Entrapment efficiency		% Filterability
	PAC	CYC			PAC	CYC	
1	1.5	13.125	97.8 ± 4.1	0.092 ± 0.090	100.0 ± 1.2	100.5 ± 1.1	100.00 ± 0.00
2	3	26.25	88.5 ± 7.6	0.080 ± 0.005	99.1 ± 1.7	98.7 ± 1.7	100.00 ± 0.00
3	4.5	39.375	161.3 ± 2.1	0.252 ± 0.042	96.0 ± 1.2	97.5 ± 1.4	90.00 ± 5.00

Based on above results, Paclitaxel concentration as 3mg/mL and Cyclophosphamide as 26.25mg/mL was finalized.

8.2.3. Optimization of Process Parameters

[A]Mixing Speed

Different mixing speeds between 250, 500 and 1000 RPM were evaluated.

[B] Mixing Time

Different mixing time between 5 minutes, 15 minutes, and 30 minutes were evaluated.

Results & Discussion: The results of mixing speed, mixing time, and temperature of liquid phase is presented below:

Table 9: Results of mixing speed, mixing time, and temperature of both phases

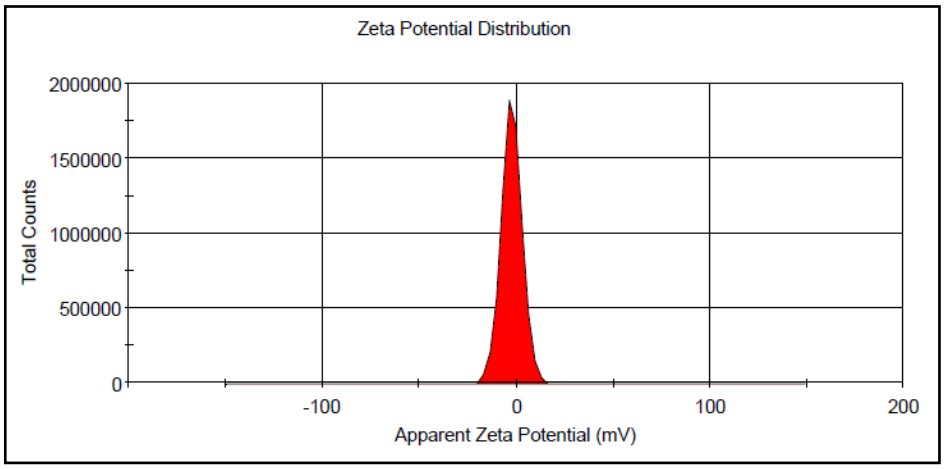
Mixing Speed (RPM)	Mixing time (minutes)	Z-Average	PDI	% Entrapment efficiency	
				PAC	CYC
250	15	151.4 ± 9.5	0.103 ± 0.05	89.2 ± 1.7	100.1 ± 1.3
500	15	92.1 ± 6.8	0.090 ± 0.07	99.7 ± 1.3	99.8 ± 1.1
1000	15	116.2 ± 10.6	0.115 ± 0.09	98.5 ± 1.5	97.9 ± 1.6
500	**5**	197.2 ± 15.9	0.336 ± 0.05	77.3 ± 1.4	96.4 ± 1.5
500	**30**	107.1 ± 11.0	0.052 ± 0.08	100.3 ± 1.2	95.0 ± 1.7

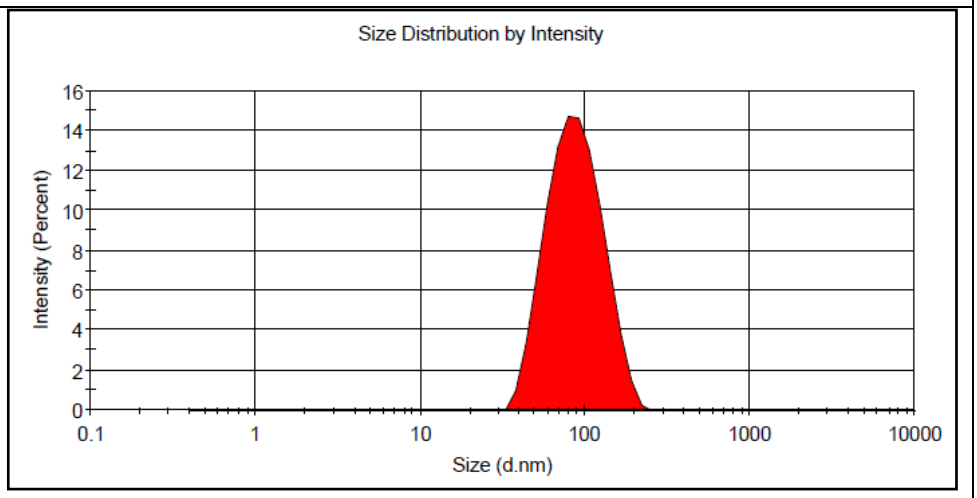
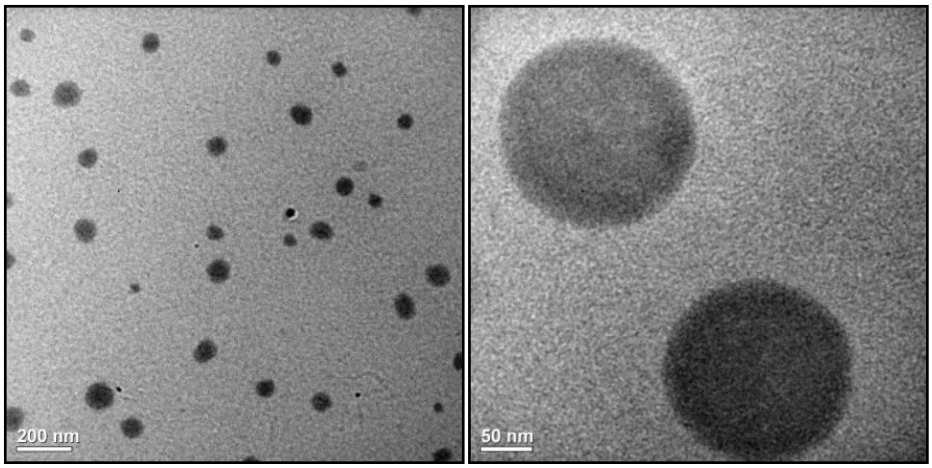
Based on above results, Stirring speed of 500 RPM and time of 15minutes is sufficient to produce NLCs with desirable particle size distribution and % entrapment efficiency.

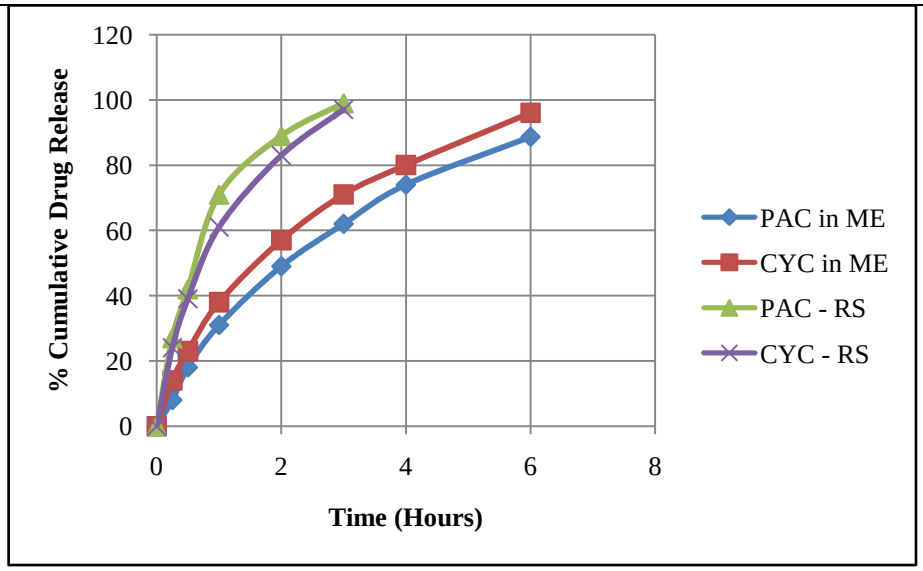
8.2.4. CHARACTERIZATION OF MICROEMULSION

Paclitaxel and Cyclophosphamide loaded Microemulsion was characterized for morphology by TEM, Globule size distribution, Zeta potential, Viscosity, Surface Tension, Dilution test. The results and discussion are as presented in Table 10:

Table 10: Results and Discussion of characterization of Microemulsion

Sr. No.	Test Name	Results and Discussion
1	Dilution test	Paclitaxel and Cyclophosphamide loaded microemulsion was easily diluted with water at 1:10, 1: 100 and 1: 250 ratios. Therefore the prepared microemulsion was oil-in-water type.
2	Viscosity	The viscosity of the optimized formulation was observed to be 1.09 ± 0.06 cps.
3	Zeta Potential	The zeta potential of the microemulsion was observed to be -2.6 ± 0.2 mV. The graph of Zeta potential is show in Figure 9.  **Figure 9: Graph of Zeta Potential of NLCs**
5	Globule size distribution	The particle size distribution of the optimized NLCs revealed that the average Z-average of optimized NLCs was obtained as 74.3 ± 3.7nm with PDI of 0.072 ± 0.004. The graph of particle size distribution is presented in Figure 10 below:

Sr. No.	Test Name	Results and Discussion
		 **Figure 10: Graph of Particle size distribution of Microemulsion**
6	Morphology by TEM	The images obtained by TEM shown in (Figure 11) revealed that the optimized microemulsion were discrete spherical oil nanocarriers. TEM images of optimized microemulsion showed distinct clear spherical droplet size (<100 nm), which was similar to the results obtained by dynamic light scattering (DLS) method.  **Figure 11: TEM Image of Optimized Microemulsion**
7	In-vitro drug release study	In-vitro release studies were performed for Reference Standard of Paclitaxel, Reference Standard of Cyclophosphamide and Paclitaxel & Cyclophosphamide loaded microemulsion. The graph of % cumulative release versus time in hours is as presented in Figure 12:

Sr. No.	Test Name	Results and Discussion
		 **Figure 12: Graph of % cumulative drug release vs. time in hours for Paclitaxel & Cyclophosphamide loaded microemulsion, Paclitaxel Reference standard and Cyclophosphamide Reference standard**
8	Sterility	At the end of incubation period of 14 days, the Petri dish containing negative control, positive control and test sample (microemulsion) were observed for any signs of microbial growth. No growth was observed in Negative control and test sample, whereas growth was observed for Positive control. Therefore the optimized microemulsion passed the test for sterility.

9. CELL LINE STUDIES

9.1. Cell viability assay

The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilised and quantified by spectrophotometric means. The assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability.

MCF-7 Cells cultured in T-25 flasks were trypsinized and aspirated into a 5mL centrifuge tube. Cell pellet was obtained by centrifugation at 300 x g. The cell count was adjusted, using DMEM-HG medium, such that 200µL of suspension contained approximately 10,000 cells. To each well of the 96 well microtitre plate, 200µL of the cell suspension was added and the plate was incubated at 37°C and 5% CO_2 atmosphere for 24 h. After 24 h, the spent medium was aspirated. 200µL of different test concentrations of test drugs were added to the respective wells. The plate was then incubated at 37°C and 5% CO_2 atmosphere for the specified time.

The plate was removed from the incubator and the drug containing media was aspirated. 200µL of medium containing10% MTT reagent was then added to each well to get a final concentration of 0.5mg/mL and the plate was incubated at 37°C and 5% CO_2 atmosphere for 3 h. The culture medium was removed completely without disturbing the crystals formed. Then 100µl of solubilisation solution (DMSO) was added and the plate was gently shaken in a gyratory shaker to solubilise the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 570 nm and also at 630 nm. The percentage growth inhibition was calculated, after subtracting the background and the blank, and concentration of test drug needed to inhibit cell growth by 50% (IC50) was generated from the dose-response curve for the cell line. [35, 83-86]

9.2. Cell Cycle Analysis by Flow Cytometry

MCF-7 human breast cancer cells were seeded into 6-well plates and incubated at 37°C for 24 hours. Cells were treated with the test compound, trypsinized, and taken into 15mL tubes. Cells were washed, fixed in chilled 70% Ethanol (-20^{o}C), washed again twice, resuspended in 400 µL PI-RNase solution per million cells and taken into 12x75mm tubes. Samples were mixed well and analysed by Cytomics FC500 Flow cytometer, Beckman Coulter, USA [87, 88].

9.3. Drug Uptake Assay and Apoptosis by Fluorescence Microscopy of Paclitaxel & Cyclophosphamide combination loaded NLCs and Microemulsion

Drug uptake assay and Apoptosis by fluorescence microscopy was performed for Paclitaxel & Cyclophosphamide combination loaded NLCs and Microemulsion. MCF-7 Human breast cancer cells were cultured in a 6-well plate at a density of 2 x 10^5 cells/2 ml and incubated in a CO_2 incubator overnight at 37°C for 24 hours. The spent medium was aspirated and washed with 1ml 1X PBS. The cells were treated with required concentration of FITC tagged NLCs/ Microemulsion in 2 ml of culture medium and then incubated for 24 hours as per the IC_{50} obtained in cell viability assay. The cells were washed two times with 1X PBS at the end of the treatment. 500µL of mounting medium was added before imaging. Cells were observed under 10X & 20X objectives of fluorescence microscope using filter cube with Excitation 470/40 and Emission 525/50 for FITC.Images were analysed using ImageJ Software v1.48.[89]

9.4. Drug uptake assay by Flow Cytometry of Paclitaxel & Cyclophosphamide combination loaded NLCs and Microemulsion

MCF-7 Human breast cancer cells were placed in a 6-well plate at a density of 3 x 10^5 cells/2 ml and incubated in a CO_2 incubator overnight at 37°C for 24 hours. The spent medium was aspirated and washed with 1ml 1X PBS. The cells were treated with required concentration of experimental test compound in 2 ml of culture medium and incubated the cells for 24 hours. One of the wells was left as untreated to be used as negative control. At the end of the treatment, the cells were washed two times with 1X PBS and harvested into 15ml centrifuge tubes and these tubes were centrifuged for five minutes at 300 x g at 25°C, further carefully aspirated the supernatant. The cells were resuspended in 0.5-1 ml of PBS and mixed well to ensure separation of individual cells. The cells were analyzed with a flow cytometer. Cells taking up FITC tagged NLCs/ Microemulsion display fluorescence with excitation and emission at 465 nm and 540 nm, respectively, and were measured in the FL1 channel (525nm) used to detect FITC [90].

10. STABILITY STUDIES

Stability is one of the critical aspects in ensuring safety and efficacy of drug products. In intravenously administered NLCs and microemulsion, for example, formation of larger particles could lead to capillary blockade and embolism so particle size and size distribution of Paclitaxel and Cyclophosphamide loaded NLCs and microemulsion needs to be closely monitored during storage. Comparative stability studies were carried out for the nanostructured lipid carriers and microemulsion at 25±2°C/ 60%±5%RH and at 2 to 8°C in accordance with ICH Q1B (R2). [91]

11. ONGOING WORK

- Stability studies
- In-vivo studies

12. REFERENCES

1. Wilkinson L, Gathani T. Understanding breast cancer as a global health concern. Br J Radiol. 2022 Feb 1; 95 1130):20211033. doi: 10.1259/bjr.20211033. Epub 2021 Dec 14. PMID: 34905391; PMCID: PMC8822551.
2. Arnold M, Morgan E, Rumgay H, Mafra A, Singh D, Laversanne M, Vignat J, Gralow JR, Cardoso F, Siesling S, Soerjomataram I. Current and future burden of breast cancer: Global statistics for 2020 and 2040. Breast. 2022 Dec; 66: 15-23. doi: 10.1016/j.breast.2022.08.010. Epub 2022 Sep 2. PMID: 36084384; PMCID: PMC9465273.
3. Stoltenberg, M., Spence, D., Daubman, B.-R., Greaves, N., Edwards, R., Bromfield, B., Perez-Cruz, P.E., Krakauer, E.L., Argentieri, M.A. and Shields, A.E. (2020), The central role of provider training in implementing resource-stratified guidelines for palliative care in low-income and middle-income countries: Lessons from the Jamaica Cancer Care and Research Institute in the Caribbean and Universidad Católica in Latin America. Cancer, 126: 2448-2457. https://doi.org/10.1002/cncr.32857
4. Shah, R. (2014). Pathogenesis, prevention, diagnosis, and treatment of breast cancer. World Journal of Clinical Oncology, 5(3), 283. doi:10.5306/wjco.v5.i3.283
5. Coleman, M. P., Et. Al. (2008). Cancer survival in five continents: a worldwide population-based study (CONCORD). The Lancet Oncology, 9(8), 730–756. doi:10.1016/s1470-2045(08)70179-7
6. Ataollahi M. R. Et. Al. (2015). Breast cancer and associated factors: a review. Journal of Medicine and Life, 8(4), 6 – 11. PMCID: PMC5319297, PMID: 28316699
7. Malvia, S., Bagadi, S. A., Dubey, U. S., &Saxena, S. (2017). Epidemiology of breast cancer in Indian women. Asia-Pacific Journal of Clinical Oncology, 13(4), 289–295. doi:10.1111/ajco.12661
8. Yardley, D. A. (2013). Drug Resistance and the Role of Combination Chemotherapy in Improving Patient Outcomes. International Journal of Breast Cancer, 2013, 1–15. doi:10.1155/2013/137414
9. Slamon, D. J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Norton, L. (2001). Use of Chemotherapy plus a Monoclonal Antibody against HER2 for Metastatic Breast Cancer That Overexpresses HER2. New England Journal of Medicine, 344(11), 783–792. doi:10.1056/nejm200103153441101
10. Wang X, Zhang H, Chen X. Drug resistance and combating drug resistance in cancer. Cancer Drug Resist. 2019;2(2):141-160. doi: 10.20517/cdr.2019.10. Epub 2019 Jun 19. PMID: 34322663; PMCID: PMC8315569.

11. Ranji, Peyman & Heydari, Zahra & Alizadeh, Ali Mohammad. (2015). Nanobiotechnological Approaches to Overcome Drug Resistance in Breast Cancer. Current cancer drug targets. 15. 10.2174/1568009615666150706102842.
12. Mansoori, B., Mohammadi, A., Davudian, S., Shirjang, S., & Baradaran, B. (2017). The Different Mechanisms of Cancer Drug Resistance: A Brief Review. Advanced Pharmaceutical Bulletin, 7(3), 339–348. doi:10.15171/apb.2017.041
13. H.Muley, R. Fadó, Rosalía R., Casals N.(2020). Drug uptake-based chemoresistance in breast cancer treatment,Biochemical Pharmacology,177,ISSN 0006-2952,https://doi.org/10.1016/j.bcp.2020.113959.
14. Yao Y, Zhou Y, Liu L, Xu Y, Chen Q, Wang Y, Wu S, Deng Y, Zhang J, Shao A. Nanoparticle-Based Drug Delivery in Cancer Therapy and Its Role in Overcoming Drug Resistance. Front Mol Biosci. 2020 Aug 20;7:193. Doi: 10.3389/fmolb.2020.00193. PMID: 32974385; PMCID: PMC7468194.
15. Laura M. Urquhart. Taxanes as a First-Line Systemic Treatment in Metastatic Breast Cancer. Breast Cancer Treatment, Metastasis, Targeted Therapies, CJON 2013, 17(1), 15-21. DOI: 10.1188/13.CJON.S1.15-21
16. Overmoyer B, Silverman P, Holder LW, Tripathy D, Henderson IC. Pegylated liposomal doxorubicin and cyclophosphamide as first-line therapy for patients with metastatic or recurrent breast cancer. Clin Breast Cancer. 2005 Jun;6(2):150-7. doi: 10.3816/CBC.2005.n.017. PMID: 16001993.
17. Prabhjot Kaur, Tarun Garg, Goutam Rath, R. S. Rayasa Murthy & Amit K. Goyal (2016) Development, optimization and evaluation of surfactant-based pulmonary nanolipid carrier system of paclitaxel for the management of drug resistance lung cancer using Box-Behnken design, Drug Delivery, 23:6, 1912-1925, DOI: 10.3109/10717544.2014.993486
18. Tońska M, Dołżonek J, Stepnowski P, Białk-Bielińska A. Hydrolytic stability of anticancer drugs and one metabolite in the aquatic environment. Environ Sci Pollut Res Int. 2021 Nov;28(41):57939-57951. doi: 10.1007/s11356-021-14360-0. Epub 2021 Jun 8. PMID: 34105071; PMCID: PMC8536627.
19. Scripture CD, Figg WD, Sparreboom A. Paclitaxel chemotherapy: from empiricism to a mechanism-based formulation strategy. Ther Clin Risk Manag. 2005 Jun;1(2):107-14. doi: 10.2147/tcrm.1.2.107.62910. PMID: 18360550; PMCID: PMC1661618.
20. Gelderblom H, Verweij J, Nooter K, Sparreboom A. Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation. Eur J Cancer. 2001 Sep;37(13):1590-8. doi: 10.1016/s0959-8049(01)00171-x. PMID: 11527683.

21. Parisa Ghasemiyeh and Soliman Mohammadi - Samani; Solid lipid nanoparticles and nanostructured lipid carriers as novel drug delivery systems: applications, advantages and disadvantages; Research in Pharmaceutical Sciences, August 2018; 13(4): 288-303
22. Sarabjotkaur, Ujjwal Nautyal*, Ramandeep Singh, Satvinder Singh, Anita Devi; Nanostructure Lipid Carrier (NLC): the new generation of lipid nanoparticles; Asian Pac. J. Health Sci., 2015; 2(2): 76-93
23. Chia-Lang Fang, Saleh A. Al-Suwayeh, and Jia-You Fang; Nanostructured Lipid Carriers (NLCs) for Drug Delivery and Targeting; Recent Patents on Nanotechnology, 2013, 7, 41-55
24. Neda Naseri, Hadi Valizadeh, Parvin Zakeri-Milani; Solid Lipid Nanoparticles and Nanostructured Lipid Carriers: Structure, Preparation and Application; Adv Pharm Bull, 2015, 5(3), 305-313 doi: 10.15171/apb.2015.043
25. Piyush Jaiswal , BinaGidwani& Amber Vyas; Nanostructured lipid carriers and their current application in targeted drug delivery; Artificial Cells, Nanomedicine, and Biotechnology, 2014; Early Online: 1–14
26. Talegaonkar S, Azeem A, Ahmad FJ, Khar RK, Pathan SA, Khan ZI. Microemulsions: a novel approach to enhanced drug delivery. Recent Patents on Drug Delivery and Formulation. 2008; 2(3):238–257.
27. Bagwe RP, Kanicky JR, Palla BJ, Patanjali PK, Shah DO. Improved drug delivery using microemulsions: rationale, recent progress, and new horizons. Critical Reviews in Therapeutic Drug Carrier Systems. 2001; 18(1):77–140.
28. Lawrence MJ, Rees GD. Microemulsion-based media as novel drug delivery systems. Advanced Drug Delivery Reviews. 2000; 45(1):89–121.
29. Callender SP, Mathews JA, Kobernyk K, Wettig SD. Microemulsion utility in pharmaceuticals: Implications for multi-drug delivery. Int J Pharm. 2017 Jun 30;526(1-2):425-442. doi: 10.1016/j.ijpharm.2017.05.005. Epub 2017 May 7. PMID: 28495500.
30. Dubey, R. (2013). Controlled-release injectable microemulsions: recent advances and potential opportunities. Expert Opinion on Drug Delivery, 11(2), 159–173. doi:10.1517/17425247.2014.870151
31. Yadav, Vishal; Jadhav, Prakash; Kanase, Kishor; Bodhe, Anjali; Dombe, Shailaja (2018). Preparation and Evaluation of Microemulsion containing Antihypertensive Drug. International Journal of Applied Pharmaceutics, 10(5), 138–146. doi:10.22159/ijap.2018v10i5.27415

32. YrjöRoos, Melting and glass transitions of low molecular weight carbohydrates, Carbohydrate Research, Volume 238, 1993,Pages 39-48, ISSN 0008-6215, https://doi.org/10.1016/0008-6215(93)87004-C.
33. Górniak A, Złocińska A, Trojan M, Pęcak A, Karolewicz B. Preformulation Studies of Ezetimibe-Simvastatin Solid Dispersions in the Development of Fixed-Dose Combinations. Pharmaceutics. 2022 Apr 22;14(5):912. doi: 10.3390/pharmaceutics14050912. PMID: 35631498; PMCID: PMC9147300.
34. Alhamhoom Y, Honmane SM, Hani U, Osmani RAM, Kandasamy G, Vasudevan R, Paramshetti S, R Dudhal R, K Kengar N, Charde MS. Study of Formulation and Process Variables for Optimization of PiroxicamNanosuspension Using 3^2 Factorial Design to Improve Solubility and In Vitro Bioavailability. Polymers (Basel). 2023 Jan 17;15(3):483. doi: 10.3390/polym15030483. PMID: 36771784; PMCID: PMC9919943.
35. H. Yeşim Karasulu, Bülent Karabulut, Erdem Göker, Tamer Güneri & Franz Gabor (2007) Controlled Release of Methotrexate from W/O Microemulsion and Its In Vitro Antitumor Activity, Drug Delivery, 14:4, 225-233, DOI: 10.1080/10717540601067760
36. Karasulu, H. Y. (2007). Microemulsions as novel drug carriers: the formation, stability, applications, and toxicity. Expert Opinion on Drug Delivery, 5(1), 119–135. doi:10.1517/17425247.5.1.119
37. Ghosh PK, Murthy RS. Microemulsions: a potential drug delivery system. Curr Drug Deliv. 2006 Apr;3(2):167-80. doi: 10.2174/156720106776359168. PMID: 16611003.
38. Üstündag-Okur, Neslihan; Gökçe, Evren Homan; Eğrilmez, Sait; Özer, Özgen; Ertan, Gökhan (2014). Novel Ofloxacin-Loaded Microemulsion Formulations for Ocular Delivery. Journal of Ocular Pharmacology and Therapeutics, 30(4), 319–332. doi:10.1089/jop.2013.0114
39. USP 43 NF 38, Monograph of Paclitaxel Injection
40. USP 43 NF 38, Monograph of Cyclophosphamide for Injection
41. Prabhjot Kaur, Tarun Garg, Goutam Rath, R. S. Rayasa Murthy & Amit K. Goyal (2016) Development, optimization and evaluation of surfactant-based pulmonary nanolipid carrier system of paclitaxel for the management of drug resistance lung cancer using Box-Behnken design, Drug Delivery, 23:6, 1912-1925, DOI: 10.3109/10717544.2014.993486
42. Swidan, Shady & Ghonaim, Hassan & Ahmed, Ahmed & Ghorab, Mamdouh. (2016). Comparative study of solid lipid nanoparticles and nanostructured lipid carriers for in vitro Paclitaxel delivery. Journal of Chemical and Pharmaceutical Research. 2016. 482-493.

43. Royce J, Wilkins R. Considerations for Sizing and SelectingSterile Buffer Filters. Merck Millipore Application Note. 2006
44. Asif Rasheed, Ph.D., Assessment of Complex Drug Product – Physicochemical Characteristics to Support In-Vitro Bioequivalence Studies, SBIA 2020: Advancing Innovative Science in Generic Drug Development Workshop, 2020
45. Valicherla, Guru R.; Dave, Kandarp M.; Syed, Anees A.; Riyazuddin, Mohammed; Gupta, Anand P.; Singh, Akhilesh; Wahajuddin, ; Mitra, Kalyan; Datta, Dipak; Gayen, Jiaur R. (2016). Formulation optimization of Docetaxel loaded self-emulsifying drug delivery system to enhance bioavailability and anti-tumor activity. Scientific Reports, 6, 26895– doi:10.1038/srep26895
46. Cho, Y.-H & Kim, Seunghae& Bae, E.K. &Mok, C.K. & Park, Jeeyeon. (2008). Formulation of a Cosurfactant-Free O/W Microemulsion Using Nonionic Surfactant Mixtures. Journal of Food Science. 73. E115 - E121. 10.1111/j.1750-3841.2008.00688.x.
47. Rahdar A, Sargazi S, Barani M, Shahraki S, Sabir F, Aboudzadeh MA. Lignin-Stabilized Doxorubicin Microemulsions: Synthesis, Physical Characterization, and In Vitro Assessments. *Polymers*. 2021; 13(4):641. https://doi.org/10.3390/polym13040641
48. Amis TM, Renukuntla J, Bolla PK, Clark BA. Selection of Cryoprotectant in Lyophilization of Progesterone-Loaded Stearic Acid Solid Lipid Nanoparticles. Pharmaceutics. 2020 Sep 19;12(9):892. doi: 10.3390/pharmaceutics12090892. PMID: 32961738; PMCID: PMC7560102.
49. Bhambere, Deepak &Gaidhani, Kunal &Harwalkar, Mallinath&Nirgude, Pallavi. (2015). LYOPHILIZATION / FREEZE DRYING – A REVIEW. World Journal of Pharmaceutical Research. 4. 516-543.
50. Wenzel T, Gieseler M, Abdul-Fattah AM, Gieseler H. Cycle Development in a Mini-Freeze Dryer: Evaluation of Manometric Temperature Measurement in Small-Scale Equipment. AAPS PharmSciTech. 2021 Apr 26;22(4):143. doi: 10.1208/s12249-021-02014-w. PMID: 33903988; PMCID: PMC8076153.
51. Verdonck, E., Schaap, K., & Thomas, L. C. (1999). A discussion of the principles and applications of Modulated Temperature DSC (MTDSC). International Journal of Pharmaceutics, 192(1), 3–20. doi:10.1016/s0378-5173(99)00267-7
52. Swidan, Shady & Ghonaim, Hassan & Ahmed, Ahmed & Ghorab, Mamdouh. (2016). Comparative study of solid lipid nanoparticles and nanostructured lipid carriers for in vitro Paclitaxel delivery. Journal of Chemical and Pharmaceutical Research. 2016. 482-493.

53. Emami J., Rezazadeh M., Varshosaz J., Tabbakhian M., & Aslani A. (2012). Formulation of LDL Targeted Nanostructured Lipid Carriers Loaded with Paclitaxel: A Detailed Study of Preparation, Freeze Drying Condition, andIn-VitroCytotoxicity. Journal of Nanomaterials, 2012, 1–10. doi:10.1155/2012/358782
54. Joshi, B. V., Patil, V. B., &Pokharkar, V. B. (2002). Compatibility Studies Between Carbamazepine and Tablet Excipients Using Thermal and Non-thermal Methods. Drug Development and Industrial Pharmacy, 28(6), 687–694. doi:10.1081/ddc-120003860
55. USP <71> Sterility test, USP 32 – NF 27
56. Sakre Sachin, AnushaBaddam, NamiraSimran, Vunnava Prasanna Lakshmi, HanumannagariAkhila, J. V. C Sharma. (2021). Review on Sterility Testing. International Journal of Pharmaceutical Research and Applications Volume 6, Issue 1 Jan-Feb 2021, pp: 581-587. DOI: 10.35629/7781-0601581587
57. Pagani O, Sessa C, Martinelli G, Cerny T, de Jong J, Goldhirsch A, Zimatore M, Cavalli F. Dose-finding study of paclitaxel and cyclophosphamide in advanced breast cancer. Ann Oncol. 1997 Jul;8(7):655-61. doi: 10.1023/a:1008211629858. PMID: 9296218.
58. Duarte D, Vale N. Evaluation of synergism in drug combinations and reference models for future orientations in oncology. Curr Res Pharmacol Drug Discov. 2022 May 12;3:100110. doi: 10.1016/j.crphar.2022.100110. PMID: 35620200; PMCID: PMC9127325.
59. Tallarida RJ. Quantitative methods for assessing drug synergism. Genes Cancer. 2011 Nov;2(11):1003-8. doi: 10.1177/1947601912440575. PMID: 22737266; PMCID: PMC3379564.
60. Foucquier J, Guedj M. Analysis of drug combinations: current methodological landscape. Pharmacol Res Perspect. 2015 Jun;3(3):e00149. doi: 10.1002/prp2.149. Epub 2015 May 20. Erratum in: Pharmacol Res Perspect. 2019 Dec;7(6):e00549. PMID: 26171228; PMCID: PMC4492765.
61. Sharma, A., & Baldi, A. (2018). Nanostructured Lipid Carriers: A Review. Journal of Developing Drugs, 7 (2), 1-12.
62. Wang, W., Chen, L., Huang, X., & Shao, A. (2016). Preparation and Characterization of Minoxidil Loaded Nanostructured Lipid Carriers. PharmSciTech .
63. Shah, K. A., Date, A. A., Joshi, M. D., & Patravale, V. B. (2007). Solid lipid nanoparticles (SLN) of tretinoin: Potential in topical delivery. International Journal of Pharmaceutics , 163-171.
64. Design, preparation and in vitro characterizations of fluconazole loaded nanostructured lipid carriers. Brazilian Journal of Pharmaceutical Sciences , 1-14.

65. Budai-Szucs, M. (2019). Design and Optimization of Nanostructured Lipid Carrier Containing Dexamethasone for Ophthalmic Use. MDPI Journal of Pharmaceutics, 11, 1-18.
66. Li Q, Cai T, Huang Y, Xia X, Cole SPC, Cai Y. A Review of the Structure, Preparation, and Application of NLCs, PNPs, and PLNs. Nanomaterials (Basel). 2017 May 27; 7(6):122. doi: 10.3390/nano7060122. PMID: 28554993; PMCID: PMC5485769.
67. Lee JS, Oh H, Sung D, Lee JH, Choi WI. High Solubilization and Controlled Release of Paclitaxel Using Thermosponge Nanoparticles for Effective Cancer Therapy. Pharmaceutics. 2021 Jul 27; 13(8):1150. doi: 10.3390/pharmaceutics13081150. PMID: 34452111; PMCID: PMC8398058.
68. Dave, Vivek & Haware, Rahul & Sangave, Nikhil & Sayles, Matthew &Popielarczyk, Michael. (2015). Drug-Excipient Compatibility Studies in Formulation Development: Current trends and techniques. AAPS, FDD Section Newsletter - January 2015.
69. Chidambaram M, Krishnasamy K. Drug-Drug/Drug-Excipient Compatibility Studies on Curcumin using Non-Thermal Methods. Adv Pharm Bull. 2014;4(3):309-12. doi: 10.5681/apb.2014.045. Epub 2014 Feb 7. PMID: 24754017; PMCID: PMC3992969.
70. Chinsriwongkul, A., Chareanputtakhun, P., Ngawhirunpat, T., Rojanarata, T., Sila-on, W., Ruktanonchai, U., &Opanasopit, P. (2011). Nanostructured Lipid Carriers (NLC) for Parenteral Delivery of an Anticancer Drug. AAPS PharmSciTech, 13(1), 150–158. doi:10.1208/s12249-011-9733-8
71. Kaur, Kamaldeep & Nautiyal, Ujjwal & Singh, Devendra. (2015). Nanostructured lipid carrier for bioavailability enhancement. International Journal of Recent Advances in Science and Technology. 2. 10.30750/ijrast.211.
72. Li, Q., Cai, T., Huang, Y., Xia, X., Cole, S., & Cai, Y. (2017). A Review of the Structure, Preparation, and Application of NLCs, PNPs, and PLNs. Nanomaterials, 7(6), 122. doi:10.3390/nano7060122
73. Wenzel T, Gieseler M, Abdul-Fattah AM, Gieseler H. Cycle Development in a Mini-Freeze Dryer: Evaluation of Manometric Temperature Measurement in Small-Scale Equipment. AAPS PharmSciTech. 2021 Apr 26; 22(4):143. doi: 10.1208/s12249-021-02014-w. PMID: 33903988; PMCID: PMC8076153.
74. Bhambere Deepak, Gaidhani A Kunal, Harwalkar Mallinath, Nirgude S Pallavi. (2015) Lyophilization/ freeze drying – A Review. World Journal of Pharmaceutical Research. 4(4), 516-543.

75. Khairnar Sandip, Kivi Rajesh, Harwalkar Mallinath, Salunkhe Kishor. (2013) A Review on Freeze Drying Process of Pharmaceuticals, International journal of Research in Pharmacy. 4(1), 76-94.
76. G R. Nireesha, L Divya, C Sowmya, N Venkateshan, Niranjan Babu, V Lavakumar. (2013). Lyophilization/ Freeze Drying-An Review. International Journal of Novel Trends in Pharmaceutical Sciences. 3(4), 87-98.
77. Jadhav R Tushar, Moon R S. (2015). Review on lyophilization technique. World Journal of Pharmaceutical Sciences. 2015, 4(5), 1906-1928.
78. Bisht Deepak, Dr. Iqbal Zeenat. (2015). Lyophilization-Process and Optimization for Pharmaceutics. International Journal of Drug Regulatory Affairs, 3(1), 30-40.
79. Shukla Soham. (2011). Freeze Drying Process: A Review. International Journal of Pharmaceutical Sciences and Research. 3(2), 3061-3068.
80. Wang Y, Wu KC, Zhao BX, Zhao X, Wang X, Chen S, Nie SF, Pan WS, Zhang X, Zhang Q. A novel paclitaxel microemulsion containing a reduced amount of Cremophor EL: pharmacokinetics, biodistribution, and in vivo antitumor efficacy and safety. J Biomed Biotechnol. 2011; 2011:854872. Doi: 10.1155/2011/854872. Epub 2011 Jan 20. PMID: 21331356; PMCID: PMC3035038.
81. Cai-Xia He, Zhong-Gui He & Jian-Qing Gao (2010) Microemulsions as drug delivery systems to improve the solubility and the bioavailability of poorly water-soluble drugs, Expert Opinion on Drug Delivery, 7:4, 445-460, DOI: 10.1517/17425241003596337
82. Sahu GK, Sharma H, Gupta A, Kaur CD (2015) Advancements in Microemulsion Based Drug Delivery Systems for Better Therapeutic Effects. Int J Pharm Sci Dev Res 1(1): 008-015. DOI: https://dx.doi.org/10.17352/ijpsdr.000003
83. MTT Cell Proliferation Assay Instruction Guide – ATCC, VA, USA www.atcc.org
84. Alley, M. C., Scudiere, D. A., Monks, A., Czerwinski, M., Shoemaker, R. II., and Boyd, M. R. Validation of an automated microculture tetrazolium assay (MTA) to assess growth and drug sensitivity of human tumor cell lines. Proc. Am. Assoc. Cancer Res., 27: 389, 1986
85. Nornoo AO, Osborne DW, Chow DS. Cremophor-free intravenous microemulsions for paclitaxel I: formulation, cytotoxicity, and hemolysis. Int J Pharm. 2008 Feb 12; 349(1-2):108-16. Doi: 10.1016/j.ijpharm.2007.07.042. Epub 2007 Aug 7. PMID: 17869459.
86. Van Meerloo, J., Kaspers, G. J. L., &Cloos, J. (2011). Cell Sensitivity Assays: The MTT Assay. Cancer Cell Culture, 237–245. doi:10.1007/978-1-61779-080-5_2
87. Pozarowski P, Darzynkiewicz Z, Analysis of cell cycle by flow cytometry. Methods Mol Biol. 2004; 281:301-11.

88. Ormerod et al., Consensus Report of the Task Force on Standardisation of DNA Flow Cytometry in Clinical Pathology. Anal Cell Pathol. 1998; 17(2): 103–110.
89. Pawley JB. (1995). Handbook of Biological Confocal Microscopy (2nd Edition). Plenum Publishing Corporation, New York.
90. Perrigue, P. M., Murray, R. A., Mielcarek, A., Henschke, A., &Moya, S. E. (2021). Degradation of Drug Delivery Nanocarriers and Payload Release: A Review of Physical Methods for Tracing Nanocarrier Biological Fate. Pharmaceutics, 13(6), 770. https://doi.org/10.3390/pharmaceutics13060770
91. ICH Harmonised Tripartite Guideline: Stability Testing Of New Drug Substances And Products Q1A(R2), Current Step 4 version dated 6 February 2003