Design, Characterization and In-vitro, In-vivo Evaluation of Paclitaxel Loaded Nanoparticulate System

THESIS

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Under the Supervision of
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CERTIFICATE

This is to certify that the thesis entitled **"Design, Characterization and In-vitro, In-vivo Evaluation of Paclitaxel Loaded Nanoparticulate System"** and submitted by **VasanthaKumar S** ID No **2007PHXF010P** for award of Ph.D. of the Institute embodies original work done by him under my supervision.

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Abstract

Novel delivery systems with selective distribution are need of the time for enhanced therapeutic efficacy with no or lesser side effects and with better patient compliances. The objective of the present research work was formulation and characterization of Paclitaxel loaded polymeric nanoparticulate drug delivery system to improve the therapeutic efficacy and selective distribution of Paclitaxel. Paclitaxel loaded nanoparticles were prepared using biodegradable and biocompatible poly caprolactone, poly (lactic-co-glycolic acid) and poly lactic acid polymers. As simple, sensitive and accurate analytical methods are essential for design of delivery systems. UV-spectrophotometric, liquid chromatographic analytical and bioanalytical methods for Paclitaxel was developed and validated in required matrix. The developed methods were simple, selective, sensitive, accurate and precise in the estimation of Paclitaxel. The analytical method was applied in the estimating of Paclitaxel in nanoparticles and in the in-vitro dissolution sample and the bioanalytical method was used to estimation Paclitaxel in plasma, liver, kidney and spleen samples in pharmacokinetic and biodistribution studies.

Before formulation, preformulation studies were performed to establish necessary physicochemical properties of Paclitaxel to develop efficient formulation. The bulk characterization, partition coefficient, solubility, stability and compatibility of Paclitaxel with excipients were studied. Paclitaxel is white crystalline powder and its solubility was less than 1 µg/mL. Paclitaxel was stable in pH 1.2 to 7.4, but it undergoes rapid degradation in pH 11.00 with high degradation rate constant. Paclitaxel was compatible with all the polymers and stabilizers used in the formulation of nanoparticles.

Paclitaxel loaded poly caprolactone, poly (lactic-co-glycolic acid) and poly lactic acid nanoparticles were prepared using nanoprecipitation and solvent evaporation method. The prepared nanoparticles were characterized for the size, size distribution, polydispersity index, zeta potential, encapsulation efficiency, drug content and in-vitro dissolution using appropriate methods. In addition the surface morphology of the prepared NPs was examined using microscopy techniques like, scanning electron microscopy, transmission electron microscopy and atomic force microscopy. The characterization result confirms that the prepared nanoparticles were stable, spherical in shape with uniform size. The drug content and encapsulation efficiency result illustrated that the prepared nanoparticles can entrap high amount of Paclitaxel in the polymer matrix. The prepared nanoparticles showed control release of drug from 42 hr to beyond 48 hr. The

critical formulation and process variables in the preparation of nanoparticles were optimized. This study result showed that, polymer amount and stabilizer concentration play important role in determining the size, drug content and drug release of nanoparticles. The stability study result showed that the prepared nanoparticles were stable.

The cytotoxicity study in MCF-7 cells clearly demonstrated Paclitaxel loaded nanoparticles were more cytotoxic than the commercial formulation. During cytotoxicity study, the morphological examination of cells incubated with Paclitaxel loaded nanoparticles showed alterations in cells shape which demonstrated the cytotoxic effect of drug loaded nanoparticles. The particle cellular uptake study in MCF-7 cells illustrates that all the prepared fluorescence poly caprolactone, poly (lactic-co-glycolic acid) and poly lactic acid nanoparticles were taken up efficiently by cancer cells. The pharmacokinetic and biodistribution behavior of the optimized formulations were studied in rats. The pharmacokinetic study result illustrates that the prepared Paclitaxel loaded nanoparticles can produce 4.5 to 4.2 fold increase in the area under the drug concentration curve in plasma when compared with Paclitaxel solution. The increase in mean residence time and elimination half life of Paclitaxel loaded nanoparticles indicated the increase in the drug residence time in systemic circulation. When compared to Paclitaxel sol, the mean residence time increased 2.8 to 3.2 fold in poly caprolactone, poly (lactic-co-glycolic acid) and poly lactic acid nanoparticles.

The clearance of Paclitaxel loaded NPs were decreased 4.5 fold when compared to the Paclitaxel sol, which implies longer retention of NPs in blood circulation. The biodistribution of Paclitaxel loaded NPs illustrate that there was increase in availability of Paclitaxel in rat liver over kidney and spleen tissue. The selected Paclitaxel loaded NPs were studied for their therapeutic efficacy in hepatocellular carcinoma model in rats. The survival curve analysis result illustrates that the treatment with nanoparticles significantly increased the survival of the rats. This study result demonstrated that the prepared poly caprolactone, poly (lactic-co-glycolic acid) and poly lactic acid nanoparticles are effective in selective delivery of Paclitaxel to liver and can produce better efficacy. On the whole, the prepared poly caprolactone, poly (lactic-co-glycolic acid) and poly lactic acid nanoparticles for Paclitaxel have great potential as targeted delivery systems for cancer treatment. Distribution profile may also vary depending on nature of polymer and their amount or proportion. The delivery of Paclitaxel using nanoparticles would be advantageous over the currently available commercial formulations.

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List of Abbreviations and Symbols

ABC	Area between the drug dissolution curve and its asymptote
AFM	Atomic force microscopy
AIC	Akaike information criterion
ALT	Alkaline transaminase
ALP	Alkaline phosphates
ANOVA	Analysis of variance
AUC	Area under the concentration versus time curve
AUDC	Area under the dissolution curve
AUMC	Area under first moment curve of concentration versus time profile
AST	Aspartate transaminase
°C	Degree centigrade
CI	Confidence intervals
C_{max}	Maximum concentration of drug in serum or tissue
Cl	Clearance
CNS	Central nervous system
CYP	Cytochrome P450 enzyme system
C6	Coumarin 6
DC	Drug content
DCM	Dichloro methane
DE	Dissolution efficiency
DEN	N-Nitrosodiethylamine
DG	Diffractogram
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DSP	Down slope purity
EE	Entrapment/Encapsulation efficiency
EPR	Enhanced permeability and retention
EP	Etoposide
FBS	Fetal bovine serum
FDS	Forced hydrolytic degradation study
FDA	Federal Drug Administration
FTIR	Fourier transform infrared spectrum
GI	Gastrointestinal tract
g	Grams
h	Reduced plate height
hr	hour
HEFF	Height equivalent to one effective plate
HETP	Height equivalent to theoretical plates
HPLC	High performance liquid chromatography
HQC	High quality control
ICH	International conference on harmonization
IC_{50}	Concentration producing 50% of the maximum inhibitory response
IM	Imatinib mesylate
IS	Internal standard
i.v	Intravenous
k’	Capacity factor

K_{deg}	Degradation rate constant
LC	Liquid chromatography
LCMS	Liquid chromatography coupled with mass spectrophotometer
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOQ	Limit of quantitation
LQC	Lower quality control
LTS	Long-term survivor
MCF-7	Breast cancer cells
MDT	Mean dissolution time
MEM	Minimal essential medium
MEST	Estimated median survival time
mg	Milligram
min	Minutes
mL	Millilitre
mm	Millimetre
MPS	Mononuclear phagocyte system
MQC	Medium quality control
MRTD	Mean residence time of the drug molecules in the dosage form
MRT	Mean residence time
MSC	Model selection criterion
MST	Mean survival time
MTL	Maximum tolerable level
MTE	Minimum therapeutic effective level
MTT	3-(4, 5-Dimethylthiazol-2yl)-2, 5, diphenyltetrazolium bromide
MXST	Maximal survival time
N	Number of theoretical plates
NC	Negative control
NDDS	Novel drug delivery systems
Neff	Effective plate number
NMR	^{1}H nuclear magnetic resonance
NPDDS	Nanoparticulate drug delivery systems
NPs	Nanoparticles
OST	Oxidative stress treatment
p-value	Significance level in statistical tests (probability of a type I error)
PBS	Phosphate buffer
PC	Positive control
PCL	Poly caprolactone
PDDS	Particulate drug delivery systems
PDI	Polydispersity index
PEG	Poly ethylene glycol
PECA	Poly (ethylcynoacrylate)
PF 68	Pluronic F68/Poloxamer 188
P-gp	P-glycoprotein
pH	Negative log to the base 10 of hydrogen ion concentration
PLA	Poly lactic acid
PLGA	Poly (lactic-co-glycolic acid)
PNPs	Polymeric NPs

PP	Propyl 4-hydroxybenzoate
PPI	Propidium iodide
PVA	Poly vinyl alcohol
QC	Quality control
R^2	Regression coefficient
RD	Relative dispersion of dissolution time
RH	Relative humidity
rpm	Revolutions per minute
SD	Standard deviation
SEM	Scanning electron microscopy
SPE	Solid phase extraction
T	Tailing factor
$t_{1/2}$	Half life
TAX	Paclitaxel
T25	Time taken to release 25 % of drug from NPs
T50	Time taken to release 50 % of drug from NPs
T90	Time taken to release 90 % of drug from NPs
TEM	Transmission electron microscopy
TST	Thermal stress treatment
UP	Upslope purity
USP	United States Pharmacopoeia
UV	Ultraviolet
Vd	Apparent volume of distribution
VDT	Variance of dissolution time
Vss	Apparent volume of distribution at steady-state
XRD	X-ray powder diffraction patterns
ZP	Zeta potential
% RSD	Percent relative standard deviation / coefficient of variation
3D	Three dimensions
σ	Standard deviation of y-intercept of regression equation

Chapter 1. Introduction

1.1 Introduction

Development in nanotechnology and nanoscience has opened up various opportunities and created potential applications in medical sciences. Interest and investment in research have been increased extensively from private and government sector for various applications in life sciences areas apart from electronics, mechanical, material sciences, physics etc. Apart from its use in other areas, nanoscience and nanotechnology have invaded pharmacy and opened up new opportunities in synthesis and drug delivery for improved therapy. Though, nanotechnology has plenty of promise, it also brings new challenges in safety and ethical considerations (1, 2). Formulation pharmacists are continuously working on novel drug delivery systems (NDDS) as conventional dosage forms have various shortcomings. New generation drugs, though lesser in numbers, have entered the market, most of them found to have drawbacks, such as poor water solubility, low gastro-intestinal permeability, high first pass metabolism, poor stability, non-selective distribution and many more (3, 4). Thus, in recent years, novel particulate drug delivery systems (PDDS), either polymer or lipid based, have been evaluated to overcome one or more of these drawbacks of conventional dosage forms. In recent times, nanoparticulate drug delivery systems (NPDDS) have been found to show promising results in overcoming several of these problems with the potential to improve therapeutic outcomes, though microparticulate systems have been studied and used extensively. Biological membranes are a major obstacle for conventional drug delivery systems, especially in cancer chemotherapy. However, NPDDS have shown better membrane permeability leading to improved therapy, in the treatment of several diseases including cancer therapy (5, 6, 7).

Nanoparticulate systems of some drugs have been reported to provide improved dissolution rate, oral absorption, bioavailability and stability, but they can also be used to target drugs, including peptide based drugs, to specific sites or organs and for extended release (8, 9). The ultimate goal of NPDDS is to provide clinically useful formulations for better therapy and patient quality of life. Chemotherapy is one area where NPDDS has shown great promise, as conventional dosage forms produce severe toxic effects due to non-selective distribution of cytotoxic drugs. The available literatures show that the NPDDS are not only useful in chemotherapy, but can be employed to deliver other class of drugs to their site of action through different routes of administration (1). Nanoparticles (NPs) can also provide a better and alternative for ophthalmic administration, because they can be retained at the application site and prolong drug release to the eye (1, 2). The NPs based tuberculosis treatment reduces the dosing frequency and increases the patient compliance. It is also possible to deliver central nervous system active drugs selectively to brain by NPDDS (3, 5, 10).

1.2 Challenges in cancer therapy

Cancer is a critical disease spread worldwide causing death of 12.5 % of the population annually and more than 11 million peoples are diagnosed with cancer every year. It has been estimated that by 2020, there will be 16 million new cancer cases every year all over the world (11). Although localized primary solid tumors can be removed successfully by surgical process, the prevention of spreading tumors and tumor metastases require extensive chemotherapy. Anti-cancer drugs are mostly associated with side effects, in particular nephrotoxicity, neurotoxicity, ototoxicity etc. due to non-selective distribution of drug to normal cells. Strident efforts have been made to reduce side effects by means of biological and pharmacological strategies.

Cancer chemotherapy is a delicate balance between response and toxicity, while under-dosing undermines effective therapy and over-dosing results in excessive toxicity. As the cytotoxic action is not selective for malignant cells, but also affects normal cells, non- selective distribution of drug in the body will inevitably cause significant side/toxic effects. It is a great challenge to formulation pharmacists to balance the two by designing novel delivery systems for selective distribution. Inherently, cancer cells are more susceptible to chemotherapy treatment than the normal cells, but most of the anti-cancer drugs are non-selective which results in serious side effects, some of them are life threatening. In general, more than 90 % of the administered anti-cancer drugs distribute to unwanted place (i.e. normal cells), hence there is a requirement for spatial and temporal drug delivery.

NPDDS promises to overcome the drawbacks of chemotherapeutic treatment by modifying the biodistribution and pharmacokinetics of the drug. Particulate and colloidal systems like NPs and liposomes have been studied as a physical approach to alter and improve the biodistribution, pharmacokinetic and pharmacodynamic properties of various types of drug molecules. They have been used to protect the drug entity in the systemic circulation, to restrict access of the drug to chosen sites and to provide drug release at a controlled or sustained rate to the site of action. NPDDS can be selective and effective in localization of pharmacologically active ingredient at pre-selected targets maintaining a therapeutic concentration for extended period of time, restricting its access to non-target areas, thus maximizing the effectiveness of the drug (12). Numerous investigations have shown that both the tissue and cell distribution profile of anti-cancer drugs can be controlled or modified by their entrapment in NPs (13).

1.3 NPDDS in chemotherapy

In general, NPs are polymeric or lipidic sub micron-sized colloidal pharmaceuticals, in which, drugs may be embedded in a polymeric/lipidic nanomatrix, or dissolved, encapsulated, entrapped in the polymeric/lipidic matrix or adsorbed on their surface. Specifically for anti-cancer drugs, NPs modify the biodistribution and pharmacokinetic character of drugs and hence reduce the systemic side effects accrued due to non-specific delivery of anti-cancer drugs to normal cells. Tumor-selective targeted drug delivery systems have become one of the most important advances of the 21^{st} century leading to improvements in the cure rate of advanced stage cancers (1, 14). The NPDDS can increase the half life ($t_{1/2}$) of drugs up to 10 times in tissue and blood, leading to less frequent administrations of dose with increased patient compliance and quality of life (15). The $t_{1/2}$ of L- asparaginase, when used for the treatment of lymphocytic leukemia, is between 8-30 hr and requires daily administration for 4 weeks. By contrast, treatment with Poly ethylene glycol (PEG)-L-asparaginase conjugate (Oncaspar®, $t_{1/2}$ approx 14 days) requires only one infusion every 2 weeks (16, 17). Different NPs, like lipid NPs, micelles, nanospheres, nanocapsules, niosomes, nanoemulsion, and nanosuspension have been formulated and their applications have also been evaluated (13, 18, 19).

The cancer chemotherapy began in the early 1940s and efforts are continuing to provide better drugs and better therapy. Several new drugs are at different phases of clinical trials. Presently, efforts are being made to enhance the therapeutic effectiveness by modified delivery systems or formulations. NPDDS has received tremendous attention from researchers for its potential to target cancer cells. NPDDS can overcome the drawbacks of the conventional cancer chemotherapy by providing a continuous controlled supply of drug selectively to the cancer cells and without the development of drug resistance. Treatment with NPDDS allows the oncologist to administer the cytotoxic drugs in lower doses because of its selective delivery, with the opportunity to further enhance the dose if required (20).

NPDDS can be designed and developed selectively depending on the intended route of administration as nanoemulsion or nanosuspension. The oncologists may prefer the simple drug NPs to target organs like liver, lungs, kidney and spleen, because they have the inherent ability to target reticulocyte endothelial system organs (21). In some cases, ligand molecules may be coated on the surface of NPs for the active docking to particular macrophage receptors in cancer cells, triggering phagocytosis. If the target tissue is not a part of the reticulocyte endothelial system, then the initial opsonization process has to be inhibited. This can be done by coating the NPs with substances like PEG or polysorbates-80, which prevent capture by opsonin proteins. This results in an increase in the circulation time of the particles in the blood stream and gives time to the NPs to

identify the target area and increase the therapeutic efficacy and decrease systemic toxicity (21). It has been found that Poly (lactic-co-glycolic acid) (PLGA) NPs coated with biodegradable Poly lactic acid (PLA)-PEG copolymer, are less susceptible to hepatic uptake than uncoated PLGA NPs (22). The di-block and tri-block copolymer (e.g. PEG-PLGA, methoxy-PEG-PLA, PLA-PEG-PLA NPs) NPs evade the reticulocyte endothelial system and have extended circulation times. There is a significant decrease in drug distribution to liver and spleen for the same copolymer NPs. Thus, NPDDS avoiding the reticulocyte endothelial system result in increased circulation half-life and enhanced therapeutic efficacy of anti-cancer drugs (23, 24). Maeda et al., reported styrene-maleic acid copolymer-conjugated neocarzinostatin as a tumor-targeted drug delivery system, producing an enhanced permeability and retention (EPR) effect on solid tumors (15, 25). The EPR-effect appears to be a universal phenomenon in solid tumors which warrants the development of NPDDS/nanomedicines. The NPDDS, which target tumors, are a novel strategy for improving drug performance by exploiting the patho-physiological uniqueness of tumors (26, 27).

The desired pharmacological action of anti-cancer drugs is achieved not only by targeting the cell surface, but further by working it to reach specific cell organelles (endosomes, lysosomes, mitochondria, endoplasmic reticulum and nucleus). Hence, the NPDDS provide an improved option for chemotherapy. The NPDDS can provide better cancer treatment through dual action of providing site specific drug delivery at cancer cells with control release, overcoming the major impediment of multidrug resistances.

1.4 Different types of NPs in drug delivery

NPDDS can be classified into solid lipid nanoparticles, nanospheres, nanocapsules, liposomes, polymersomes and micelles, based on the methods of preparation. If solid nanoparticles are prepared by incorporating drug in lipid, they are called solid lipid nanoparticles (SLN), whereas nanospheres are matrix systems in which the drug is embedded throughout the solid polymers. Nanocapsules are vesicular systems in which the drug alone or drug, confined to an aqueous or oily drops, is surrounded by a single polymeric membrane. Nanocapsules are usually used to encapsulate lipophilic drugs. If the polymeric membrane is of multiple layers then it is called a polymersome. Polymeric NPs (PNPs) are typically prepared from biodegradable polymers to avoid accumulation of the polymer matrix following repeat dosing (28, 29).

1.5 Polymers used in NPs preparation

The polymers used to prepare NPs should be biodegradable and have maximum elimination from body in short period of time or before the next dose, allowing for repeat administration of

formulation. It should be non toxic for long-term use and non immunogenic. If it is undergoing any degradation then the degradants should also have the above said properties (15, 16, 25-27). NPs can be prepared from different source of materials like proteins, polysaccharides, synthetic polymers which includes homopolymers [pre-polymerized and polymerized in process (monomers polymerization)] and copolymers (amphiphilic block copolymers). Different polymers used for NPs preparation are, the natural polymers like, proteins (gelatin, albumin, lectins, legumin, vicilin and casein) and polysaccharides (alginate, dextran, chitosan, agarose and pullulan), the synthetic homopolymers-polymerized in process [poly (alkyl cyanoacrylate) e.g. alkyl –methyl, ethyl, isobutyl, butyl, hexyl, isohexyl, poly (alkyl methacrylate) e.g. alkyl- methyl, poly (styrene) and poly (vinylpyridine)], the synthetic homopolymers-pre-polymerized [poly (ε-caprolactone) (PCL), poly (lactic acid), poly (lactic-co-glycolic acid), poly (methacrylate), poly (β-hydroxybutyrate), ethyl cellulose and cellulose acetate phthalate], amphiphilic block copolymers **[**methoxypolyethylene glycol-b-poly (lactic acid), methoxypolyethylene glycol-b-poly (caprolactone), methoxypolyethylene glycol-b-poly ((lactic-co-glycolic acid), polyethylene oxide-poly (propylene oxide)- poly ethylene oxide (PEO), polyethylene glycol-poly (β-benzyl-L-aspartate), poly (acrylic acid)-b-polystyrene] and stabilizers in NPs (17-22).

1.6 Method of preparation of NPs

PNPs are efficient alternate delivery systems to overcome the problems associated with cancer chemotheraphy. It has been reported by many scientist that the tumor accumulation of the drug encapsulated in PNPs was increased by several fold when compared to pure drug (5-9). In general, NPs are prepared by processes such as, solvent evaporation, solvent diffusion/displacement, reverse salting-out and droplet gelation, emulsification and polymerization, dispersion polymerization, interfacial condensation polymerization and interfacial complexation. The solid lipid nanoparticle is prepared by emulsification and solvent evaporation techniques. The simplest method of NPs preparation is nanoprecipitation.

1.6.1 Emulsification and solvent evaporation process

In general, PLA, PLGA and Poly caprolactone (PCL) NPs can be prepared by using this method with particle size larger than 250 nm. This method is used for preparing lipophilic drug loaded NPs extensively. Drug and polymer are dissolved or dispersed in the water-immiscible solvent like methylene chloride, chloroform, ethyl acetate. This solution or dispersion is added drop wise or injected at optimized rate to aqueous phase containing stabilizer. During the addition of organic phase to aqueous phase, the aqueous phase is stirred/homogenized which helps in reducing the

particle size, and then the solvent is evaporated by different techniques which hardens the NPs and which is a very critical step in NPs preparation. The formed nanoparticle suspension is lyophilized to harvest the particles. Snehalatha et al., prepared etoposide loaded PCL NPs by emulsification solvent evaporation technique using Pluronic F 68 as stabilizer, the diameter of the particles were 257 ± 3.96 nm and the encapsulation efficiency (EE) was 80.15 ± 1.01 % (13).

1.6.2 Emulsification solvent diffusion/displacement process

As the name implies diffusion of organic solvent in to the aqueous phase is the key step in emulsion solvent displacement method. The organic solvent should be partially soluble in water. Formulation scientist has wide range of solvents to select for this method, like benzyl alcohol, 2-butanone, methyl acetate, propylene carbonate, ethyl acetate, isopropyl acetate, methyl acetate, methyl ethyl ketone and isovaleric acid. This method is used to prepare NPs of size around 150 nm for poorly water soluble drug using PLA, PLGA, PCL and Eudragit® as polymers. Initially thermodynamic equilibrium of both phases has to be carried out for the successful preparation of NPs by emulsion diffusion technique. The polymer solvent (organic phase) has to be saturated with the aqueous phase and the aqueous phase has to be saturated with the organic phase. Once the crude emulsion is formed, hardening of NPs has to be carried out by a method, which results in diffusion of additional organic solvent from the organic phase contained in the dispersed droplets and it lead to precipitation of polymer as NPs (30).

1.6.3 Emulsification-reverse salting-out method

In this method, water miscible acetone is emulsified with aqueous phase by dissolving high concentration of salts or sucrose. Magnesium chloride, calcium chloride and magnesium acetate are used because of there high salting out effect in aqueous phase. When acetone is added to aqueous phase, because of the presence of large quantity of electrolyte which hold water molecules for their own solubilization, the miscibility property of water to acetone changes which results in emulsion droplet formation. The precipitation of polymer from the emulsion is induced by adding large quantity of water, which results in sudden drop of the concentration of salt or sucrose in the continuous phase of the emulsion and hence inducing the organic solvent migrates out of the emulsion droplets, this process is called reverse salting out (30).

1.6.4 NPs by polymerization

NPs prepared by in situ polymerization uses monomer alkycyanoacrylate to produce poly (alkycyanoacrylate) NPs. This polymer can encapsulate both lipophilic and hydrophilic drugs and used to prepare nanospheres and nanocapsules containing aqueous or oily core. Authors prepared

imatinib mesylate (IM) loaded poly (ethylcynoacrylate) (PECA) NPs by in situ polymerization technique. (30).

1.6.5 Nanoprecipitation

As the name implies, it is precipitation of the polymer along with drug in NPs size. It is the simple, fast, reproducible, less time consuming and economical method among all the methods of preparation of NPs. This method is also called as solvent displacement method and it is the most extensively explored method for NPs preparation. The basic requirement for this method is polymer, stabilizer, polymer solvent (acetone, ethanol, dimethylformamide (DMF), Dimethylsulfoxide (DMSO)) which is miscible with aqueous phase and the aqueous phase has to be non solvent to polymer. Some times the aqueous phase water is replaced with ethanol, methanol and propanol. During the preparation, the aqueous phase has to be stirred, followed by addition of polymer solution acetone (drop wise/injected/poured) in to the aqueous phase. To increase the precipitation process some time with acetone, water or ethanol will be added. Once the organic solvent is added to aqueous phase, NPs form instantaneously because of the rapid diffusion of acetone in to the aqueous phase. Because of the instantaneous process, nanoprecipitation method provides very fine 200 nm particle sizes with narrow distribution through the colloidal suspension (31). The nanoprecipitation method was extensively used to encapsulate lipophilic drugs and the encapsulation of hydrophilic drug is also possible with this method but the drug content (DC) found to be very less (less than 10 %).

In a recent work (32) nanoprecipitation method results were compared with emulsion-based technique of NPs preparation and they found that nanoprecipitation method is a good alternative to the classical and widely used double emulsion method for encapsulating lipophilic molecules. They used DMSO as organic solvent instead of the routine acetone and they used ethanol as the non solvent for polymer PLA and PLGA. The 9-nitrocamptothecin loaded PLGA NPs were prepared for targeted drug delivery by nanoprecipitation method. The results of optimized formulation showed a narrow size distribution with average diameter of 207 ± 26 nm and DC of more than 30 %. The in-vitro drug release profile showed a sustained release of 9-nitrocamptothecin up to 160 hr. All the above results showed that NPs prepared by nanoprecipitation are having the spatial (localizing and selectivity) and temporal (release control) controlled drug delivery (33).

1.7 Critical steps in NPs preparation

1.7.1 Concentrating, Hardening and Drying

The main task in NPs preparation is the size of particles (because of opsonization and targeting) and it's DC. If DC is low then larger quantity of carrier polymers and other excipients will also reach the system and can cause hypersensitivity reactions. Thus the volume of suspension that has to be administered in order to reach the therapeutic concentration of the drug becomes too high. The nanoparticle has to be concentrated to reduce the volume of formulation to be administered to the patients and to reduce the systemic exposure of other excipients in the formulation. The concentration process plays main role in particle size and its aggregation in the final formulations. There are several methods to concentrate the prepared NPs such as centrifugation, lyophilization, evaporation and dialysis (30).

1.7.2 Evaporation

Concentration by evaporation is usually performed by roto- evaporator. Based on the solvent and polymer used for the NPs preparation the temperature and vacuum are fixed in the instrument and operated to get the desired volume of nanosuspension. In this method large quantities of volatile organic solvent and a part of water are removed. During this process the polymer layer of the formed particles will be solidified in the aqueous system where the polymer is insoluble.

1.7.3 Lyophilization

This method is used to concentrate and to increase the stability of the prepared nanoparticulate formulations. In general, during this process the nanosuspension is transformed into a dry product, to avoid microbiological contamination, premature polymer degradation, physiochemical instability and loss of drug activity. To avoid stress during the lyophilization process special excipients are added to the nanosuspension before freezing. These excipients can have both action of cryoprotectant (freezing stress) and lyoprotectant (drying stress). Some of the very frequently used cryo or lyoprotectant are glucose, sucrose, lactose, mannitol, sorbitol, aerosol, poly (vinyl pyrrolidone), glycerol, Poly vinyl alcohol (PVA) and dextran.

1.7.4 Centrifugation

In this method, concentration of NPs suspension is done by normal centrifugation and ultracentrifugation. Normal centrifugation at low gravity force can remove aggregates and large particles from the polymeric nanoparticle suspension. But this method will not warranty to remove all particles above the nanometer size in the formulation. Ultracentrifugations (high speed centrifugation) can sediment particles with slight higher density than water. Ultracentrifugations are performed at 100,000-110,000 x g for 30 to 45 min to get NPs pellet. This pellet can be reconstituted to the desired volume of dispersion medium.

1.7.5 Dialysis

Nanosuspension concentration by dialysis can be performed by using different cellulose membrane with various molecular weight cut off. In simple dialysis method, the concentration of the suspension is performed against a polymer solution. This causes an osmotic stress, which produces a displacement of water from the nanosuspension towards the counter-dialysis solution. Vauthier et al., compared simple dialysis method with the evaporation, lyophilization and ultracentrifugation for concentrating the NPs formulation. The dialysis method results showed that the amount of water removed can be controlled and it gives reproducible results (34, 35).

1.8 NPs characterization and its important

Even though the Federal Drug Administration (FDA) has not released any specific guidelines for NPDDS, the following characterizations are usual for nanomedicine formulations; particle size and its distribution, surface morphology (surface charge/zeta potential) and surface properties (poly-dispersity index, PDI), DC/drug entrapment efficiency and in-vitro drug release evaluation. The size, distribution, mean size and PDI of the NPs are identified using laser light scattering or photon correlation spectroscopy. Particles morphology is examined by microscopy techniques, such as, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM). Photon correlation spectroscopy, X-ray photon correlation spectroscopy, Fourier transform infrared spectroscopy, nuclear magnetic resonance spectroscopy and electrophoretic mobility can be used to examine surface properties of NPs, such as, zeta potential (ZP) and surface hydrophobicity. The drug release from NPs is mainly governed by diffusion and biodegradation processes. The methods normally used to study the in-vitro release of NPs include the use of side-by-side diffusion cells with an artificial or biological membrane, the dialysis bag diffusion technique, the reverse dialysis sac technique, ultra-centrifugation, ultra-filtration and /or centrifugal ultra –filtration (30).

1.8.1 Particle Size

Particle size plays a major role in determining the in-vivo fates of NPs. Researchers have demonstrated that opsonization and subsequent recognition and phagocytosis by macrophages of mononuclear phagocytic system are strongly correlated with NPs size (21, 36). Opsonin cannot bind to the smaller particles which have a high radius of curvature and hence, the circulation time of smaller NPs is very high and its clearance rate is low. Gaur et al. observed that the hydrophilic poly (vinyl pyrrolidone) NPs of 35 nm diameter show less than 1 % uptake by the spleen and liver, and even after 8 hr of injection 5 -10 % of NPs remain circulating in the blood stream (37). So, particles with smaller diameters can circulate for longer and have improved ability to target their

site of action. If NPs are administered through the intravenous (i.v) route, smaller particles, less than 20-30 nm, are eliminated by renal excretion and larger particles, greater than 200 nm, will be removed by opsoninization leading to localization in the liver, kidney, lung, spleen and to a lesser extent in bone marrow. Hence the ideal size for targeted drug delivery is between 70 and 200 nm (38, 39).

1.8.2 Surface properties

Surface properties of NPDDS are critical in determining their drug delivery potential as these properties govern the overall in-vivo performance of the drug delivery system. These properties also modulate the in-vitro performance such as stability, drug entrapment efficiency and drug release kinetics. The specific surface area, surface charge and surface hydrophobicity are very important as these govern the physiochemical and electrostatic interactions with biological membranes and the overall biodistribution of drug loaded NPs (40, 41).

1.8.3 Entrapment efficiency (EE) and drug content (DC)

Ideally nanomedicines should have high EE and DC to reduce the volume of nanosuspension required to be administered. Numerous different terms have been used to represent the drug content and the efficiency of the preparation method. These include EE, NPs recovery, DC, process efficiency, loading capacity, association efficiency and drug incorporation efficiency. The entrapment of drug into the NPs can be determined directly or indirectly. In the indirect method the prepared NPs are recovered or separated by ultracentrifugation (100,000 g, 25 min) and the supernatant is analyzed for the free drug content. In the direct method the incorporated drug in NPs is determined by dissolving the NPs in a suitable solvent, followed by analysis of drug content (13). The EE can be calculated by

$$EE\ (\%) = \frac{\text{Amount of drug in NPs}}{\text{Initial amount of drug taken}} \times 100$$

To determine the amount of drug in the sediment, specified volume of the prepared nanoparticle suspension was taken and centrifuged at 45000 x g for three cycles for 10 min /cycle. The sediment NPs was taken and processed to determine the drug content in NPs. It can also be determined by indir

$$EE\ (\%) = \frac{\text{Total amount of drug} - \text{Free drug}}{\text{Total amount of drug}} \times 100$$

The DC in NPs can be determined by taking required amount NPs and dissolving with suitable solvent, filtered and the drug content was determined by

$$DC\ (\%) = \frac{\text{Amount of drug in NPs}}{\text{Amount of NPs}} \times 100$$

1.9 Fate of NPDDS in biosystem

NPs identified by the immune system undergo rapid phagocytosis by the macrophages of the mononuclear phagocyte system (MPS). MPS is one of the most important biological barriers to targeted drug delivery systems, because the delivery system is removed from the body before reaching the target and performing their expected therapeutic action (21). It has been observed that smaller size NPs (‹ 200 nm) and hydrophilic surface, obtained by adsorption of surfactant/ hydrophilic molecules (PEG) or by using block / branched copolymer, which are amphiphilic block copolymers nature, can reduce the opsonization reaction and subsequent clearance by macrophages (21). This led to development of Stealth ^{TM} NPs, which are characterized by a prolonged half-life in the blood compartment (17). The amphiphilic block copolymer is of interest to researchers to formulate NPs because of its hydrophilic surface nature. It has also been observed that this nanoparticle modulates the activity of the efflux pump, P-glycoprotein (P-gp) (19). Polymers which are normally used to shield the NPs are polysaccharides, polyacrylamide, poly (vinyl alcohol) (PVA), poly (N-vinyl-2-pyrrolidone), PEG and PEG-containing copolymers such as poloxamers, poloxamines, and polysorbates. A review of the literature indicates that PEG and PEG-containing copolymers are most effective and very commonly used polymer to prepare long-lived circulating NPs (24).

1.10 Applications of NPDDS in drug delivery

A significant amount of work has been done in developing polymeric tumor-targeted NPDDS for anti-cancer drugs and promising results were reported with improved solubility and better therapeutic efficacy with selective distribution (30, 31, 39).

1.10.1 Enhanced Plasma half-life

In our lab it was observed that after i.v and oral administration of radio-labeled etoposide loaded PCL and PLGA NPs, the serum residence time of etoposide was increased. Also observed was a low distribution of etoposide to the heart, NPs are likely to produce no or low cardiac toxicity (13). After i.v. and oral administration of free etoposide and drug loaded NPs, NPs produced a higher area under the curve ($AUC_{0\text{-}inf}$), mean residence time (MRT) and $t_{1/2}$ when compared with free

etoposide. Further, it was observed that NPs with PLGA 85/15 has enhanced $AUC_{0\text{-inf,}}$ MRT and $t_{1/2}$ when compared to PCL NPs suggesting that the polymer plays an important role in distribution profile (13, 39).

1.10.2 Solubility enhancement

The solubility study result of PCL NPs show that the solubility of griseofulvin after encapsulation decreased (12.71 ± 0.32 mg/mL) when compared to the micronized griseofulvin (30.56 ± 0.26 mg/mL). This is due to the fact that drug molecules adsorbed and entrapped into NPs. The dissolution study reveals that the dissolution rate of griseofulvin was enhanced by recourse to encapsulation (100 % drug released in 15 min) compared to that of micronized griseofulvin (25 % release in 15 min) (32). Blouza et al., prepared spironolactone-loaded PCL nanocapsules using nanoprecipitation method to increase the solubility and dissolution rate of spironolactone. Spironolactone is low soluble (28 µg/mL at 25 ° C) and slow dissolution rate potassium sparing diuretic used in premature infants to reduce the lung congestion. The prepared NPs have high drug-concentration in the liquid preparation (1.5mg/mL) allowing minimizing the preparation volume administered for children medication. The dissolution study at sink condition showed that the 100 % drug was released in 20 min (42).

1.10.3 Peptide drug delivery

The PNPs is the promising way to improve the stability, solubility and the oral bioavailability of peptide and proteins. A new drug nanocarrier, chitosan-PEG nanocapsules were prepared for the oral administration of peptides by the solvent displacement technique. The in-vivo study result showed that the nanocarrier enhanced and prolonged the intestinal absorption of peptide. The permeability study in Caco-2 cells show that the intestinal absorption of the peptide was increased when it was delivered in NPs. Chitosan NPs containing a peptide (Z-DEVD-FMK) caspase inhibitor with particle diameter ranging from 313-412 nm were prepared and this prepared positively charged NPs have a loading capacity of 0.46 % and the highest extent of release (65 % at 24h) (43).

1.10.4 Selective delivery to brain

Wilson et al., prepared and studied polysorbate 80-coated poly (n-butylcyanoacrylate) NPs of anti-Alzheimer's drug tacrine to target brain (44). The study reported that tacrine concentration in the brain increased by 4.07 fold when compared to the free drug tacrine. The pharmacokinetic and biodistribution study of i.v. administered etoposide loaded tripalmitin positively charged NPs showed that NPs produced a higher brain concentration (0.07% of injected dose/organ) when compared to negatively charge SLN and etoposide solution. The prepared nitrendipine loaded SLN

for brain targeting, has found to be taken up to a greater extent by brain and hence the concentration of nitrendipine was higher and maintained for 6 hr as compared to suspension (3 hr) (30). Xiong et al., prepared a nanosuspensions, average particle size of 300 and 650 nm, containing nimodipine (a calcium-channel blocker for the treatment of senile dementia and subarachnoid haemorrhage related vasospasm) to target the brain and the results were compared with nimodipine ethanol formulation and Tween 80 coated NPs. The i.v. biodistribution study demonstrated that the 300 nm nanoparticle effectively increased brain drug concentration and reduced drug concentration in the liver, spleen and lungs, indicating that the 300 nm particles are not taken up by Kupffer cells as are the 650 nm NPs (18).

1.10.5 Selective delivery to lungs

NPs targeted to lungs are used to treat patients with pulmonary diseases including cystic fibrosis, lung cancer, asthma, and chronic pulmonary infections such as tuberculosis (30). The pulmonary delivery of drug through nanotechnology, to get deposited in respiratory tract depends on the particle size, density and surface properties of the NPs. Literature reported that particles with diameter ranging from 100-500 nm are suitable candidate for the pulmonary delivery to treat respiratory diseases. Recent report showed that after 24 hr of inhalation of NPs in rat model, the ^{13}C-labelled NPs with a particle size of 26 nm translocated to the liver more than 50 %. Gelatin and polybutylcyanoacrylate spray-dried powder NPs shows excellent delivered to the lungs via carrier particles that dissolve after coming in contact with the aqueous environment of the lung epithelium (30). After 20 IU/kg pulmonary administration of insulin SLN, the insulin level was increased to 170µIU/mL and glucose level was reduced to 39.41 % after 4 hr. This shows that the SLN was distributed in the lung alveoli and a prolonged release of insulin was observed in both plasma insulin and glucose profile. Now a day's pulmonary delivery is used to deliver drug loaded nanoparticulate formulation to CNS. The NPs give way for the drug to reach the systemic circulation and to target CNS in low dose and less frequent administration (30). The pulmonary drug loaded NPs translocate to the extrapulmonary sites and to other target site by crossing epithelia of the respiratory tract into the interstitium.

The biodistribution study of PECA NPs by oral route also revealed similar findings as observed in the i.v. biodistribution study, with an increased concentration in lungs (41.96 µg/g) and increased MRT (27.33 hr) and elimination half life (27.33 hr). The pharmacokinetic and biodistribution studies confirmed that the biodistribution of the IM can be modulated efficiently by forming NPDDS of IM. Though PECA and PLGA NPs showed enhanced but different level of distribution

in brain, whereas distribution in the lungs did not vary, indicating different profile of distribution in brain and in lungs presumably due to the different polymers employed (30). Snehalatha et al showed that etoposide loaded PCL and PLGA NPs were found to reach the lungs at relatively higher levels than the free drug when administered to rats, suggesting that such formulations may be useful in the treatment of lung cancer (39).

1.10.6 Selective delivery to liver and bone

The pharmacokinetic and biodistribution studies were performed with radio-labeled free drug etoposide, empty NPs, drug loaded PLGA NPs and drug loaded PCL NPs. All the nanoparticle formulations showed higher distribution to liver and bone and longer circulating time than the drug alone. The study showed that, after 24 hr post injection PLGA (1.005 %) and PCL (0.332 %) NPs amount of etoposide in bone were found to be 15.02 and 4.96 times more than free etoposide (0.066 %) respectively (13).

The biodistribution studies also showed that the radioactivity levels of the nanoparticle formulations in tissues/organs were significantly higher than those of etoposide alone except in the heart. Therefore using etoposide loaded PLGA and PCL NPs can reduce the accumulation of etoposide in the heart, which may lead to reduced cardiac toxicity. In tumor induced mice it was observed that tumor uptake of nanoparticulate etoposide was much higher than free drug. The free etoposide disappeared faster from the liver than the formulations indicating that the nanoparticulate etoposide may distribute more widely and stay for a longer time than drug alone. Relatively high radioactivity was found in bone for NPs than free etoposide. This is one more useful indicator that such preparations may be of value in treating bone related malignancies (13).

1.10.7 NPs for topical application

The stratum corneum is the main barrier for percutaneous absorption of topically applied drugs. Particulate drug delivery systems can be used for dermatological application to improve the therapeutic action and reduce systemic side effects. The horny layer of the skin functions as barrier to prevent the entry of hydrophilic compounds and allows the passage of lipophilic substance selectively. Complete stripping of the horny layer increases the uptake of acyclovir (logP – 1.76) absorption 440- folds, indicating that this barrier is clearly most effective against hydrophilic compounds. Highly lipophilic compound can penetrate via the hair follicle and through the follicular pathway (30**)**.

Miyazaki et al., prepared indomethacin loaded poly n-butylcyanoacrylate nanocapsules to promote its systemic action through topical application. The in-vitro permeation study in rat skin showed

that the poly n-butylcyanoacrylate based nanocapsules were able to permeate rat skin. The fluorescence photomicrography study in rat skin showed that the NPs can penetrate through the stratum corneum and reach the epidermis (30). They showed that the permeation of the drug through rat skin was mostly due to permeation of the intact nanocapsules. The study reported higher plasma drug concentration (2.24 µg/mL) in 6 hr in the case of poly n-butylcyanoacrylate nanocapsule formulation compared with gel formulation (0.88 µg/mL) which is in agreement with in-vitro results (45-48).

The reason for the penetration of NPs through the stratum corneum and epidermis into the systemic circulation is attributed to the small size, lipophilic nature of the polymers, hydrophilic and hydrophobic surface characteristics of the material. Sometimes penetration enhancers (e.g. benzyl benzoate/oleic acid) are used in the nano-formulation to enhance the penetration of the drug. Drugs, loaded in lipid carriers, appear to enhance dermal absorption. The nano size carriers can make very close contact and adhere with corneocyte clusters and furrows between corneocyte islands, which provides larger surface to the drug molecules to penetrate the barrier (49, 50).

1.11 Toxicity of NPs

NPs can be administered by oral, pulmonary, nasal, i.v, intraperitoneal, intramuscular, intrathecal routes. Dermal and ocular applications are other interesting routes of administration of NPs. Once the NPs are administered to patients (humans) or rodents, based on the route of administration the NPs absorption starts. First interaction starts with the biological components (proteins, cells i.e. macrophages or tissue cells) for absorption. If the drug delivery is through passive targeting then distribution of the particles through out the body takes place where NPs interaction with all biological membrane or organs, based on the organs the clearance takes places from the whole body. Elimination of NPs from the body decides the accumulation behavior of the NPDDS in organs. Many research paper shows that the chemical composition and size of the particles influences the excretion of NPs (51).

It is not necessary that all NPs have to be eliminated; some will be accumulated in the body or organs. If the exposure and accumulation of NPs is less, then toxicity may not be high, but high or chronic/repeated exposure of NPs results in more amount of NPs accumulate in organs, which is very toxic to the body. There is no abundant data for the accumulation of NPs in-vivo and its toxicity behavior to biosystem. Even some time the NPs can enter into cells and nucleus and they stay for long time, creating the immunological toxicity. Apart from the amount of NPs in the biosystem, the charge, structure, solubility, chemical and physical properties decides the cellular

response to NPs. Hence each system can produce its own dynamic and kinetic toxicity. In case of gastrointestinal (GI) absorption the positively charged particles are absorbed more efficiently than the neutral and negative charged particles (52, 53). In contrast, the ulcerated tissue has high concentration of positive charged proteins hence the negative charged NPs can be adhering to the tissue more efficiently than the other charged particles (30, 54).

Once the passive targeted NPs (no hydrophilic coat) reach the blood, it is freely available to interact with membranes of the body, the unwanted interaction and distribution provides the adverse effect and severe toxicity to the individuals. In case of oral NPs they reach the blood after absorbed throughout the GI barrier, it gets distributed to kidney, liver (some NPs accumulates in the liver because of its first pass metabolism), spleen, lungs and brain. It has been reported that the i.v injected NPs is distributed to lungs, colon, bone marrow, liver, spleen and lymphatics where as the inhalation NPs is distributed to lungs, spleen, liver, heart and brain.

There is no general and universal toxicity data for all case of NPs. So each NP systems have to be considered as individual dosage forms and the toxicity profile should be documented. The toxicity study protocol of the NPs should be very stringent, efficient and reproducible, if it is used for drug delivery, food industry and for diagnosis (55, 56). The current toxicity studies are conducted by in-vitro cell lines, where the cell viability, some specific markers or protein secretion are observed for the cytotoxic action of the NPs. The in-vitro data is not a substitute for the whole body in-vivo study, the in-vitro data is a preliminary data and it gives general mechanism of toxicity. In case of in-vivo toxicity study, the change of organ morphology, serum biochemical parameters, hematological changes and histopathological examinations are performed for the dosed animal samples. Indirect method of identifying the toxicity of NPs can be performed by biodistribution study. If the toxicity of the drug is know then the selective delivery of the drug can be monitored by biodistribution study. Etoposide has cardiac toxicity hence the distribution of the blank NPs and drug loaded NPs to heart can be monitored (39). The distribution of NPs (organ or biomatrix samples) can be monitored by radio labeling, electron microscopy analysis and drug content in the targeted or non-targeted organ can be determined by different liquid chromatographic methods. The quantitative analysis of NPs inside the body or biological matrix has to be very efficient (validated) for the determination of dose-response toxicity.

The polymeric NPs are explored in a very great way in the last two decades for its therapeutic benefits, which results, some formulations are in clinical trials, few are with approval for clinical use and some are in preclinical investigations. Even though the toxicity profile of the polymer or lipid used in NPs preparation is known it is necessary to have data in hand regarding the NPs

toxicity in-vivo and in-vitro, because the properties of the chemicals/polymers as such and the NPs are totally different. Many researchers showed that the NPs avoid the unwanted toxic effects because of its selective delivery and its selective bioaccumulation. The liposomes and polymeric NPs of doxorubicin or etoposide avoid the accumulation of these particles in heart and hence avoid the cardiac toxicity associated with the pure drug (39, 57).

1.12 Objective of the project

Inherently, cancer cells are more susceptible to chemotherapy than the normal cells, but most of the anti-cancer drugs are non-selective which results in serious side effects, some of them are life threatening. In general, more than 90 % of the administered anti-cancer drugs distribute to other places (i.e. normal cells). Thus, there is a need for spatial and temporal drug delivery in cancer chemotheraphy. NPDDS offers controlled and targeted release of the encapsulated drug, hence results in high therapeutic efficacy with low side effects (7, 8). It is expected that in the next decade almost 50% of drug delivery and design approaches will be shift to NPDDS (1). PNPs are an efficient alternate delivery system for cancer therapy to overcome the problems associated with cancer treatment.

It has been reported by many scientists that the tumor accumulation of the drug encapsulated in NPs was increased by several fold when compared to pure drug, but the increase in accumulation is only 5 % of the total administered dose (1). This shows that there is wide scope to work in the development of drug loaded polymeric NPs to further increase the percent of drug reaching the tumor from the administered dose, for the various cancer treatments. This will decrease the dose related unwanted side effects and reduce the treatment cost. Alteration in the physiochemical properties of NPs play significant role in the penetration behavior of the NPs in the respective cancerous cells (8).

In the present research, attempts have been made to formulate and characterize Paclitaxel loaded polymeric NPs with improved and selective distribution and enhanced therapeutic efficacy. Paclitaxel loaded NPs were prepared using biodegradable and biocompatible polymers PCL, PLGA and PLA. Prior to preparation and characterization, some preformulation studies of Paclitaxel were performed to develop an efficient formulation. Paclitaxel loaded PNPs were prepared using nanoprecipitation and solvent evaporation method, the ideal methods to load hydrophobic drug in NPs. The prepared PNPs were characterized for their size, particle size distribution, PDI and surface charge/ZP by photon correlation spectroscopy using Zetasizer. The surface morphology of the prepared PNPs were examined using microscopy techniques, SEM,

TEM and AFM. The EE, DC and in-vitro dissolution studies were performed for the prepared PNPs using standard procedures. Formulation of drug loaded NPs are mainly influenced by amount of polymer/stabilizer, concentration of stabilizer and amount of Paclitaxel. The influences of all these parameters on characterization NPs like size, PDI, EE, DC, ZP, % recovery of NPs and in-vitro release were studied and analyzed in order to optimize the parameters for delivering Paclitaxel effectively.

The optimized Paclitaxel loaded PCL, PLGA and PLA NPs were subjected for stability study. The cytotoxicity and cellular uptake efficiency of the prepared Paclitaxel loaded NPs were studied in suitable cancer cell lines by standard in-vitro procedure. The therapeutic efficacy and selective biodistribution of Paclitaxel loaded NPs were studied in rats.

The proposed research work required simple and sensitive analytical and bioanalytical methods to estimate Paclitaxel in different samples like, bulk powder, formulations, in-vitro dissolution samples, stability samples and biosamples. So, high performances liquid chromatography (HPLC) and UV (ultraviolet) - spectrophotometry methods were developed and validated for Paclitaxel estimation.

References

1. Couvreur P, Vauthier C. (2006). Nanotechnology: intelligent design to treat complex disease. Pharm Res, 23, 1417-1450.
2. Aston R, Siebert RS, Canham L, Ogden J. (2005). Nanotechnology application for drug delivery. Pharm Technol Eur, 17, 21-28.
3. Gill S, Lobenberg R, Ku T, Azarmi S, Roa W, Prenner EJ. (2007). Nanoparticles: characteristics, mechanisms of action, and toxicity in pulmonary drug delivery- a review. J Biomed Nanotechnol, 3,107-119.
4. Grau MJ, Kayser O, Muller RH. (2000). Nanosuspension of poorly soluble drugs-reproducibility of small scale production. Int J Pharm, 196, 155-157.
5. Osborne DW, Ward AJ, Neil KJ. (1991). Microemulsions as topical delivery vehicles: in-vitro transdermal studies of a model hydrophilic drug. J Pharm Pharmacol, 43, 450-454.
6. Kemken J, Ziegler A, Muller BW. (1992). Influence of supersaturation on the pharmacodynamic effect of bupranolol after dermal administration using microemulsion as vehicle. Pharm Res, 9, 554-558.
7. Trotta M. (1999). Influence of phase transformation on indomethacin release from microemulsion. J Control Release, 60, 399-405.

8. Dillen K, Vandervoort J, Mooter GV, Ludwig A. (2006). Evaluation of ciprofloxacin-loaded Eudragit RS 100 or Rl 100/PLGA nanoparticles. Int J Pharm, 314, 72-82.
9. Amidi M, Romeijn SG, Borchard G, Junginger HE, Hennink WE, Jiskoot W. (2006). Preparation and characterization of protein-loaded N-trimethyl Chitosan nanoparticles as nasal delivery system. J Control Release, 111,107-116.
10. Gao X, Wu B, Zhang Q, Chen J, Zhu J, Zhang W, Rong Z, Chen H, Jiang X. (2007). Brain delivery of vasoactive intestinal peptide enhanced with the nanoparticles conjugated with wheat germ agglutinin following intranasal administration. J Control Release, 121, 156-167.
11. Stewart BW, Kleihues P. (2003). World Cancer Report- International Agency for Research on Cancer (IARC), Non-serial Publication, World Health Organization, Geneva, Switzerland.
12. Cumminngs J, Allan L, Smyth J. (1994). Encapsulation of mitomycin C in albumin microspheres markedly alters pharmacokinetics, drug quinine reduction in tumor tissue and antitumor activity. Biochem Pharmacol, 47, 1345-1356.
13. Snehalatha M, Venugopal K, Saha RN. (2008). Etoposide-loaded PLGA and PCL nanoparticles I: preparation and effect of formulation variables. Drug Deliv, 15, 267-275.
14. Miklos GL. (2005). The human cancer genome project-one more misstep in the war on cancer. Nat Biotechnol, 23, 535-537.
15. Maeda H, Bharate GY, Daruwalla J. (2009). Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect. Eur J Pharm Biopharm, 71, 409-419.
16. Graham ML. (2003). Pegaspargase: a review of clinical studies. Adv Drug Deliv Rev, 55, 1293-1302.
17. Harris JM, Chess RB. (2003). Effect of pegylation on pharmaceuticals. Nat Rev Drug Discov, 2, 214-221.
18. Xiong R, Lu W, Yue P, Xu R, Li J, Chen T, Wang P. (2008). Distribution of an i.v. injectable nimodipine nanosuspension in mice. J Pharm Pharmacol, 60, 1155-1159.
19. Letchford K, Burt H. (2007). A review of the formation and classification of amphiphilic block copolymer nanoparticulate structure: micelles, nanospheres, nanocapsules and polymersomes. Eur J Pharm Biopharm, 65, 259-269.
20. Skipper HE, Schabel FM, Wilcox WS. (1964). Experimental evaluation of potential anticancer agents XIII: on the criteria and kinetics associated with 'curability' of experimental leukemia. Cancer Chemothr Rep, 55, 1-111.

21. Owens ED, Peppas NA. (2006). Opsonization, biodistribution and pharmacokinetics of polymer nanoparticles. Int J Pharm, 307, 93-102.
22. Stolik S, Dunn SE, Garnett MC, Davies MC, Coombes AG, Taylor DC, Irving MP, Purkiss SC, Tadros TF, Davis SS. (1994). Surface modification of poly (lactide-co-glycolide) nanospheres by biodegradable poly (lactide)-poly (ethylene glycol) copolymers. Pharm Res, 11, 1800-1808.
23. Gref R, Minamitake Y, Peracchia MT, Domb A, Trubetskoy V, Torchilin V, Langer R. (1997). Biodegradable long-circulating polymeric nanospheres. Science, 263, 1600-1603.
24. Otsuka H, Nagasaki Y, Kataoka K. (2003). PEGylated nanoparticles or biological and pharmaceutical applications. Adv Drug Deliv Rev, 55, 403-419.
25. Maeda H, Sawa T, Konno T. (2001). Mechanism of tumor-targeted delivery of macromolecular drug including the EPR effect in solid tumor and clinical overview of the prototype polymeric drurg SMANCS. J Control Release, 74, 47-61.
26. Langer R. (1998). Drug delivery and targeting. Nature, 392, 5-10.
27. Duncan R. (2006). Polymer conjugates as anticancer nanomedicines. Nat Rev Cancer, 6, 688-701.
28. Discher DE, Eisenberg A. (2002). Polymer vesicles. Science, 297, 967-973.
29. Hassan AS, Sapin A, Lamprecht A, Emond E, Ghazouani El, Maincent P. (2009). Composite microparticles with in-vivo reduction of the burst release effect. Eur J Pharm Biopharm, 73, 337-344.
30. Saha RN, Vasanthakumar S, Benda G, Snehalatha M. (2010). Nanoparticulate drug delivery systems for chemotheraphy. Mol Membr Biol, 27, 215-223.
31. Kollipara S, Girish B, Snehalatha M, Saha RN. (2010). Application of rotatable central composite design in the preparation and optimization of poly (lactic-co-glycolic acid) nanoparticles for controlled delivery of paclitaxel. Drug Dev Ind Pharm, 36, 1377-1387.
32. Zili Z, Sfar S, Fessi H. (2005). Preparation and characterization of poly-caprolactone nanoparticles containing griseofulvin. Int J Pharm, 294, 261-267.
33. Vyas SP, Khar RK. (2001). Targeted and Controlled Drug Delivery. 1st ed. CBS Publisher and Distributor, New Delhi, India, 150-160.
34. Vauthier C, Bouchemal K. (2008). Method for the preparation and manufacture of polymeric nanoparticles. Pharm Res, 26, 1025-1058.
35. Vauthier C, Cabane B, Labarre D. (2008). How to concentrate nanoparticles and avoid aggregation?. Eur J Pharm Biopharm, 69, 466-475.

36. Champion JA, Katare YK, Mitragotri S. (2007). Particle shape: new design parameters for micro and nanoscale drug delivery carriers. J Control Release, 121, 3-9.
37. Gaur U, Sahoo SK. (2000). Biodistribution of fluoresceinated dextran using novel nanoparticles evading reticuloendothelial system. Int J Pharm, 202, 1-10.
38. Moghimi SM, Hunter AC, Murray JC. (2001). Long-circulating and target-specific nanoparticles: theory to partice. Pharmacol Rev, 53, 283-318.
39. Snehalatha M, Venugopal K, Saha RN, Babbar AK, Sharma RK. (2008). Etoposide loaded PLGA and PCL nanoparticles II: biodistribution and pharmacokinetic after radiolabeling with Tc-99m. Drug Deliv, 15, 277-287.
40. Hasani S, Pellequer Y, Lamprechi A. (2009). Selective adhesion of nanoparticles to inflamed tissue in gastric ulcers. Pharm Res, 26, 1149-1154.
41. Lamprecht A, Schafer U, Lehr CM. (2001). Size dependent targeting of micro-and nanoparticles carriers to the inflamed colonic mucosa. Pharm Res, 18, 788-793.
42. Blouza IL, Charcosset C, Sfar S, Fessi H. (2006). Preparation and characterization of spironolactone-loaded nanocapsules for paediatric use. Int J Pharm, 325, 124-131.
43. Aktas Y, Andrieux K, Alonso MJ, Calvo P, Gursoy RN, Couvreur P, Capan Y. (2005). Preparation and in-vitro evaluation of chitosan nanoparticles containing a capase inhibitor. Int J Pharm, 298, 378-383.
44. Wilson B, Samanta MK, Santhi K, Sampath KKP, Paramakrishnan N, Suresh B. (2008). Targeted delivery of tacrine into the brain with polysorbates 80-coated poly (n-butylcyanoacrylate) nanoparticles. Eur J Pharm Biopharm, 70, 75-84.
45. Sapra P, Moase EH, Ma J, Allen TM. (2004). Improved therapeutic responses in a xenograft model of human B lymphoma (Namalwa) for liposomal vincristine versus liposomal doxorubicin targeted via anti-CD19 IgG2a or Fab fragments. Clin Cancer Res, 10, 1100-1111.
46. Kawakami K, Kawakami M, Puri RK. (2002). IL-13 receptor-targeted cytotoxin cancer therapy leads to complete eradication of tumors with the aid of phagocytic cells in nude mice model of human cancer. J Immunol, 169, 7119-7126.
47. Nakase M, Inui M, Okumura K, Kamei T, Nakamura S, Tagawa T. (2005). p53 gene therapy of human osteosarcoma using a transferring-modified cationic liposome. Mol Cancer Ther, 4, 625-631.
48. Wang X, Yang L, Chen ZG, Shin DM. (2008). Application of nanotechnology in cancer therapy and imaging. CA Cancer J Clin, 58, 97-110.

49. Zamboni WC. (2005). Liposomal, nanoparticle, and conjugated formulations of anticancer agents. Clin Cancer Res, 11, 8230-8234.
50. Li SD, Chen YC, Hackett MJ, Huang L. (2008). Tumor-targeted delivery of siRNA by self-assembled nanoparticles. Mol Ther, 16, 163-169.
51. Lewinski N, Colvin V, Drezek R. (2008). Cytotoxicity of nanoparticles. Small, 4, 26- 49.
52. Florence AT. (2005). Nanoparticle uptake by the oral route: Fulfilling its potential?. Drug Discov Today Technol, 2, 75-81.
53. Hussain N, Jaitley V, Florence AT. (2001). Recent advances in the understanding of uptake of microparticulates across the gastrointestinal lymphatics. Adv Drug Deliv Rev, 50, 107-142.
54. Hasani S, Pellequer Y, Lamprechi A. (2009). Selective adhesion of nanoparticles to inflamed tissue in gastric ulcers. Pharm Res, 26, 1149-1154.
55. Hoet P, Hohlfeld IB, Salata OV. (2004). Nanoparticles-known and unknown health risks. J Nanobiotechnol, 8, 12.
56. Service RF. (2003). Nanomaterials show signs of toxicity. Science, 300, 243.
57. Batist G, Ramakrishnan G, Rao CS, Chandrasekharan A, Gutheil J, Guthrie T, Shah P, Khojasteh A, Nair MK, Hoelzer K, Tkaczuk K, Park YC, Lee LW. (2001). Cardiotoxicity and preserved antitumour efficacy of liposome-encapsulated doxorubicin and cyclophosphamide compared with conventional doxorubicin and cyclophosphamide in a randomized, multicenter trial of metastatic breast cancer. J Clin Oncol, 19, 1444-1454.

Chapter 2. Drug Profile

2.1 Introduction

Paclitaxel is a unique natural diterpene pseudoalkaloid, isolated from the bark of pacific or western yew tree (*Taxus brevifolia*). Paclitaxel is one of the most effective antitumor agents developed in the past four decades and it is the first drug identified by the US National Cancer Institute in 1967. Paclitaxel is the first line chemotherapeutic agent for breast, lung and ovarian cancer and a second line drug of choice for Kaposi sarcoma in HIV patients. Paclitaxel shows cytotoxicity on various tumor cell lines, MCF-7, BT4C rat glioma, C6 glioma, 4T1 murine epithelial breast cancer, human epithelial colorectal adenocarcinoma and human hepatocellular carcinoma, HepG2 and HuH-7 (1-5).

Paclitaxel has poor solubility in water (less than 1 µg/mL) and also low solubility in other pharmaceutical solvents used for i.v administration (Table 2.1). The drug does not form any salt and solubility is pH independent as it has no ionizing group (Fig. 2.1). The drug has narrow therapeutic index and comes under BCS class IV drug and poor permeability (log P 3.5). Paclitaxel is substrate for several enzymes and P-glycoprotein leading to poor bioavailability (<10 %). Thus, Paclitaxel is an ideal representative drug for any formulation scientist to work with for better delivery and improved therapeutic efficacy (6-8).

Table 2.1 Physico-chemical properties of Paclitaxel

S.NO	Parameters	Description
1	Generic name	Taxol
2	CAS registry number	33069-62-4
3	Empirical formula	$C_{47}H_{51}NO_{14}$
4	Molecular weight	853.92 g/mol
5	Melting point	213-220°C
6	Appearances	White to off-white crystalline powder
7	Log P	3.5
8	Water solubility	Paclitaxel is highly lipophilic drug which is practically insoluble in water. The reported aqueous solubility of Paclitaxel was 0.7 µg/mL. Paclitaxel is highly soluble in triacetin (75 mg/mL), ethanol (~39 mg/mL) and 75 % PEG 400 (31 mg/mL)

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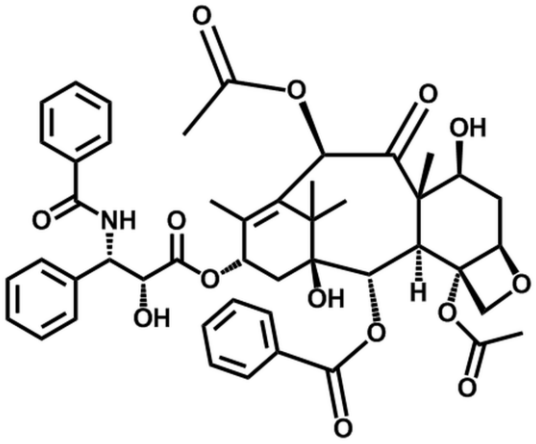


Fig. 2.1 Chemical structure of Paclitaxel

2.2 Mechanism of action

Paclitaxel is the preferred drug because of its unique mechanisms of action; (a) promoting the formation of microtubules (Polymerization) from tubulin dimers, even in the absence of factors that are normally required for microtubule assemble (e.g. guanine triphosphate) and (b) stabilizing the microtubules by binding to the N-terminal 31 amino acid of the beta-tubulin subunit in the microtubules rather than to tubulin dimers, hence prevent depolymerization of microtubules.

In normal cell cycle, microtubules play important role in the formation of the mitotic spindle during cell division and it is involved in many vital interphase functions including, maintenance of shape, motility, signal transmission and intracellular transport. The microtubules formed in the presence of Paclitaxel (Fig. 2.2) are extraordinarily stable and dysfunctional. These microtubules gets accumulated in cells and thereby causing the death of the cells by disrupting the normal microtubule dynamics required for cell division and vital interphase processes. In cancer growth, progression and metastasis are angiogenesis dependent. Anti-angiogenic property of Paclitaxel inhibits vasculature, making the environment unfavorable for cell growth and proliferation. The above cascade action stops cell replication in the late G2 and M phase of the cell cycle (9-11).

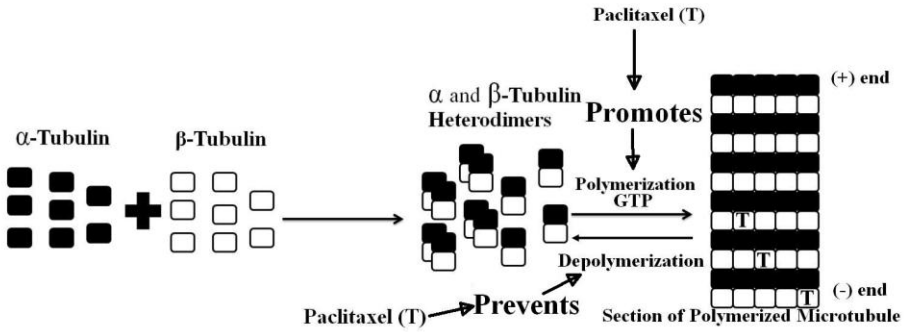


Fig. 2.2 Paclitaxel mechanism of action

2.3 Pharmacokinetics and metabolism

In humans, when Paclitaxel was administered at a dose of 175 mg/m^2 for 3 hr, mean distribution phase half-lives were reported to be, $t_{1/2\alpha}$ = 16 min and $t'_{1/2\beta}$ = 140 min respectively. The other pharmacokinetic parameters were found to be, CL = 12.69 l/hr/m^2, C_{max} = 4.27 µmol/l, AUC = 16081 µmol/l hr, Vss = 99.25 l/m2, urinary excretion, <10 % of dose and fecal excretion 70 % of dose respectively. Adults who received a 24 h infusion of Paclitaxel (135 mg/m^2) achieved C_{max} range of 0.053– 0.077 µmol/l and when the duration of i.v infusion was decreased from 24 to 3 hr, the pharmacokinetic profile of Paclitaxel became nonlinear because drug elimination and tissue binding was saturated (2, 9).

Paclitaxel major pathway of elimination is hepatic metabolism followed by biliary excretion and less than 10 % of the Paclitaxel is eliminated unchanged through the kidney. The cytochrome P450 enzymes, CYPs 3A4, 3A5, 1A2, and CYP2C8 in the liver play important role in the metabolism of Paclitaxel. The metabolic disposition of Paclitaxel is through hydroxylation at the C6 and the C3′-para position leading to the formation of 3 major metabolites, 6α-hydroxy-paclitaxel, 3′-p-hydroxy-paclitaxel and 6α, 3′-p-hydroxy-paclitaxel, the metabolites of Paclitaxel is inactive or much less cytotoxic than the parent compound Paclitaxel. These major metabolites were not found in rat and mice, where Paclitaxel is excreted unchanged in feces. It can be expected that patients with impaired liver function will suffer from enhanced toxicity due to changes in metabolism and elimination (9, 12).

2.4 Pharmacodynamics

Paclitaxel has been found to be highly useful against breast, ovarian, lung and several other tumour types.

2.4.1 Ovarian cancer

Paclitaxel at a dose of 135 and 175 mg/m^2 for 24 hr was approved by regulatory for drug refractory and recurrent ovarian cancer. Paclitaxel is also used as a second line drug for the palliative treatment of recurrent or refractory ovarian carcinoma. Cisplatin (75 mg/m^2) was the first agent to be used in combination with Paclitaxel at 135 mg/m^2/24 hr infusion for ovarian cancer. When compared to Cyclophosphamide (750 mg/m^2) and Cisplatin (75 mg/m^2) combination, Paclitaxel and Cisplatin combination was superior in terms of survival of patients. Carboplatin has also been combined with Paclitaxel in several studies for ovarian cancer, where less myelosuppression, especially thrombocytopenia was observed and this combination is also effective in lung cancer patients (13, 14).

2.4.2 Breast cancer

Significant antitumor activity in women with metastatic breast cancer was observed when Paclitaxel was administered at 200-250 mg/m^2 for 24 hr. Holmes and her colleagues (15) reported that Paclitaxel have a response rate of 56% in 25 women with metastatic disease, 14 of whom had received prior treatment for metastases. Since then there have been numerous clinical studies of Paclitaxel in the treatment of patients with breast cancer. In general, response rates have been higher (32 to 62%) in patients for whom Paclitaxel was first line metastatic therapy than in those receiving this treatment (21 to 48%) as second line or subsequent therapy (9, 10).

2.4.3 Lung cancer

Paclitaxel as monotherapy/combination therapy was evaluated in previously untreated patients with advanced non–small-cell lung cancer and small-cell lung cancer, at high starting doses, 200 to 250 mg/m^2 with 24 hr infusions. Response rates with this approach were 21% and 24%, but neutropenia was severe. Subsequently, Paclitaxel at 135 to 225 mg/m^2 doses over 1 to 3 hr infusion have been studied in a similar population where response rate activity was in the range of 25% with less myelosuppression. In various studies, Paclitaxel has also been combined with Cisplatin, Carboplatin, Ifosfamide and Vinorelbine for lung cancer treatment. Paclitaxel at 250 mg/m^2 for 24 hr with granulocyte colony stimulating factor has demonstrated activity in patients with locally recurrent and metastatic squamous cell carcinoma of the head and neck who have received no prior chemotherapy (9).

2.5 Dose, dosing schedule and precautions

In general, Paclitaxel is given at a dose of 135 or 175 mg/m^2 as infusion for every 3 weeks. The dose and infusion schedule varies, based on the condition and severity of the patients. Paclitaxel can be used at 135 to 250 mg/m^2 dose for 3 to 96 hr for breast cancer treatment as single agent. In case of ovarian cancer, Paclitaxel can be used at 110 to 250 mg/m^2 dose for 3 to 24 hr infusion. As a single agent trial, Paclitaxel can be administered at 175 to 250 mg/m^2 for 3 to 24 hr infusion for non-small-cell and small-cell lung cancer. Paclitaxel is prescribed for squamous cell carcinoma of the head and neck at 175 to 250 mg/m^2 with or without granulocyte colony stimulating factor for 3 to 24 hr infusion. The maximum tolerated dose of Paclitaxel administered by 3 hr infusion to patients with solid tumors was found to be 225–240 mg/m^2 (1, 9).

2.6 Development of resistance to Paclitaxel

Development of resistance of cancer cells to anti-cancer drugs is very common during chemotheraphy and thus requires increase of the dose to maintain efficacy. Paclitaxel develops

resistance by, multidrug resistance-1, drug efflux transporter, breast cancer resistance protein, over expression of oncogene c-erb-B-2, modification of apoptosis signaling sensitivity and b-tubulin expression. (16-18).

2.7 Drug-Drug interactions

When Paclitaxel is co-administered with drugs whose major elimination is through hepatic pathway or by cytochrome P450 enzyme systems, the metabolism of Paclitaxel is expected to be modified. Non-antineoplastic drugs which are specific substrates of these enzymes may therefore have an impact on the pharmacological and pharmacokinetic profiles, toxicity and antitumour efficacy of Paclitaxel. In addition, sequence dependent toxicity was observed when Paclitaxel is given over 24 hr or longer with cisplatin, cyclophosphamide and doxorubicin. In some study, decrease in the clearance of Paclitaxel was observed when administered after cisplatin and in addition myelosuppression is more severe when Paclitaxel precedes cyclophosphamide. Paclitaxel when administered with carboplatin, there was no change in the pharmacokinetics of both the drugs, but thrombocytopenia decreased. Paclitaxel shows incompatibilities with Amphotericin B, Chlorpromazine HCl, Hydroxyzine HCl, Methylprednisolone sodium succinate and Mitoxantrone HCl. When maximum tolerated dose of R-verapamil is co-administered with Paclitaxel, patients show hypotension and bradycardia (9, 19, 20).

2.8 Toxicity

2.8.1 Hematological toxicity

Neutropenia is the principal hematological toxicity of Paclitaxel (21) and its onset is usually on day 8 to 10 after treatment. In such conditions, complete recovery occurs by day 15 to 21. Paclitaxel does not irreversibly damage immature hematopoietic cells and hence it is not a cumulative effect. Paclitaxel produces severe neutropenia in patients when it is administered above 200 mg/m^2 for 24 hr. In general, granulocyte colony stimulating factor is commonly given to prevent the complications of neutropenia when the dose is above 200 mg/m^2. The maximal tolerated dose without granulocyte colony stimulating factor is 175 to 200 mg/m^2 for 24 hr infusion (9).

2.8.2 Neurotoxicity

Paclitaxel induces peripheral neuropathy, after 24 to 72 hr treatment with dose greater than 250 mg/m^2. In case of conventional dose (135 to 250 mg/m^2) neurotoxicity occur only after multiple courses. In addition, optic nerve disturbances, transient myalgia, and myopathy have been noted with high doses of Paclitaxel (9).

2.8.3 Cardiac toxicity

Paclitaxel causes disturbances in cardiac rhythm in addition to myocardial infarction, cardiac ischemia, atrial arrhythmias and ventricular tachycardia. During Paclitaxel treatment, cardiac monitoring is not necessary routinely but it is desirable for patients who may not be able to tolerate the Paclitaxel related potential bradyarrhythmic effects. In addition, patients with atrioventricular conduction defects or ventricular dysfunction should be monitored for their cardiac functions during Paclitaxel therapy. As like other chemotherapeutic agents, Paclitaxel induces reversible alopecia of the scalp and hair loss all over the body with cumulative therapy (9).

2.9 Delivery systems and problems

Paclitaxel is currently marketed as Taxol® (Bristol-Myers Squibb Co, NY, USA) i.v solution (5 mL, single-dose vials) by dissolving Paclitaxel (6 mg mL^{-1}) in Cremophore EL and dehydrated alcohol in the ratio of 1:1 v/v. Cremophore EL is a low molecular weight surfactant (BASF, NJ, USA) which is used in high amount in Taxol® as solubility enhancing vehicle (9). In order to minimize the hypersensitivity reactions of Taxol® vehicle, patients are administered high dose of dexamethasone (adrenocortical hormone), diphenylhydramine (antihistamine) and cimetidine (H2 antagonist) in advance, which is an additional burden for the patients and increase the cost of treatment. Treating cancer patients is a clinical challenge, moreover these formulation related issues creates additional pressure to the oncologist while treating them (22).

In drug discovery, the development cost of a new drug may be about $250-300 million and it takes about 12-15 years to reach the market, whereas an existing drug molecule can get a second life with novel drug delivery systems that can be developed in half the time and with only 20 % of cost of a new drug discovery. This made the formulation scientist to work for alternative novel drug delivery systems and hence there is plenty of literature in the last 5 years in the development of novel Paclitaxel formulations, liposomes, nanoparticles, micelles, microemulsion, cyclodextrin complex, prodrug for both oral and i.v. route (23). The outcome of the growing demand, interest and investment in the development of alternate formulation for Paclitaxel results in novel biocompatible and biodegradable Paclitaxel formulations, Abraxane®, (Abraxis BioScience Inc) solvent free, albumin bound 130 nm NPs and Xyotax®, a poly (L-glutamic acid)-based Paclitaxel conjugate in the clinical use. Abraxane has been approved by FDA in 2005 for the treatment of metastatic breast cancer. But still it is not the complete alternative for the current problems what Taxol is facing. In the phase III clinical trial, patients who are in the Abraxane receiving group

developed neutropenia and sensory neuropathy (24). Hence further research is required for alternative formulation which should be reasonable to the patients.

2.10 Conclusion

Paclitaxel is one of the most effective anti-cancer drugs developed in the past four decades. Paclitaxel has been clinically investigated extensively and more mature clinical trial data were available, including several first-line combination therapy trials. Paclitaxel shows antitumor activity on various tumor cell lines, MCF-7, BT4C rat glioma, C6 glioma, 4T1 murine epithelial breast cancer, human epithelial colorectal adenocarcinoma cells and HCC cell lines, HepG2 and HuH-7 etc. The wide range of activity makes Paclitaxel most effective antitumor agent. In the commercial formulation, Cremophore EL is used in excess to dissolve Paclitaxel, which produces hypersensitivity reactions to patients. Thus, there is a need for making better delivery systems to deliver Paclitaxel safely and efficiently.

Reference

1. Brittain HG. Profiles of drug substances, excipients, and related methodology. ln: Jauhari S, Singh S, Dash AK, editors. Paclitaxel. UK: Academic Press; 2009. 299-344.
2. Dhillon T, Stebbing J, Bower M. (2005). Paclitaxel for AIDS-associated Kaposi's sarcoma. Expert Rev Anticancer Ther, 5, 215-219.
3. Brunetti AE, Attilio G, Vito L, Carla M, Angela S, Ilaria M, Nicola S. (2012). Complete response to second line paclitaxel every 2 weeks of eyelid Kaposi sarcoma: a case report. Ophthal Plast Reconstr Surg, DOI: 10.1097/IOP.0b013e3182776f32.
4. Kim SY, Kim DH, Lee HJ, Seo YJ, Lee JH, Lee Y. (2011). Treatment of disseminated classic type of Kaposi's sarcoma with paclitaxel. Ann Dermatol, 23, 504-507.
5. Liang L, Lin SW, Dai W, Lu JK, Yang TY, Xiang Y, Zhang Y, Li RT, Zhang Q. (2012). Novel cathepsin B-sensitive paclitaxel conjugate: higher water solubility, better efficacy and lower toxicity. J Control Release, 160, 618-629.
6. Sivacharan K, Bende G, Snehalatha M, and Saha RN. (2010). Application of rotatable central composite design in the preparation and optimization of poly (lactic-c*o*-glycolic acid) nanoparticles for controlled delivery of paclitaxel. Drug Dev Ind Pharm, 36, 1377-1387.
7. Panchagnula R. (1998). Pharmaceutical aspects of paclitaxel. Int J Pharm, 172, 1-15.
8. Biswas S, Dodwadkar NS, Deshpande PP, Torchilin VP. (2012). Liposomes loaded with paclitaxel and modified with novel triphenylphosphonium-PEG-PE conjugate possess low

toxicity, target mitochondria and demonstrate enhanced antitumor effects in-vitro and in-vivo. J Control Release, 159, 393-402.

9. Rowinsky K, Donehower R. (1995). Paclitaxel (Taxol). N Engl J Med, 332, 1004-1014.
10. Yao H, Ju R, Wang X, Zgang Y, Li R, Yu Y, Zhang L, Lu W. (2011). The antitumor efficacy of functional paclitaxel nanomicelles in treating resistant breast cancers by oral delivery. Biomaterials, 32, 3285-3302.
11. Dhanikula AB, Panchagnula R. (1999). Localized paclitaxel delivery. Int J Pharm, 183, 85-100.
12. Huizing MT, Giaccone G, van Warmerdan LJC. (1997). Pharmacokinetics of paclitaxel and carboplatin in a dose escalating and dose sequencing study in patients with non-small cell lung cancer. J Clin Oncol, 15, 317-329.
13. Sarosy G, Kohn E, Stone DA. (1992). Phase I study of taxol and granulocyte colony-stimulating factor in patients with refractory ovarian cancer. J Clin Oncol, 10, 1165-1170.
14. Kohn EC, Sarosy G, Bicher A. (1994). Dose-intense taxol: high response rate in patients with platinum-resistant recurrent ovarian cancer. J Natl Cancer Inst, 86, 18-24.
15. Holmes FA, Walters RS, Theriault RL. (1991). Phase II trial of taxol, an active drug in the treatment of metastatic breast cancer. J Natl Cancer Inst, 83, 1797-1805.
16. Cabral F, Wible L, Brenner S, Brinkley BR. (1983). Taxol-requiring mutant of Chinese hamster ovary cells with impaired mitotic spindle assembly. J Cell Biol, 97, 30-39.
17. Horwitz SB, Lothstein L, Manfredi JJ, Mellado W, Parness J, Roy SN, Schiff PB, Sorbara L, Eheb R. (1986). Taxol: mechanisms of action and resistance. Ann NY Acad Sci, 466, 733-744.
18. Gupta RS. (1983). Taxol resistant mutants of chinese hamster ovary cells: genetic biochemical, and cross-resistance studies. J Cell Physiol, 114, 137-144.
19. Nannan PVR, Huizing MT, Willemse PHB. (1997). Hepatic metabolism of paclitaxel and its impact in patients with altered hepatic function. Semin Oncol, 24, 34-38.
20. Huizing MT, Sewberath MVH, Pieters RC. (1995). Taxanes: a new class of antitumor agents. Cancer Invest, 13, 381-404.
21. Rowinsky EK, Eisenhauer EA, Chaudhry V, Arbuck SA, Donehower RC. (1993). Clinical toxicities encountered with paclitaxel (Taxol). Semin Oncol, 20, 1-15.
22. Zhan C, Bing Gu, Cao Xie, Jin Li, Liu Yu, Weiyue Lu. (2010). Cyclic RGD conjugated poly (ethylene glycol)-co-poly (lactic acid) micelle enhances paclitaxel anti-glioblastoma effect. J Control Release, 143, 136-142.

23. Dhanikula B, Singh DR, Panchagnula R. (2005). In-vivo pharmacokinetic and tissue distribution studies in mice of alternative formulations for local and systemic delivery of paclitaxel: gel, film, prodrug, liposomes and micelles. Curr Drug Deliv, 2, 35-44.
24. Soma D, Kitayama J, Konno T, Ishihara K, Yamada J, Kamei T, Ishigami H, Kaisaki S, Nagawa H. (2009). Intraperitoneal administration of paclitaxel solubilized with poly (2-methacryloxyethyl phosphorylcholine-co n-butyl methacrylate) for peritoneal dissemination of gastric cancer. Cancer Sci, 100, 1979-1985.

Chapter 3. Analytical and Bio-analytical Methods

3.1 Introduction

In course of research and development of any drug delivery systems, estimation of drug is a fundamental practice and for this, the formulation scientists need a suitable and highly sensitive analytical method. The analytical method significantly influences the product development process. A simple, rapid, accurate, precise and stability indicating method will make the product development process faster with greater success. Therefore development of analytical method is very much important before starting designing any drug delivery systems (1).

Estimation of drug content in bulk, drug loaded NPs and there respective drug release in the dissolution medium is a routine work in the development of NPDDS. Further, estimation of drug in plasma and other biological samples is another important aspect of product development. Hence, there is a need for more simple and sensitive analytical method and procedure to determine Paclitaxel in different matrix samples. Chromatographic analysis technique is very basic and sensitive method, explored extensively in the research and development of any drug delivery systems (2-3). In the present work, apart from UV-spectrophotometric method, liquid chromatographic (LC) analytical and bioanalytical method was developed and validated for the estimation of Paclitaxel in bulk and other samples. The developed methods were successfully applied for the estimation of Paclitaxel in NPs, dissolution samples and in different biomatrix.

3.2 Method I: UV-Spectrophotometric method

3.2.1 Experimental

a) Materials and Methods

A generous gift sample of Paclitaxel (assay 99.95 %) was provided by Getwell Pharmaceuticals, Gurgaon, India. Solvents acetonitrile, ethanol and dimethylsulfoxide (DMSO) used were of HPLC grade from Spectrochem, Mumbai, India. All other chemicals used were of analytical grade procured from S.D. Fine Chemicals Ltd, Mumbai, India. PCL (molecular weight 40, 000), PLGA (50:50, inherent viscosity 0.15-0.25dL/g (lit), average molecular weight 5,000-15,000), PLA (inherent viscosity 0.55-0.75dL/g (lit), average molecular weight 75,000-120,000) and Pluronic F 68 (PF68, average molecular weight ~ 8,400) were purchased from Sigma-Aldrich chemicals, Bangalore, India.

b) Instrument

A double-beam Jasco (Tokyo Japan) UV-spectrophotometer model V630 and for inter-instrument repeatability study**,** a double-beam Jasco (Tokyo Japan) UV–VIS–NIR (near IR) spectrophotometer model V570 was used.

3.2.2 Method development and validation

a) Preparation of stock, calibration curve and quality control (QC) standards

A master stock solution of Paclitaxel (1mg/mL) was prepared in ethanol. To get desired calibration curve standards, 6-34 µg/mL, different aliquots (30–170 µL) were taken from master stock solution into a series of standard 5 mL volumetric flask and volume made up through addition of acetonitrile and water, 85:15 % v/v. On three consecutive days of validation**,** three separate series of eight calibration curve standards were prepared fresh and their absorbance was recorded in fixed wavelength mode at 229 nm keeping diluent as blank. QC samples were prepared at three concentrations, lower (LQC 6 µg/mL) medium (MQC 18 µg/mL) and higher (HQC 30 µg/mL) from the master stock solution like the calibrator sample.

b) Selectivity

Selectivity is performed by spiking 50 and 100 % of Paclitaxel as per the label claim (6 mg/mL) into the PCL placebo NPs and the spectrum was compared with the freshly prepared pure drug calibrator sample 6 and 18 µg/mL (n=5). The overlaid spectrum and accuracy in terms of % recovery confirm the selectivity of the method. Paired t-test was performed at 95 % level of significances to the spiked sample and to the pure drug sample.

c) Precision

Precision of the developed method was tested with repeatability with intra-day precision and reproducibility by intermediate (inter-day) precision. Intra-day precision experiment was performed by analyzing six separate QC samples within a day on three different occasions, which gives the precision with in the same concentration (n=6) in a day and between the same set of concentrations (n = 18) in a day. Inter-day precision was performed with the QC samples between days for three days (n=18). In addition, UV-spectrophotometric method was validated for inter-instrument repeatability where six set of QC samples were analyzed using a different instrument with same specification. The precision of the developed method was represented by actual mean concentration and % RSD.

d) Accuracy

In addition to the intra and inter day accuracy study, the accuracy of the developed method was supported by studying the Paclitaxel recovery by two unique techniques, the placebo spiking and standard addition using NPDDS. Placebo spiking was performed by adding a known amount of drug from standard solution at five different concentrations, 6, 9, 12, 15, 18 µg/mL to placebo nanoparticles prepared by nanoprecipitation using PCL as polymer and PF 68 as stabilizer. Standard addition was done with three concentrations (6, 12 and 18 µg /mL) of pure drug solutions

to pre-analyzed (10 and 12.2 µg/mL) drug loaded nanoparticles. The accuracy results were represented by mean absolute recovery, % RSD and % Bias.

e) Sensitivity

The sensitivity of the developed UV-Spectrophotometric method was represented by limit of detection (LOD) and limit of quantitation (LOQ). The LOD and LOQ were determined using the standard formula $3.3\ \sigma s^{-1}$ and $10.00\ \sigma s^{-1}$ respectively, where s- is the slope of the calibration curve and σ- is the SD. of y-intercept of regression equation or SD of intercept.

f) Robustness and stability study

Robustness of the developed UV- Spectrophotometric method was determined by making a small deliberate change to the optimized internal parameter. In this study the diluent composition acetonitrile: water was changed from 85: 15 % v/v to 80: 20 % v/v (Diluent-1) and 90:10 % v/v (Diluent-2) respectively. The robustness result was represented by mean % recovery and % RSD.

The stability of Paclitaxel in the diluent medium (acetonitrile and water 85:15) at 4°C was monitored using the validated UV-Spectrophotometric method. The QC samples were prepared fresh and there respective spectrum was recorded using the developed method. Now all the QC samples were stored at 4°C at a predetermined time point of 8 and 72 hr, once again the spectrum was recorded. The overlay of the 8^{th} and 72^{nd} hr spectrum, over the fresh QC sample spectrum demonstrates the stability of Paclitaxel in the selected diluent. The stock solution stability of Paclitaxel in ethanol at 4°C was determined for one month at 18 µg/mL and it was represented by % recovery.

3.3 Application of the developed method - analysis of formulation

3.3.1 Estimation of Paclitaxel in in-house prepared drug loaded NPs

The developed method was applied to determine the drug content of Paclitaxel in the prepared PLGA, PCL and PLA NPs formulations. The samples were processed in triplicate as per the sample processing procedure given below.

a) Sample preparation and procedure

An accurately weighed amount of freeze dried placebo/ Paclitaxel loaded NPs equivalent to 1 mg of Paclitaxel was taken in 50 mL of volumetric flask. The particles were digested with 25 mL of acetonitrile by ultrasonication using the ultra sonic bath (6.5 L Toshibha Laboratory testing instruments Delhi India) for 30 min and then the volume was made up to 50 mL with acetonitrile. The processed samples were centrifuged in cooling compufuge CPR 24 (REMI India) at 12000

rpm for 20 min at 4°C. The supernatant was suitably diluted and then analyzed by the developed method.

3.4 Result and Discussion

3.4.1 Linearity

The typical best-fit linear regression equation for the method was obtained after mathematical treatment (least square method) of the results by plotting the absorbance vs. the concentration (Fig. 3.1). The obtained regression equation was, absorbance = 0.0363 x concentration of Paclitaxel (µg /mL) + 0.0233. The goodness of fit of regression equation in the selected concentration range, 6-34 µg/mL for Paclitaxel was linear with significant regression coefficient $r^2 = 0.9941$. The absorption recorded for the calibration curve concentrations 6-34 µg/mL (n=9) shows very low % RSD. The back calculated concentration of the obtained absorbance using regression equation illustrated low % bias and error (Table 3.1). The statistical analysis, confidence interval, standard deviation and standard error further confirmed the excellent linearity of Paclitaxel in the concentration range of 6-34 µg/mL (Table 3.2). The ANOVA results of the developed UV method showed that the calculated F-value, 1.95×10^{-4}, was found to be lower than the critical F- value 2.09 at 95% level of significant which supports the best-fit linear equation. The test of the intercept was performed using the t-statistic at 95% confidence interval and the t_{df} value of 0.929 showed that the intercept was not significantly (0.05 level) different from zero**,** thus**,** once again supports the best fit linear equation.

3.4.2 Selectivity

The developed method was found to be selective and it differentiates Paclitaxel from the other excipients substances in the NPs formulation samples (Fig. 3.2). The method produced response only to Paclitaxel at 229 nm (Fig. 3.1 and 3.2A) where it is monitored and quantified in the formulation samples studied. The experimental result of selectivity showed that spectrum of Paclitaxel spiked to PCL placebo NPs at two different concentrations 6 and 18 µg/mL overlaid with the freshly prepared calibrator sample spectrum of the same concentrations respectively. The % recoveries of the analyzed samples were 99.88 ± 1.02 and 100.23 ± 0.45 respectively. These indicated that the excipients in the NPs formulations do not interfere in the estimation of Paclitaxel. The paired t-test result of the developed method showed that the calculated t-value 1.88 was less than the critical t–value 4.30, indicat**e** that there is no significant (P=0.05) differences between mean absorbance/peak area of Paclitaxel spiked PCL NPs and pure drug calibrator samples.

Table 3.1 Calibration data of the developed UV-spectrophotometric method

Concentration (µg/mL)	Average abs ± SD. (229 nm [a])	% RSD[b]	Actual con.[c] (µg/mL)	%Bias	% Error
6	0.234 ± 0.002	0.88	5.81	-3.17	0.36
10	0.385 ± 0.003	0.74	9.95	-0.47	0.30
14	0.538 ± 0.003	0.56	14.18	1.25	0.26
18	0.701 ± 0.005	0.72	18.67	3.71	0.30
22	0.835 ± 0.003	0.36	22.35	1.58	0.15
26	0.905 ± 0.005	0.54	24.29	-6.58	0.22
30	1.135 ± 0.002	0.21	30.62	2.05	0.09
34	1.265 ± 0.011	0.90	34.21	0.62	0.37

[a] Average of nine separate determinations with standard deviation, [b] Percent relative standard deviation, [c] Predicted concentration is calculated from average absorbance

3.4.3 Accuracy

Results of the accuracy study showed % RSD less than 2.29 with mean % recovery range from 99.62 to 102.60 and the % relative error range from 0.39 to 1.08 respectively. The mean absolute % recovery of Paclitaxel in case of placebo spiking ranged from 99.65 to 101.56 with low % RSD (0.21) for the highest concentration, 18 µg/mL and % RSD of 2.30 for the lowest concentration, 6 µg/mL. The mean % recovery of standard addition method was in the range of 99.83 to 102.62 with % RSD less than 2.23. This indicated that the Paclitaxel recovery was high at each time of analysis and the developed extraction procedure was efficient in extracting Paclitaxel and the developed method was accurate in determining Paclitaxel. This was supported by the low % bias range obtained for each technique of the developed method, 0.21 to -0.89 and 0.01 to -0.19 respectively. The important inference from this accuracy study was that there was no significant interference of the excipients (PF 68, PCL, PLGA and PVA) at wavelength 229 nm used for estimation of drug.

3.4.4 Precision

The repeatability study results (% RSD) of the developed UV method ranged from 0.22 to 2.63 respectively. The intra-day precision (% RSD) of the QC samples on three different occasions with in a day were between 0.27 to 2.53. In case of inter-day precision the % RSD were in the range of 1.02 to 1.93. This study result showed that there was no significant variation in absorbance, which demonstrates that the entire calibration range, 6-34 µg/mL has very good repeatability and reproducibility. The repeatability of the QC samples set on each day of validation was less than

Table 3.2 Summary of statistical data of optical characteristics and validation parameters of the developed spectroscopic method

Parameters	Observations
Calibration range	6-34 μg mL^{-1}
Linearity (Regression coefficient)	$r^2 = 0.9941$
Regression equation	absorbance (AU) = 0.0363×Conc.(μg/mL) + 0.0233
Confidence interval of slope (S.E.[a])	0.03613 to 0.03650 (8.92×10^{-5})
Confidence interval of intercept (S.E.)	0.02030 to 0.02632 (1.43×10^{-3})
Standard deviation of intercept ($Ś_c$)	2.68×10^{-4}
Standard deviation of slope	4.28×10^{-3}
t-value for intercept[b] (tab = 2.11)	0.929 (P-value 0.39)
Calculated *F*-value (critical *F*-value)[c]	1.95×10^{-4} (2.09)
Standard error of estimate	2.96×10^{-2}
Limit of detection	0.39 μg/ mL
Limit of quantification	1.18 μg/ mL
Absolute recovery	99.62 – 102.60 %
Precision (%RSD)	Repeatability - 2.63 % (intra-day)
	Intermediate Precision - 1.93 % (inter-day)
System suitability (Optical characteristics)	Molar Absorptivity - 3.15×10^4 L M^{-1} cm^{-1}
	Specific Absorptivity - 3.70×10^{-2} mL μg^{-1} cm^{-1}
	Sandell's sensitivity - 2.70×10^{-2} μg cm^{-2}
Specificity and Selectivity- t_{Cal} (t_{Crit})[d]	1.88 (4.30)
Robustness (mean % recovery ± S.D.)	100.62 ± 1.80 at 232 nm
	101.35 ± 1.61 at 233 nm

[a] Standard error of mean, [b] calculated at 0.05 level of significance, using the test of the intercept ($t_{df} = | C - \alpha | / Ś_c$), [c] Calculated based on one-way ANOVA test at P = 0.05 level of significance, [d] Calculated at P = 0.05 level of significance (d.f. 2) based on paired t-test.

2.63 and the LOQ was always less than 1.95 μg/mL with least % RSD of 0.98 respectively. The concentration range of QC samples during inter-day precision were 5.79 to 6.20 18.21 to 18.22 and 30.34 to 30.61 μg /mL respectively with % RSD ranged from 1.02 to 1.93. The inter-instrument repeatability (% RSD) of the method was in the range of 0.21 to 2.30.

3.4.5 Sensitivity

The LOD and LOQ of the developed method were found to be 0.39 and 1.18 μg/mL respectively. The developed calibration curve of the method has showed high magnitude of slope (0.0363 ±

0.00027), less SD of intercept (0.0043) and low standard error of mean slope (0.00009) and intercepts (0.0014). The developed UV method was found to be very sensitive**,** even a small change in concentration can be detected.

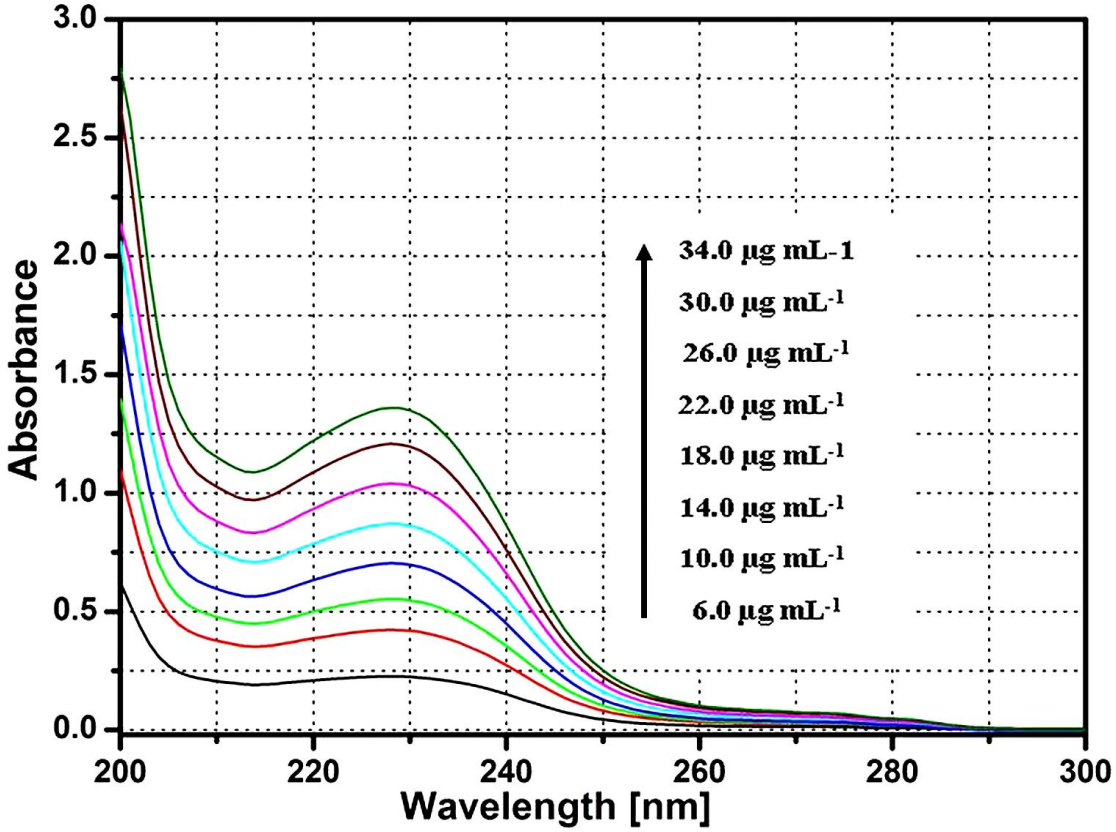


Fig. 3.1 UV-visible absorption spectra of calibration points of paclitaxel

3.4.6 Robustness and stability study

A deliberate change in composition of solvent system or diluent in estimation of Paclitaxel at 229 nm in UV-method showed that there was no significant change in absorbance at the selected variation of concentrations of solvent. The mean % recovery ranged from 99.93 to 102.66 and 99.64 to 102.84 with SD less than 1.25 and 1.75 and % RSD less than 1.26 and 1.70 respectively. The stability of Paclitaxel in the selected diluent was monitored at 4°C by the method. The superimposed spectrum of the 8^{th} and 72^{nd} hr samples over the fresh sample spectrum (Fig. 3.2 B) of the selected QC samples indicated that Paclitaxel is stable over the evaluated time 72^{nd} hr and temperature 4 °C in the selected diluent acetonitrile: water 85:15 %v/v. The stock solution stability result suggested that Paclitaxel was well stable with % recovery of 101.21 ± 0.45 in ethanol for one month at 4 °C.

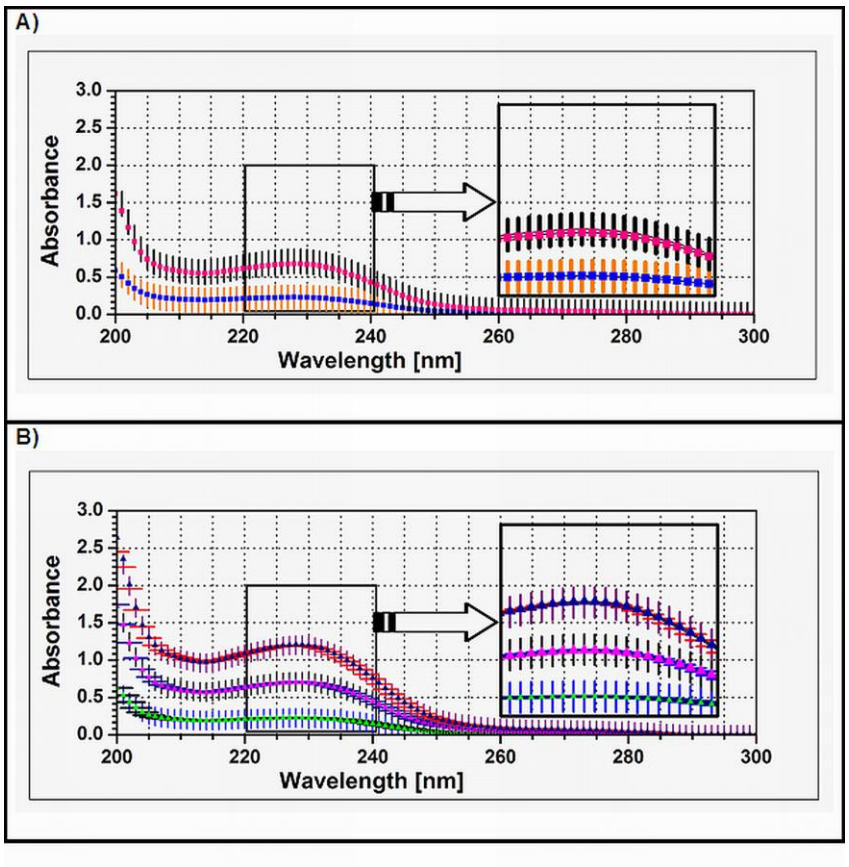


Fig. 3.2 Overlaid selectivity indicating UV-visible absorption spectra of Paclitaxel (ı pure drug at two concentrations 6 and 18 µg/mL; ■ 50 and 150 % drug spiked placebo sample; zoom view of the selectivity absorption spectra is showed in inset) (A), overlaid stability indicating UV-visible absorption spectra of quality control samples (6, 18 and 30 µg/ml) of Paclitaxel in diluent medium (acetonitrile and water 85:15) at 0^{th} hr 8^{th} hr and 72^{nd} hr. (ı Paclitaxel at 0^{th} hr; — Paclitaxel at 8^{th} hr; and ▲ Paclitaxel at 72^{nd} hr zoom view of the spectra is showed in inset spectrum) (B)

3.4.7 Application of the developed method - analysis of formulation

a) Estimation of Paclitaxel in in-house prepared drug loaded NPs

The drug content of Paclitaxel loaded PCL and PLGA NPs determined by the method were found to be 100.89 ± 0.56 and 100.02 ± 0.04 with low % bias of 0.25 to 0.33 respectively. The recovery of Paclitaxel from the NPs by the developed method was close to 100%, which indicated the accuracy of the method in determining Paclitaxel in polymeric NPs. The one-way ANOVA test result showed that there is no significant (95 %) different in concentration determination between the NP formulations and pure drug, this is confirmed by less calculated F-value than the F_{crit}. The F_{calc} for UV method was 0.35 which is less than the F_{crit} 3.88, showing that the developed method was suitable for estimation of Paclitaxel in NPs formulations with high accuracy and precision.

3.5 Method II: LC analytical method

3.5.1 Experimental

a) Materials and Methods

Same as mentioned in UV-spectrophotometric method

b) Instrument

The UFLC (Ultra Fast Liquid Chromatography) system (Shimadzu prominence Japan) consisting of a pump (LC-20AD) with integrated system controller auto sampler (SIL-20AC) and variable wavelength UV detector diode array detector (SPD-M20A). The chromatographic separation of Paclitaxel was carried out in reverse phase Luna C8(2) end capped 250 x 4.60 mm column with 5µm sphere shape particles, 100Å pore size, 400 m2/g surface area, 13.5 % carbon load and calculated bonded phase coverage of 5.50 µmole/m^2 (Phenomenex®USA).

3.5.2 Method development and validation

a) Preparation of stock, calibration curve and QC standards for LC method

Paclitaxel master stock solution of 1 mg/mL in DMSO was prepared and stored in glass ampoules at -20°C. This stock solution was further serially diluted to get working standard from which nine calibration curve standards (10-1500 ng/mL) were prepared. On each day of validation QC samples were prepared at 10, 25, 500 and 1500 ng /mL from independent stock and working standard like the calibration samples. The injection volume of 50µL is used for all HPLC analysis.

b) Selectivity

The selectivity of the developed HPLC method at LLOQ (10 ng/mL) was determined by placebo and spiked-placebo analysis technique. In this method, on three consecutive days chromatograms of the placebo PCL NPs formulation were compared with the fresh chromatogram of drug spiked at LLOQ level in placebo PCL NPs formulation standards and QC standard LLOQ. In addition, the % recovery of Paclitaxel was compared between QC standard LLOQ and drug spiked in placebo PCL NPs. On the other hand the placebo chromatogram was compared with the diluent chromatogram. The PCL NPs formulation standard samples were processed for analysis by the procedure given in the sample preparation and all the samples were prepared in triplicate on each day of analysis.

c) Forced degradation study

i) Oxidative stress treatment (OST)

Paclitaxel was subjected to oxidation by treating with 6 % v/v hydrogen peroxide (HPO). To the 2 mg of drug taken in 5 mL of volumetric flask, 2 mL of HPO (6 % vv) was added and vortexed in

multi holder vortex mixer (SPINIX Multilab India) for 2 min. This 1 mg/mL drug solution was kept at 25°C for 12 hr**,** in a temperature controlled orbital shaking incubator chamber (MAC Macro scientific works (R) Delhi India). The oxidized drug solution was analyzed by the developed HPLC method.

ii) Forced hydrolytic degradation study (FDS)

Paclitaxel was subjected to hydrolysis in acidic and alkaline condition by a microwave based fast and effective technique (1). In a 5 mL volumetric flask, 5 mg of Paclitaxel was mixed with 5 mL of 0.1 N HCl or 0.1 N NaOH for acidic/alkaline hydrolysis. The mixtures were subjected to microwave radiation (LG microwave appliance LG Grill intellowave MG-555F frequency 2450 MHz 2650 W 230v-50 Hz LG Electronics India Pvt.Ltd.) for 20 min by 10 cycle with 2.45 GHz 300W and 80 % intensity. During the radiation treatment, care has been taken to avoid bubbling of solution. Once the treatment was over, the samples were allowed to cool and then neutralized to stop further reaction and then analyzed by the developed HPLC method.

iii) Thermal stress treatment (TST)

Paclitaxel was subjected to thermal stress treatment (4) in liquid state by exposing the drug solution to 90°C in a temperature controlled oven (MAC Macro scientific works (R) Delhi India). Drug solution of 1 mg/mL was prepared in ethanol and exposed to the above temperature for 12 hr and then the sample was suitably diluted and analyzed by the developed method. The selectivity and specificity of the developed method were tested by observing the retention time of Paclitaxel in the degraded sample through chromatogram and by calculating the percent Paclitaxel degraded.

The HPLC method further confirms the selectivity and specificity by constructing counter view graph and ratiogram.

d) Precision

Precision of the developed LC method was determined by repeatability with intra-day precision and reproducibility by intermediate (inter-day) precision. Intra-day precision experiment was performed by analyzing nine separate QC samples within a day on three different occasions. Inter-day precision was performed with the QC samples between days for three days (n=27). The precision of the developed method was represented by actual mean concentration and % RSD.

e) Accuracy

In addition to the intra-day and inter-day accuracy, developed LC method was supported by studying the Paclitaxel recovery by two unique techniques, placebo spiking and standard addition. The placebo spiking was performed by adding a known amount of drug from standard solution at five different concentrations 50, 75, 100, 125 and 150 % of label claim respectively to placebo

PCL NPs. The standard addition was done by adding known amount of drug at five different concentrations 50, 75, 100, 125 and 150 % of label claim from standard solution to the pre-analyzed (150-450 ng/mL) Paclitaxel loaded NPDDS. The accuracy results were represented by mean absolute recovery, % RSD and % Bias.

f) Sensitivity

The sensitivity of the developed LC method was represented by LOD and LOQ. The LOD and LOQ of the developed method were obtained by slope calculation and by signal-to-noise ratio. The LOD and LOQ were determined using the standard formula $3.3\ \sigma s^{-1}$ and $10.00\ \sigma s^{-1}$ respectively, where 's' is the slope of the calibration curve and 'σ-' is the SD of y-intercept of regression equation or SD of intercept. In signal-to-noise ratio method, the LOQ determination was performed on five different days (n=5) by spiking an aliquot from working standard to diluent to get the desired concentration of the lowest calibrator. The results with precision, RSD less than 20 %, accuracy of 80-120% and signal-to-noise ratio of 10:1 confirms the LOQ of the developed method.

g) System suitability

The repeatability or routine analysis of the developed method within the validated limits was checked by system suitability parameters. The parameters used in this study are Capacity factor (k), Tailing factor (T), No of theoretical plates, Height equivalent to theoretical plates (HETP), Effective plate number (Neff), Height equivalent to one effective plate (HEFF) and Reduced plate height (h). All the above parameters were calculated using the standard formula (1).

3.6 Application of the developed method- analysis of formulation

3.6.1 Estimation of Paclitaxel in in-house prepared formulations

The developed LC method was applied to determine the drug content of Paclitaxel in the prepared PLGA and PCL NPs formulations. In addition it is also used to find the amount of Paclitaxel released from the respective NPs formulation in the dissolution medium. The dissolution study was performed in phosphate buffer pH 7.4 with 1 % Tween 80 at 37 ± 0.5°C. The samples were processed in triplicate as per the sample processing procedure given in UV method.

3.7 Result and Discussion

3.7.1 Chromatographic separation

From the preliminary study, millipore water and acetonitrile mixture were chosen for the separation of Paclitaxel in C8 column. Even though ammonium acetate buffer has shown good separation, millipore water was selected as buffer creates additional pressure on the column and

takes more time to wash the column. The Luna C8 column provides significantly improved performance over the traditional C8 phases due to high surface coverage and slightly low carbon load than original Luna C8 (14.75%).

Paclitaxel was estimated selectively, specifically and sensitively at 229 nm by isocratic reverse-phase HPLC method at 1 mL/min flow rate. To decrease the cost of analysis flow rate kept 1 mL/min, lower than reported methods (4, 5). The optimized mobile phase of the developed HPLC method was made acetonitrile and millipore water (60:40 %v/v) which provided moderate and quick retention time (6.66 ± 0.18 min) with better peak properties (Fig. 3.3) and selectivity/specificity for Paclitaxel and degradation products (Fig. 3.4).

In literature there are reports using very complicated and time consuming extraction procedure to estimate Paclitaxel in the NPs and in the in-vitro dissolution sample (8-10). The developed method does not use any complicated extraction procedure to estimate Paclitaxel in NPs and dissolution samples. In the present method it was well established with chromatograms, counter view plot, peak purity determination and ratiogram, even in the presence of Paclitaxel degradation products, Paclitaxel was selectively and specifically estimated and separated from the degradation peaks. In the present method, there were no interferences of excipients (polymers and stabilizers) in the estimation of Paclitaxel were clearly shown by the standard addition and placebo spiking method and their respective chromatograms (Fig. 3.5).

3.7.2 Linearity

The typical representative chromatogram of Paclitaxel calibration curve was illustrated in Fig. 3.3. The obtained best-fit linear regression equations for the concentration range 10-1500 ng/mL was, peak area (mV s) = 211.59 × concentration of Paclitaxel (ng/mL) – 792.26 with regression coefficient (r2) = 0.9999. The % bias ranged from 0.70 to -2.51 for the entire calibration curve concentrations. This low % bias supports the selected linear model with univariant regression and it also shows the goodness of fit (Table 3.3). The % error calculated for the entire range of calibration points were between 0.02 to 0.53 (Table 3.4). The goodness of fit was further supported with the low standard error of estimate (5.72) and low standard error of mean (126.48) respectively. Slope value fell within the 95 % confidence interval of slope limits (Table 3.3) hence the linearity of the developed LC method could be practically in the range of 0-1500 ng /mL.

The present developed method linearity range (10-1500 ng/mL) was very sensitive than the reported methods (5, 11). The linearity range of method reported by Anupama et al (11) for determination of Paclitaxel in amphiphilic polymer matrix was 2-50 µg/mL which is less sensitive to the present developed method. The statistical results illustrate that the calculated F-value (3.11 ×

10^{-5}) was found to be lower than the critical F- value of 2.41 at 95% level of significant which supports the best-fit linear equation. The test of the intercept was performed using the t-statistic at 95% confidence interval the t_{df} value of 6.11x 10^{-6} indicated that the intercept was not significantly (0.05 level) different from zero**,** thus**,** once again supports the obtained best-fit linear regression equations.

3.7.3 Selectivity

The paired t-test result of the developed method showed that the calculated t-value, 0.83 was found to be less than the critical t–value 2.78, which indicate that there was no statistical significant (P=0.05) differences between mean peak area of Paclitaxel spiked PCL NPs and pure drug calibrator samples. The base shift overlay chromatogram (Fig. 3.6) clearly explains that there were no interferences from the excipients used in the PCL NPs over the diluent chromatogram (Fig. 3.6D) at the RT of Paclitaxel, which confirm the selectivity of the developed method. The overlaid chromatogram (Fig. 3.6) of response of Paclitaxel at LLOQ in the calibrator sample and the drug spiked (10 ng/mL) in blank placebo NPs formulation showed that the method selectively and specifically determine Paclitaxel at 229 nm. Finally the % recovery of Paclitaxel at two different samples at the same concentration, the QC standard LLOQ (99. 96 ± 0.21) and drug spiked placebo NPs formulation samples (100.01 ± 0.21) confirmed that the method was selective and specific in determining Paclitaxel in presences of expected excipients at LLOQ levels.

3.7.4 Forced degradation study- peak purity curve and ratiogram

A forced degradation studies were conducted to generate degradation products of Paclitaxel to check for the selectivity and specificity of the developed method. All the degradation samples were analyzed by the developed method and chromatogram were recorded up to 60 min. Paclitaxel is a complex molecule which has numerous hydrolysable ester groups and with a strained oxetane ring which undergoes cleavage in acid catalyzed conditions (12). The presence of ester, amide, acetyl, acetoxy ester, free hydroxyl groups makes Paclitaxel very prone to hydrolysis and oxidation under different stress conditions applied. As per guidelines**,** the degradation study protocols were optimized to make sure that the 100% of Paclitaxel will not be degraded in the given set of stress conditions (13). The developed method not only helps in separating the Paclitaxel from the degradation product but also helps us to determine the drug accurately with high sensitivity (LLOQ, 10 ng/mL) and specificity. The novel microwave forced hydrolytic degradation by acidic and alkaline condition generated hydrophilic and hydrophobic degradation products. Hydrophilic products were eluted at 2.35, 3.55, 3.86, 4.08, 4.95, 5.68, 7.39, 9.61 and 15.06 min and hydrophobic products were eluted at 2.34, 3.87, 6.33 and 6.73 min respectively.

The thermal stress of Paclitaxel generated hydrophilic and hydrophobic degradation products eluting hydrophilic products at 2.68 min and hydrophobic products at 7.64 and 9.11 min respectively. The oxidative stress treatment of Paclitaxel generated only hydrophilic degradation products which eluted at 4. 10 min. The developed HPLC method separates Paclitaxel from all the degradation products completely which was clearly shown by the counter view graph (Fig. 3.4) and ratiogram (Fig. 3.5).

There was very less degradation only 19.62 % of Paclitaxel, during oxidative stress treatment among the forced hydrolytic degradation by acidic (91.04 %), alkaline (92.97 %) and thermal stress (67.51 %). The peak purity curve of all the degradation samples clearly showed that the similarity curve did not intersect the threshold curve**,** hence there are no impurities or degradants coeluting with Paclitaxel at the estimated wavelength. The peak purity index (PPI) and single point threshold (SPT) of the degradation samples, FDS-HCL (0.995600 and 0.880975), FDS-NaOH (0.999577 and 0.723763), TST (0.999986 and 0.946592) and OST (1.0000 and 0.992731) were always close to one and the PPI was always greater than the SPT which shows the absences of degradation product co-eluting with Paclitaxel at 229 nm in the developed method. The positive MPPI of all the degradation samples, FDS-HCL, FDS-NaOH TST and OST were 114035, 275813, 53393 and 7268 respectively which shows the specific quantification of Paclitaxel in the degraded sample by the developed method. In addition, the contour view plot (Fig. 3.4) shows that the degradation peaks does not overlap with Paclitaxel peak and the optimum wavelength (229 nm) where Paclitaxel was well separated from the degradation peaks. The developed HPLC method allow analyst to record UV spectra (Fig. 3.4) corresponding to specific time period when the sample is analyzed chromatographically. The recorded UV spectrum of the entire degraded sample (Fig. 3.4 D, G and H) is similar to the pure Paclitaxel (Fig. 3.5 E) at the detection wavelength 229 nm. The three point view results of peak purity supports the total peak mode results in determining Paclitaxel specifically by the developed HPLC method. In case of three point view, the upslope purity (UP) value of all the degradation samples, 0.635258, 0.749008, 0.992718 and 0.118460 was always greater than down slope purity (DSP) value 0.116591, 0.563228, 0.323086 and 0.084852 respectively.

This results in positive 3 point purity (PP) value 0.375925, 0.656118, 0.657902 and 0.101656 of all the degradation samples, FDS-HCL, FDS-NaOH TST and OST respectively. The rectangular ratiograms (Fig. 3.5) obtained for the entire degradation sample in selected wavelength 229 nm at the Paclitaxel RT shows that only Paclitaxel was determined specifically by the developed HPLC method. The degradation of Paclitaxel was performed only to show the selectivity and specificity

of the developed HPLC method and hence the degradation products were not separated in the present study. The structural elucidation of the degradation product is out of scope of the paper, but FTIR spectrum for the degraded samples was taken. The FTIR result showed that, Paclitaxel was degraded partially in all the stress conditions. The pure Paclitaxel was selectively and specifically separated and estimated by the developed HPLC method.

3.7.5 Accuracy

The accuracy results of the developed HPLC method showed % RSD less than 0.64 with mean % recovery range from 98.63 to 102.62 and the % relative error ranged from 0.07 to -1.37 respectively (Table 3.5). The mean absolute % recovery of Paclitaxel in case of placebo spiking ranged from 99.81 to 100.37 with low % RSD of 0.04 for the concentration 450 ng/mL and the highest % RSD of 0.21 for the concentration, 150 ng/mL respectively. The mean % recovery of standard addition method was in the range of 99.94 to 100.13 respectively with % RSD less than 0.199 respectively. The overlay chromatogram (Fig. 3.6) of the blank placebo NPs formulation standards and the blank diluent chromatogram indicated that there was no interference from the excipients at the retention time (6.64 ± 0.31) of Paclitaxel at 229 nm. In Fig. 3.6 the overlay chromatogram of 300 ng/mL and 100 % addition of label claim once again confirm that there was no interference from the excipients in determining Paclitaxel by the developed LC method. As per literature review this is the first work which describes the accuracy of the developed method in terms of standard addition and placebo spiking for Paclitaxel determination.

3.7.6 Precision

The repeatability study results (% RSD) of the developed HPLC method ranged from 0.06 to 2.35 respectively. The intra-day precision of the QC samples on three different occasions with in a day was always less than 1.14 and with least % RSD of 0.03. In case of inter-day precision the % RSD was in the range of 0.13 to 1.74 respectively. The repeatability of the QC samples set on each day of validation was less than 2.35 respectively. In case of LOQ it was always less than 1.18 with least % RSD of 0.63. This once again showed the sensitivity of the method at the level of LOQ with acceptable limit of % RSD. The inter-instrument repeatability (% RSD) of the developed LC method was in the range of 0.21 to 2.30 respectively. The intra-day and inter-day precision results (% RSD) are very low as compared to Badea et al (10) 1.33 to 3.53 and Anupama et al (11) 1.06 to 1.77 and 0.77 to 1.53 respectively. This study results clearly shows that the developed method can be used routinely in any analytical lab.

Table 3.3 Summary of statistical data of validation parameters of the developed LC method

Parameter	LC Method
Calibration range	10-1500 ng mL^{-1}
Linearity (Regression coefficient)	r^2 = 0.9999
Regression équation	Peak area (mV s) = 211.59 × conc.(ng/mL) – 792.26
95 % Confidence interval of slope (S.E.[a])	210.47 to 212.71 (0.437)
95 % Confidence interval of intercept (S.E)	593.33 to 991.19 (77.42)
Standard deviation of intercept ($Ś_c$)	189.64
Standard deviation of slope	1.07
Slope without intercept	210.78
t-value for intercept [b] (tab = 2.57)	6.11x 10^{-6} (P-value 0.999)
Calculated *F*-value (critical *F*-value) [c]	3.11 × 10^{-5} (2.41)
Standard error of estimate	5.72
Limit of detection	4 ng/ mL (2.96 ng/ mL) [A]
Limit of quantification	10 ng/ mL (8.96 ng/ mL) [B]
Absolute recovery	98.63 – 102.62 %
Precision (%RSD)	Repeatability - 2.35 %
	Intermediate Precision - 1.74 %
Specificity and Selectivity- t_{Cal} (t_{Crit})[d]	0.83 (2.78)
Robustness (mean % recovery ± S.D.)	100.25 ± 0.80 (50:50 % v/v)
	100.24 ± 1.01 (55:45 % v/v)

[a] Standard error of mean, [b] Calculated at p= 0.05 level of significance, using the test of the intercept ($t_{df} = | C - \alpha | / Ś_c$), [c] Calculated based on one-way ANOVA test at P = 0.05 level of significance, [d] Calculated at P = 0.05 level of significance (d.f. 4) based on paired t-test, A-Based on the formula 3.3 σs^{-1}, B-Based on the formula 10 σs^{-1}, where σ- standard deviation of intercept and s-slope of the calibration curve

3.7.7 Sensitivity

The LOD, 4 ng/mL of the developed method satisfies the signal to noise ratio 2:1. The calibration point starts from LOQ (10 ng/mL) as it satisfies the signal to noise ratio condition, 10:1, with precision less than 0.12 % RSD and accuracy 98.63 ± 0.12 %. In addition, the peak properties like retention time 6.64 ± 0.31; peak area 1278.87 ± 3.89 and T ≤ 2 of Paclitaxel at LOQ were not affected during the accuracy and precision study. In Badea et al (10) method LOQ and LOD were

found to be 240 and 72 ng/mL which is high when compared to the present method. The sensitivity of the present HPLC method was high when compared to the recently published method (11, 13) whose LOD and LOQ was 0.17 and 0.5 µg/mL respectively.

3.7.8 System suitability and Paclitaxel stability

The developed LC method was found to be suitable in terms of system performances as per the system suitability evaluation parameter results. The k´ of Paclitaxel in the selected column and mobile phase was 3.36, which was greater than 2 and it showed that the Paclitaxel spends enough time to get optimum resolution with the selected stationary phase versus the mobile phase. The k´ value above 1.00 showed that the Paclitaxel was well separated from the solvent peak. Highest number of theoretical plates 11016.6 showed the sharpness of the peak and therefore the efficiency of the column. The higher the number of plate, the column efficiency is more. The column efficiency was supported by the smaller HETP 0.023. The T=1.25, which is less than 2 shows that the Paclitaxel peak was symmetrical. The other system suitability parameters like Neff (6518.7), HEFF (0.038) and h (2203.32) demonstrate the suitability and repeatability of the developed method in the respective conditions.

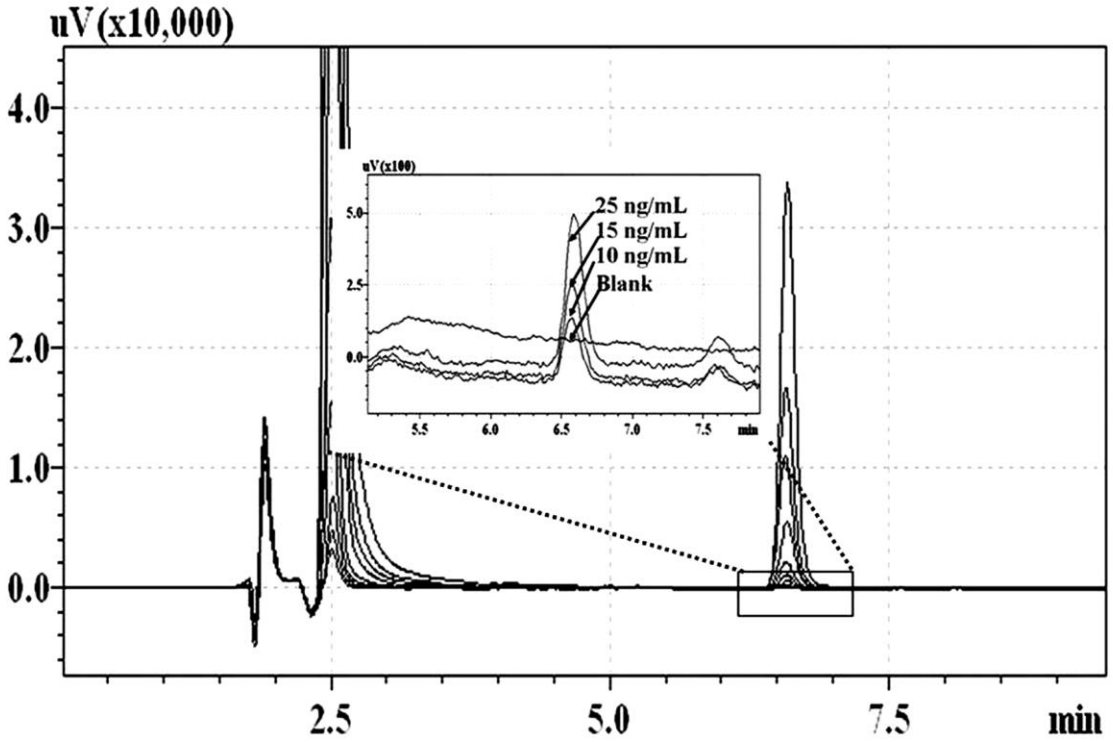


Fig. 3.3 Overlaid chromatograms of calibration points (10-1500ng/mL), the figure inside is the base shift zoom view of 10, 15 and 25 ng/mL concentration

3.7.9 Application of the developed method- analysis of formulation

a) Estimation of Paclitaxel in in-house prepared formulation

The drug content of Paclitaxel loaded PCL and PLGA NPs formulations determined by the developed HPLC method were found to be 101.06 ± 0.75 and 100.03 ± 0.05, with low % bias, indicating the accuracy of the method. The one-way ANOVA test result showed that, F_{calc} for LC method was 0.69 which is less than the F_{crit} 3.88. The mean SS for the developed method was 5.41 x 10^{-3} and 1.55 x 10^{-2} and 3.21 x 10^{-2} and 4.61 x 10^{-2} respectively between and within the group. In literature there are very complicated, time consuming and sophisticated procedures to estimate Paclitaxel content in the polymeric NPs.

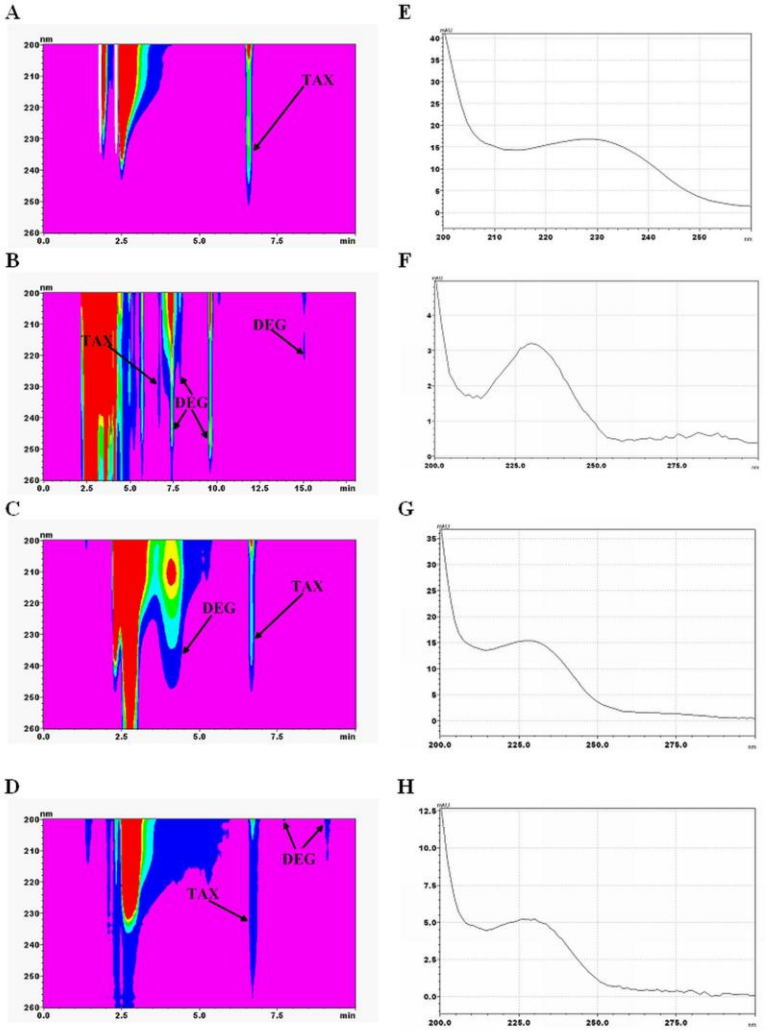


Fig. 3.4 Representative HPLC method, counter view plot and UV spectrum of degradation sample, counter view plot of Standard 750 ng/mL (A) counter view plot of FDS-HCL (B) counter view plot of OST (C) counter view plot of TST (D) UV spectrum of 750 ng/mL (E) UV spectrum of FDS-HCL (F) UV spectrum of OST (G) UV spectrum of TST (H)

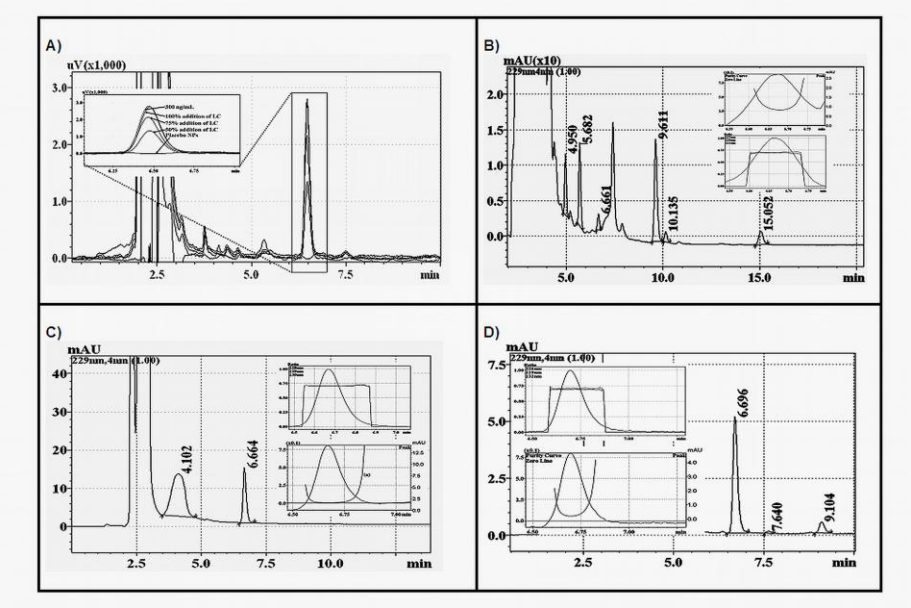


Fig. 3.5 Representative overlaid chromatogram of placebo spiking and standard addition with zoom view (A), representative chromatogram of acid hydrolytic (100 mM HCl) degradation of Paclitaxel with their peak purity curve and ratiogram (B), representative chromatogram of forced oxidative (6 % v/v H_2O_2) degradation of Paclitaxel with their peak purity curve and ratiogram (C), representative chromatogram of thermal stress degradation of Paclitaxel with their peak purity curve and ratiogram (D)

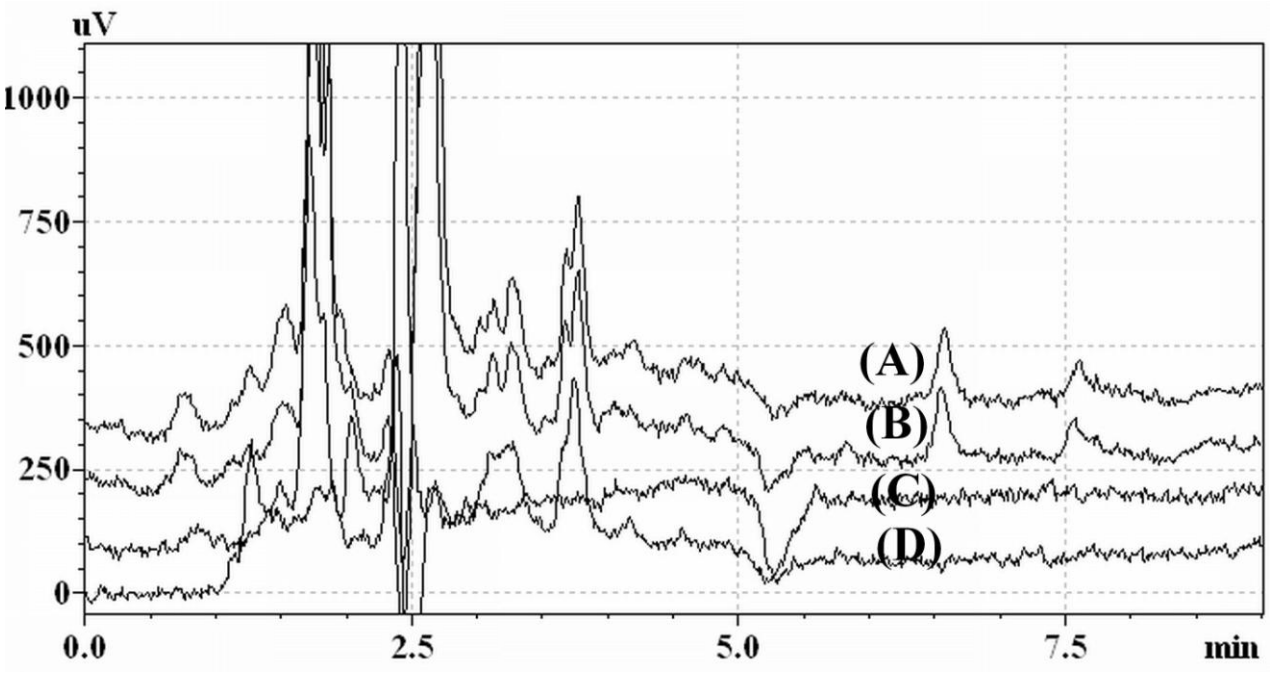


Fig. 3.6 Base shift overlay representative chromatogram demonstrating the selectivity of Paclitaxel determination in presences of excipients, calibration standard LLOQ 10 ng/mL (A) Paclitaxel (10 ng/mL) spiked in blank placebo NPs formulation standards (B) blank placebo NPs formulation standard (C) diluent (D)

Table 3.4 Calibration curve of Paclitaxel by the LC method

Concentration. (ng/mL)	Average absorbs. ± SD. (229 nm [a])	% RSD	Actual con.[b] (ng/mL)	%Bias	% Error
10	1294.67 ± 2.58	0.20	9.86	-1.37	0.08
15	2310.33 ± 19.96	0.86	14.66	-2.25	0.35
25	4449.33 ± 33.66	0.76	24.77	-0.91	0.31
50	9861.33 ± 43.74	0.44	50.35	0.70	0.18
100	19835.17 ± 259.86	1.31	97.49	-2.51	0.53
250	51344.33 ± 18.95	0.04	246.40	-1.44	0.02
500	107776.67 ±199.13	0.18	513.11	2.62	0.08
750	156603.83 ±349.69	0.22	743.87	-0.82	0.09
1500	316469.83 ±1860.76	0.59	1499.42	-0.04	0.24

[a] Average of six separate determinations with standard deviation, [b] Predicted concentration is calculated from average peak area, using regression equation

Zhang et al (14) used complicated and sophisticated sample processing procedure to extract Paclitaxel from NPs and dissolution samples, which is time consuming. Hence, this procedures and method may not be suitable for routine analysis.

Extensive literature search showed, some researchers used liquid-liquid extraction method to extract Paclitaxel from NPs. It has been found that liquid-liquid extraction method had poor efficiency of extraction and it exhibited concentration dependent recovery. Other reported methods (15-22) used complicated extraction procedures like, evaporating the extracted solvent system by using nitrogen stream, using mixture of extracting solvent with buffer and using special solvent system like tetrahydrofuran (29-32). In another report, 1 mL of acetonitrile was added to PLGA NPs and extract Paclitaxel from NPs for 16 hr, the extraction procedure is very simple but the time to extract Paclitaxel is high, hence this method may not be suitable for routine analysis (9). In the present method NPs are digested with acetonitrile with 30 min sonication and Paclitaxel was estimated using the developed LC method with good accuracy, suggesting suitability for routine analysis.

Table 3.5 Accuracy study of LC-method by placebo spiking and standard addition techniques

Product	Technique	Con. of drug in formulation [d] (ng/mL)	Amount of drug added (% of label claim)	Mean calculated Con. (ng/mL)	Total amount recovered ± SD (µg/mL)	Mean absolute recovery (%) (± SD)	% RSD	% Bias[e]
Nanoparticles	Placebo spiking [a, c]	-	50	149.72 ± 0.32	299.44 ± 0.64	99.81 ± 0.21	0.21	-0.19
		-	75	225.18± 0.26	450.37 ± 0.51	100.08 ± 0.11	0.11	0.08
		-	100	301.56 ± 0.93	603.13 ± 1.87	100.37 ± 0.31	0.31	0.37
		-	125	375.15 ± 0.15	750.29 ± 0.29	100.04 ± 0.04	0.04	0.04
		-	150	450.06 ± 0.16	900.13 ± 0.33	100.01 ± 0.04	0.04	0.01
	Standard addition[b]	450.11 ± 0.33	50	299.96 ± 0.19	599.91 ± 0.38	99.99 ± 0.063	0.063	-0.01
		375.25 ± 0.11	75	299.90 ± 0.07	599.81 ± 0.13	99.97 ± 0.022	0.022	-0.03
		299.92 ± 0.11	100	299.88 ± 0.13	599.76 ± 0.26	99.96 ± 0.044	0.044	-0.04
		225.09 ± 0.20	125	300.39 ± 0.60	600.77 ± 1.19	100.13 ± 0.199	0.198	0.13
		150.13 ± 0.06	150	299.82 ± 0.28	599.64 ± 0.56	99.94 ± 0.094	0.094	-0.06

[a] Each value is result of nine separate determinations [b] Each value is result of six separate determinations [c] In-house placebo PCL nanoparticle preparation [d] In-house drug loaded PCL nanoparticle preparation containing 1 mg of equivalent paclitaxel [e] % bias is calculated from the % recovery

3.8 Method III: Bioanalytical LC method

3.8.1 Experimental

a) Materials and Methods

Same as mentioned in UV-Spectrophotometric method

b) Instruments

The instrument used is same as the LC-analytical method. LichroCART 250-4, HPLC-Cartridge, Lichrospher 100 RP-18 e (5 µm), Lot. L57062233 double end-capped RP-HPLC column (MERCK, Germany) fitted with guard column of the same material was used for the separation. All calculations were performed using peak area ratio ($R_U = r_u/r_{is}$), where r_u is peak area of Paclitaxel obtained from a chromatogram and r_{is} is peak area of IS obtained from a chromatogram.

3.8.2 Method development

a) Preparation of stock and standard solution

A master stock solution of 2 mg/ml was prepared in DMSO and stored in glass ampoules at -20° C. Secondary stock solution of 60 µg/mL was prepared by taking aliquot from primary stock and this stock solution was further serially diluted to get working standard solutions in the range of 30-0.1 µg/mL. All the dilutions were made by acetonitrile and water 50:50% v/v. The secondary stock and working solution were prepared fresh daily. The master stock solution of propylparaben (PP) the internal standard (IS) was prepared at a concentration of 100 µg/mL in acetonitrile and stored at -20° C between uses. The working standard of 0.125 µg/mL was prepared from master stock daily in acetonitrile. The precipitation solvent (acetonitrile) containing PP is made ice cold at -80° C for sample processing. The concentration of IS in all samples were maintained at 1 µg/mL. All the stock solutions were prepared freshly on the day of validation and care was taken to store Paclitaxel stock in glass ampoules.

b) Sample collection and preparation

Rat blood samples, approximately 1.5 mL from each animal were withdrawn from more than 15 healthy animals by cardiac puncher under diethyl ether anaesthesia with disposable syringe (Dispovan, India) with 26 G needle. After blood collection, all animals were recovered from anaesthesia. The blood was collected into 2 mL polypropylene microtubes (Tarsone, India) containing EDTA-Na_2 (0.1 mL of 10% solution for 5mL of plasma) for preparation of plasma and the care has been taken to mix the blood with EDTA-Na_2. After centrifugation at 12000 rpm for 15 min at 4° C, plasma were pooled into one tube and stored at -20° C and thawed every time before analysis. Liver, kidney and spleen samples were also collected from rats, stored at -20° C before analysis.

c) Sample processing

To 100 µL plasma samples (drug spiked/real time sample), 800 µL of ice cold acetonitrile containing IS (0.125 µg/mL) was added. This solution was vortexed for 2 min in multi holder vortex mixer (SPINIX Multilab, India) and centrifuged in cooling compufuge CPR 24 (REMI, India) at 12000 rpm for 10 min at 4° C. The total sample processing time before evaporation is 12 mins. The plasma protein precipitated and collected as pellet at the bottom of the tube. The supernatant was taken, dried using a vacuum concentrator (MAXI dry lyo, Heto vacuum centrifuge, Germany) and dry residue was reconstituted with 100 µL of 50:50% v/v acetonitrile and water. The reconstituted solution is made in mobile phase, to avoid possible baseline drifts. The injection volume of 75µL is injected into HPLC. Similarly, liver, kidney and spleen samples were also processed same way for estimation of Paclitaxel in the respective organ samples. Partial validation was carried out in liver, kidney and spleen samples.

d) System suitability parameters

The column efficiency for the separation of Paclitaxel and PP was evaluated by calculating number of theoretical plates (N), k´ and T using the standard formula

3.8.3 Method validation

a) Selectivity and peak purity

Selectivity of the method was studied by investigating the interference from various endogenous matrix components (mainly proteins) and exogenous substance which may come in contact with the sample during the process. Blood from six different rats were collected for this study and the blood samples were processed for plasma and stored at -20° C until analysis. Similarly liver, kidney and spleen samples were also processed to study the selectivity of the method. Six individual samples of drug and IS free plasma (blank), samples with IS (zero samples) and LLOQ (10 ng/mL) were processed individually and analysed by the proposed method.

The obtained chromatograms of blank samples were compared against analytical, calibration standards and real time i.v. pharmacokinetic samples for investigating interference in determination. The condition for LLOQ, the peak area of compound co-eluting with the Paclitaxel or IS should not exceeds 20 % of the Paclitaxel peak area at LLOQ or 5 % of the IS area. The selectivity is further confirmed by peak purity determination by using the ratiograms and purity curve. The ratiograms is constructed by plotting, ratio of absorbance/response of Paclitaxel at two different wavelengths (231 and 235) over retention time (8.0 ± 0.25), the rectangular ratiograms show that the peak is pure.

b) Linearity and quality control samples

Calibration standards in drug free rat plasma were prepared at concentrations 10, 25, 50, 100, 250, 500, 750 and 1500 ng/mL of Paclitaxel from respective working stocks. Calibrator

samples were prepared by spiking 95µL of blank rat plasma with 5 µL of the respective Paclitaxel working stock solutions in 1.5 mL polypropylene micro centrifuge tubes, which satisfied the limit of 5% addition of organic solution to plasma. The calibration samples, consist of a blank sample (matrix sample processed without IS), a zero sample (matrix sample processed with IS) and seven non-zero samples including LLOQ. QC samples were prepared at concentrations 10, 25, 50, 500 and 1500 ng/mL from respective working stock as like the calibrator samples. On each day of validation calibration standards and QC samples were prepared fresh and analysed. Similarly, calibration standards were prepared for liver, kidney and spleen at concentrations 25-1500, 25-750 and 25-750 ng/mL, respectively. The QC sample for liver was 25, 750 and 1500 ng/mL and for kidney and spleen, it was 25, 250 and 750 ng/mL.

c) Determination of LLOQ and LOD

The LLOQ determination was performed on five different days (n=5), by spiking an aliquot of blank rat plasma (95µL) with Paclitaxel (5 µL) at concentration of the lowest calibrator with precision less than 20 %, accuracy of 80-120% and signal-to-background noise ratio greater than 6:1. The LOD was defined as the lowest concentration of Paclitaxel that the method can detect with signal to noise ratio greater than or equal to 3.

d) Recovery

The determination of the processing method efficiency was done by calculating the recovery of Paclitaxel in spiked plasma samples. The recovery was calculated by comparing the Paclitaxel peak area of the spiked plasma samples (extracted sample) with their respective aqueous samples. By the same method recovery of the IS was calculated. The concentration used to study the recovery of IS was 1 µg/mL. Paclitaxel recovery study was carried out in all calibration points. All the prepared calibration standards were subjected to sample processing and analysed by the proposed method. Recovery of Paclitaxel form liver, kidney and spleen were also calculated.

e) Intra-day and inter-day precision and accuracy

The precision and accuracy were determined by taking five concentrations (QC samples) in the range of calibration curve, with five determinations per concentration. The intra-day precision and accuracy were determined by analyzing the spiked QC plasma samples prepared within a day on three different occasions. The inter-day precision and accuracy were determined by analyzing the spiked QC samples prepared on three different days. On each day of validation separate calibration curve were constructed to determine the calculated concentration or actual concentration of the prepared samples. After concentrations were calculated by using the regression equation, % RSD was calculated using the mean value and SD., the % Bias was

calculated from the calculated concentration and known concentration (concentration prepared) and % recovery was calculated by using the standard formula at each concentration of the QC samples. The limit for precision is that, % RSD value should not exceed 15% except for LLOQ, where it should not exceed 20 %. The low percentage relative error indicated the accuracy of the proposed method.

f) Stability

During the pharmacokinetic study, the collected blood samples were processed for separating plasma and then stored in -20° C. Thus, it was necessary to find the stability of Paclitaxel and PP in the biosamples at the storage condition. The stability study are conducted in following heads, freeze and thaw stability, short-term stability, long-term stability, post-preparative stability and stock solution stability. On each day of stability study, separate calibration standards were processed and analysed with the stability samples. All stability study was conducted as per US-FDA guidelines in five QC standards.

i) Freeze and thaw stability

The five QC standards were prepared in plasma and stored at -20° C for 24 hr and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 18 hr under same conditions. The freeze-thaw cycles were repeated for four more times, after which the samples were analysed with the proposed method on the fifth cycle. Hence, Paclitaxel and IS stability in plasma were determined for five freeze and thaw cycles. The stability of Paclitaxel was determined by calculating % Bias and % recovery. Each concentration was measured in triplicate.

ii) Short and long-term stability

The short term stability was conduced up to 24 hr in room temperature (25° C ± 0.5), based on the expectation that Paclitaxel in plasma will be maintained at this temperature for a maximum of 24 hr. The selected QC standard were thawed at room temperature, then processed and analysed at 1, 6, 12 and 24 hr.

The long term stability time points were selected by considering the time between the date of first sample collection and the date of last sample analysis. The time points chosen for long term stability were 7, 15, and 30 days. On the respective time points the samples were thawed unassisted at room temperature, processed and analysed by using the proposed method. The concentration of all stability samples were compared to the mean of back calculated values of the fresh QC standards at the appropriate concentration. As like freeze – thaw stability study five QC standards were analysed in triplicates.

iii) Post-preparative stability

The main aim of this study is to determine the stability of Paclitaxel and the PP in the reconstitution solution (50:50 % v/v acetonitrile and water), during their resident time in the autosampler (18° C ± 0.2). The time points were selected based on the anticipated run time for the batch size in validation samples. The- time points selected for this study were 1, 3 and 5 days. The QC samples were prepared by spiking respective aliquots from working stock to the plasma, then all the samples were processed and loaded to the autosampler and analysis was done as per the time points.

iv) Stock solution stability

The stability of Paclitaxel and PP in the stock solution is evaluated at room temperature and at -20°C for one month. Paclitaxel stability in DMSO (dimethyl solfoxide) is monitored at two QC standard concentrations (500 and 1500 ng/mL). PP stability in acetonitrile was evaluated at 100 µg/mL. Each determination was performed in duplicate.

3.8.4 Application of the developed method- analysis of in-vivo samples

The developed bioanalytical HPLC method was used to estimate Paclitaxel in plasma and tissue samples in pharmacokinetic and biodistribution studies (Chapter 7).

3.9 Results and Discussion

3.9.1 Chromatographic separation

Many bioanalytical methods have been developed to determine Paclitaxel in biosamples (plasma, serum and tissue) for the past one decade, like HPLC-UV (19-24), immunoassays (25), capillary electrophoresis (26) and LCMS-MS (27-30). The immunoassay method lacks the specificity of the HPLC although it possesses higher sensitivity. Capillary electrophoresis needs only a small amount of samples, but it does not have the sensitivity as HPLC-UV / LC-MS has using micro-samples. LC-MS/MS is more sensitive and selective in the determination, but costly and not suitable for routine and simple analysis (24). There are some HPLC- UV methods reported in literature for the estimation of Paclitaxel in plasma with sophisticated and tedious procedure. A simple, sensitive and routine bioanalytical method was developed and validated to estimate Paclitaxel in different biological samples.

During the chromatographic separation, in order to get good resolved peaks for Paclitaxel and PP, different aqueous phases were tried while keeping organic phase (acetonitrile) constant. Acetonitrile (55:45 % v/v) with ammonium acetate buffer or millipore water as aqueous phase was selected with respect to peak symmetry (T = 1.02). Based on ease of preparation water was selected as aqueous phase. Paclitaxel does not have any ionising group; hence the mobile phase pH does not influence much on separation. The mobile phase used in this method is very simple in preparation when compared to those previously reported (22, 24, 31) and the extraction procedure is also very simple when compared to Fruscio et al (32) procedure, which

has multiple extraction steps. The optimized mobile phase was acetonitrile and millipore water (55:45 v/v), which provided moderate and quick retention time, with better peak properties, resolution and selectivity for Paclitaxel and PP. The retention times for Paclitaxel and IS were 8.0 ± 0.25 and 5.3 ± 0.15 min respectively, with 7.70 ± 0.15 min resolution.

For estimation of Paclitaxel in biosamples reported methods used several chemicals as IS like N-cyclohexy benzamide, cephalomannie, d5 Paclitaxel, 2'-methyl Paclitaxel, docetaxel, butyl paraben, n-hexyl p-hydroxy benzoic acid, dimethyl-4-4'-dimethoxy 5, 6, 5', 6'-dimethylene dioxy diphenyl- 2, 2' dicarboxylate and glafenine free base. But most of them are expensive, lacks commercial availability, synthesized especially for analysis purpose, with specificity problem or reproducibility difficulty (19-24). PP is used as IS for the first time in the estimation of Paclitaxel, which is a most frequently used preservative (33) and readily available than those used in previously published methods. PP, chemically propyl 4-hydroxybenzoate, is poorly soluble in water, very commonly available in laboratories than other parabens. PP is freely available in high purity, has detection response at 233nm as like Paclitaxel. The solubility of PP in organic solvents is less (Paclitaxel is highly lipophilic drug) than the butylparaben and PP is most commonly available pharmaceutical excipient than the hexyl ester of 4-hydroxybenzoic acid (33). Hence PP was selected as IS among the parabens. PP is adequately separated from the other components of plasma (Fig. 3.7).

Paclitaxel and IS were separated by isocratic reverse phase HPLC with diode array UV detection at 233nm. Paclitaxel lacks strong UV absorption, detection at the more permissive wavelength of 227nm requires the use of large volumes (0.4-1mL) of sample. There are many endogenous substances from biomatrix with similar lipophilicity as that of Paclitaxel, which also have strong absorption at 227 nm and they get strongly retain in C18 column (24, 30). Because of the above two reason Paclitaxel was determined at 233nm where endogenous substance absorption is less (Fig. 3.8 A) and hence estimated with high accuracy and precision. In this method we used only 800 µL of ice cold acetonitrile as extracting solvent (average % recovery 100.6 ± 3.2) to estimate Paclitaxel (LLOQ 10 ng/mL) from micro volume rat plasma. The extraction procedure described is very economical (only 800 µL of extracting solvent) than the reported method using solid phase extraction (SPE) and liquid-liquid extraction (LLE). In the reported LLE (20, 22) and SPE (24) for the estimation of Paclitaxel in biomatrix, used more than 1 mL of extracting solvent but in the present method, only 800 µL of ice cold acetonitrile [less than this volume result in reduction in extraction efficiency (% recovery)] is used for extraction and estimation of Paclitaxel from plasma. The sample processing (before evaporation step) takes very less time (12 min,) and there is no time consuming step as like freeze the aqueous layer in the freezer in LLE and multiple extraction steps in SPE (23). In the

reported method there are many complicated and sophisticated steps before the supernatant is concentrated by evaporation. By using LLE, it has been reported that the liquid solvents causes interference of endogenous compounds with Paclitaxel and with insufficient efficiency of extraction to permit routine application. It is well know that the LLE has more inconsistent variation in extraction between samples. In our method, the simple protein precipitation purified the matrix and helps to estimate Paclitaxel and PP with high sensitivity (low signal-to-noise ratio to obtain LOD of 5 ng/mL and LLOQ of 10 ng/mL) and specificity (Fig. 3.8) at 233 nm. There was no interference from the matrix during total analysis time of 10 min and even there were no high interfering peaks up to 30 min. By using the simple protein precipitation method Paclitaxel was extracted efficiently from tissue samples, liver, kidney and spleen with good % recovery.

Most of the methods developed for separation of Paclitaxel from biological samples, used multiple steps of liquid – liquid extraction with large volume (2-10 mL) and mixture of extracting solvents. Some of the method has pre/post washing with n-hexane, double extraction, complicated reconstitution solution, and there are more assay methods used solid phase extraction. All the above process makes the method more complicated and difficult for routine analysis.

3.9.2 Method validation

a) Selectivity and peak purity

Fig. 3.7 A to F illustrate representative chromatogram of a blank rat plasma, zero sample, control plasma spiked with 10, 500 and 1500 ng/mL of Paclitaxel and plasma sample of i.v. bolus pharmacokinetic study. Chromatogram of blank sample and zero samples revealed that there was no interfering peak present in the eluting window of Paclitaxel and IS. Further, the real time i.v. pharmacokinetic chromatogram confirms that there was no interference from metabolites or degradation products or other exogenous xenobiotics in the near vicinity of Paclitaxel and IS. There was no co-eluting peak, > 20% of the Paclitaxel at LLOQ and >5 % of the area of IS at their respective retention time. These results confirm the selectivity of the developed method for extracting Paclitaxel from micro-volume rat plasma. The chromatograms recorded at the elution time at different wavelengths (229-235 and 256) shows the peaks homogeneity of Paclitaxel and PP (Fig. 3.8 B) with good overlay of peak shape and retention match.

In the obtained rectangular ratiograms, the ratio of response (area/absorbance) at two selected wavelengths (231 and 235) are less than one (Fig. 3.8 C-E) and it's constant across the elution time. The rectangular ratiograms shows that the Paclitaxel is quantified with high selectivity at 233 nm. The peak purity index and single point threshold (Fig. 3.8 F) value is always close to

one (1.0000 and 0. 0.987749). The positive minimum peak purity index value (12250) also shows the purity of peak (Paclitaxel) and it's selective. The obtained purity curve data shows selectivity of the method in determining the Paclitaxel.

Likewise, there were no interferences from the tissue samples eluting at the retention time of Paclitaxel, which confers the selectivity of the method in estimating Paclitaxel in tissue samples. There is no interfering/co-eluting/ co-migrants endogenous or exogenous or metabolite substance at the selected wavelength. indicating the efficiency of the precipitation method and selectivity of determining Paclitaxel at 233nm.

b) Linearity and quality control samples

Calibration curves were prepared on each day of analysis by an IS addition method for known concentration of Paclitaxel in rat plasma samples. Calibration curve were constructed by plotting peak area ratio vs concentrations. The typical best-fit linear regression equation for the calibration curve in the range of 10 -1500 ng/mL, peak area ratio = 0.0084 x concentration of Paclitaxel (ng/mL) – 0.1208, r^2 = 0.9999. Goodness of fit of regression equation for Paclitaxel in rat plasma was linear with high mean regression coefficient of 0.9997 ± 0.0003, the standard deviation of slope and intercept were found to be 0.0007 and 0.0525 respectively (Table 3.6) with low standard error of estimate (4. 66). ANOVA test (one-way) was performed for peak area ratio obtained at individual concentration levels and calculated F-value was low than critical F- value at 95% level of significant, which supports the best-fit linear equation. The best-fit linear regression equation of calibration curve of liver, kidney and spleen samples were, peak area = 206.31 x concentration of Paclitaxel (ng/mL) – 388.24 (r^2 = 0.9999.), peak area = 205.42 x concentration of Paclitaxel (ng/mL) – 292.15 (r^2 = 0.9999.) and peak area = 205.80 x concentration of Paclitaxel (ng/mL) – 220.7 (r^2 = 1) respectively. The low % bias range from 0.16 to -3.89, 0.45 to -3.51 and 0.23 to -3.46 for the entire calibration curve concentrations of the tissue sample, supportd the selected linear model with univariant regression and it also shows the goodness of fit. The low % error calculated for the entire range of calibration points of tissue samples were between 0.19 to 2.64, 0.42 to 3.49 and 0.004 to 3.37.

c) Determination of LLOQ and LOD

The LLOQ on the calibration curve (10 ng/mL) was accepted as the limit of quantification as its response was more than six times to blank response or background noise. The peak obtained for LLOQ was identifiable, discrete and reproducible with precision (% RSD) of 1.79 % and accuracy (% recovery) of 100.76, the repeatability is very high as compared to Li et al, (22). The method was found to be sensitive with a high signal-to-noise ratio and acceptable precision and accuracy. By using micro-volume (100 µL) of rat plasma the LOD was found to be 5 ng/mL with signal to noise ratio greater than three, this is lower than the reported method by

Yonemoto et al (20) 7.5 ng/mL, Sparreboom et al (33) 15 ng/mL and Martin et al (33) 10 ng/mL. The LOD of present method is equal to the Wang et al (34) who used sophisticated SPE. The clinical pharmacokinetic and cytotoxicity studies reviles that the bioanalytical method with less than 43 ng/mL as quantification limit is required for the estimation of Paclitaxel in the bio matrix (5-7). Our LOD and LLOQ are satisfactory with respect to the 43 ng/mL of its cytotoxicity action. These suggest that the method is suitable for various pharmacokinetic investigations of Paclitaxel in rodents, which demands high sensitivity and repeatability. The developed method was able to detect Paclitaxel up to 24 hr (26.42 ng/mL ± 2.55) in i.v. pharmacokinetic study with 10 mg/kg dose in rat. The LLOQ and LOD of liver, kidney and spleen sample were 25 and 15 ng/mL.

d) Recovery

In general for protein precipitation, room temperature or ice cold acetonitrile or any other protein precipitating agent is used to extract the drug from biomatrix. In the present method, ice cold acetonitrile resulted in high recovery (Table 3.7) of both Paclitaxel and PP respectively. The recovery of Paclitaxel after simple single protein precipitation with ice cold acetonitrile was studied at all calibration standards in triplicates. The efficiency of extraction was found to be in the range of 95.3 ± 5.0 to 100.9 ± 1.2 %, with average recovery of 100.6 ± 3.2. The recovery of IS at 1µg/mL was 91.7 ± 2.9 %. This indicated that the processing method is efficient in extracting the Paclitaxel and IS effectively in micro-volume of rat plasma than the solid-phase extraction (% recovery 85 and 89 for 500 and 1000 ng/mL respectively, used by Wang et al (24) and Caporossi et al (35) (% recovery higher than 87 %, using 0.5 mL of plasma). The results show that the extraction efficiency of the method is consistent, precise and reproducible (% RSD < 5.36). The efficiency of extracting Paclitaxel form the tissue samples of the studied QC samples were, 93.26 to 99.58, 96.15 to 101.49 and 96.39 to 101.16 with low standard deviation ranging from 0.19 to 1.60, 0.22 to 2.24 and 0.46 to 2.24.

e) Intra-day and inter-day precision and accuracy

The intra-day precision of Paclitaxel in micro-volume of rat plasma showed % RSD less than 1.94. The percent relative standard deviation (% RSD) in inter-day precision of all QC sample were less than 2.06 for Paclitaxel. The precision results (% RSD) are very low as compared Coudore et al, (23) Li et al., (22) Yonemoto et al., (20), Kim et al., (21) and Wang et al., (24) these showed that the method is highly reproducible, hence it can be used for routine analysis of Paclitaxel in rat plasma. The intra-day accuracy study showed % Bias ranged from 0.03 to 0.78. The inter-day accuracy (% Bias) at all QC levels ranged from 0.05 to 0.76. The % recovery for intra-day and inter-day precision were in the range of 100.03 to 100.78 and 100.05 to 100.76.

f) System suitability parameters

The number of theoretical plates (N) of the column for separation of Paclitaxel and IS were 6400 and 4974, respectively. The capacity factor (k´) for Paclitaxel and IS was 5.5 and 3.3, respectively. The tailing factor for Paclitaxel (1.00 ± 0.05) and IS (0.98 ± 0.06) approaches to unity, which showed that the peaks are perfectly symmetrical. The system suitability parameters show that the method is reproducible with good resolution.

g) Stability

The results of freeze and thaw stability at all QC levels demonstrated that Paclitaxel and the IS were stable in rat plasma up to five freeze-thaw cycles (Fig. 3.9). Results are expressed in terms of % Bias and % recovery, which ranges from – 0.45 to 0.99 and 99.55 to 100.99 respectively. Guo et al (36) showed that Paclitaxel is stable (% accuracy and % CV) up to three freeze-thaw cycles and our study result showed that Paclitaxel and IS are stable up to five freeze-thaw cycles. This confirms edhat the Paclitaxel and IS was found to be stable for five freeze and thaw cycles making it suitable for subzero storage condition.

The short and long term stability results showed that Paclitaxel and IS was stable up to 24 hr in bench top conditions and for 60 days at -20°C (Fig. 3.9). The % Bias and % recovery for short term stability ranges from – 0.21 to 5.57 and 99.55 to 105.57 respectively. At all QC standards in long term stability the % Bias and % recovery ranged from – 3.13 to 2.25 and 97.73 to 102.25 respectively. The post preparative study results demonstrated that Paclitaxel and IS can be stored in 50:50 % v/v acetonitrile and water in auto sampler (18 °C ± 0.2) for up to 5 days. The % recovery of post preparative study ranged from 98.15 ± 1.54 to 102.91 ± 0.56. Gardner et al (37) showed that the Paclitaxel and d_5 Paclitaxel can be store in the auto sampler (4 ° C) for 24 hr. We showed that the Paclitaxel and PP can reanalyzed even after five days at 18 °C ± 0.2. This makes the analyst to reanalysis the samples if required in situations like machine failure, which is very common for the researchers who work with HPLC. Hence this post preparative stability results gives confident to reanalysis the Paclitaxel rat plasma sample up to five days.

The % RSD calculated for all stability samples were well within the acceptable range of ± 20 % at LLOQ and ± 15 % at all concentration levels. These confirm that the Paclitaxel and IS were stable under various processing and storage conditions stated in the method. The stock solution stability data shows that Paclitaxel is stable at room temperature (mean % recovery 99.99 ± 0.57) and at -20 °C (mean % recovery 99.95 ± 0.59) in DMSO for one month. IS was found to be stable in acetonitrile during a period of one month with mean percent recovery of 98.97 ± 1.32. As per our knowledge this is the first complete report with all stability study in micro volume rat plasma for Paclitaxel.

Table 3.6 Slope and intercept of calibration curve of Paclitaxel in micro-volume rat plasma

Calibration curve[a]	Slope	Intercept	Regression coefficient
1	0.0084	-0.1208	0.9999
2	0.0083	-0.0560	0.9999
3	0.0074	-0.1151	0.9998
4	0.0083	-0.1215	0.9998
5	0.0070	0.0093	0.9999
6	0.0085	-0.0974	0.9997
7	0.0069	-0.1002	0.9992
8	0.0085	-0.1663	0.9993
Mean	0.0079	-0.0960	0.9997
SD.	0.0007	0.0525	0.0003
% RSD	8.741	-	0.028

[a] Each calibration curve is obtained using eight points

3.9.3 Application of the developed method- analysis of in-vivo samples

The validated method was successfully applied to estimate Paclitaxel in rat plasma and tissue samples as reported in Chapter 7.

A)

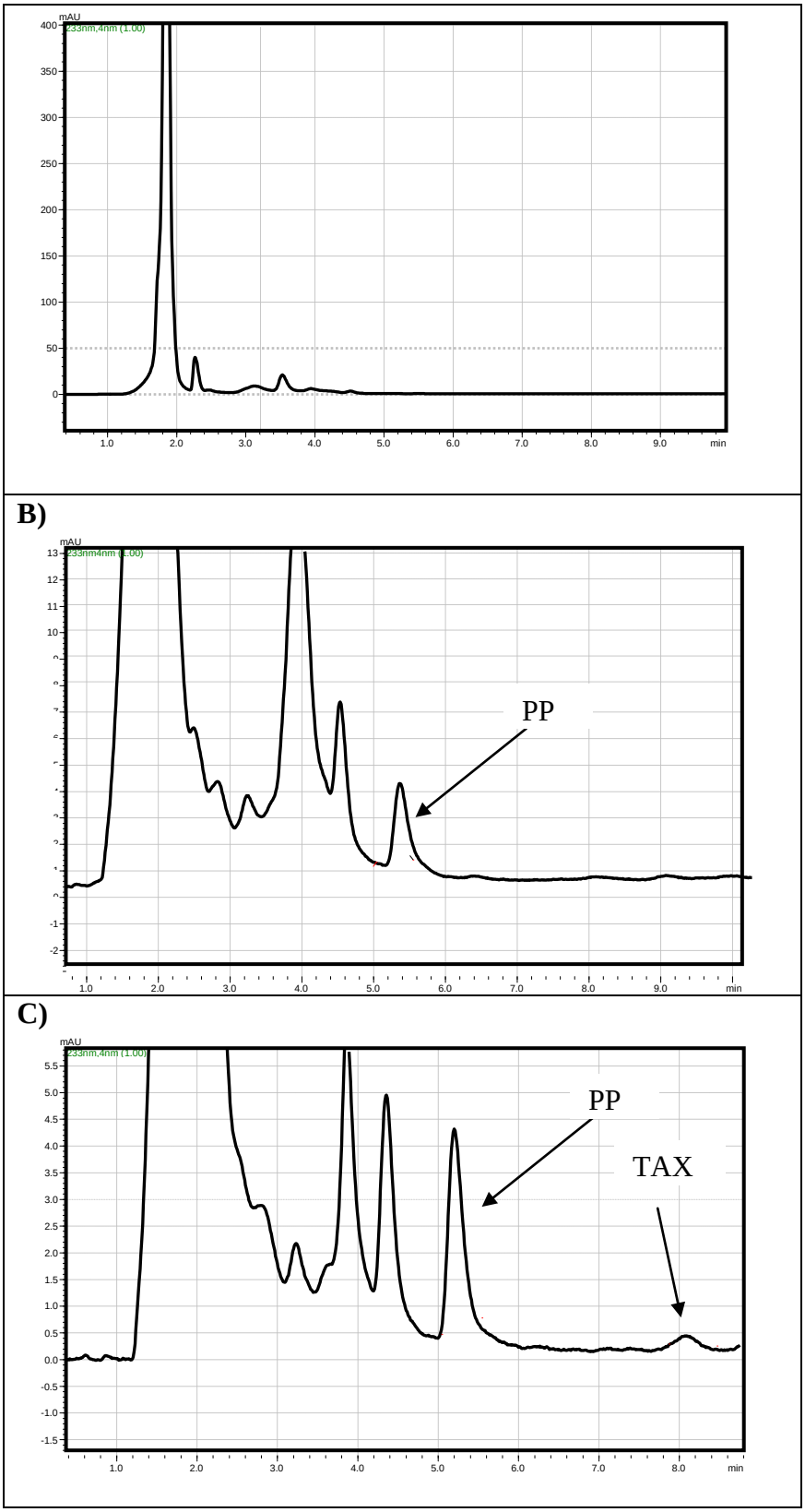


Fig. 3.7 Representative chromatograms of Paclitaxel and PP, blank sample (A) zero sample (B) control plasma spiked with 25 ng/mL (C) Continues…

D)

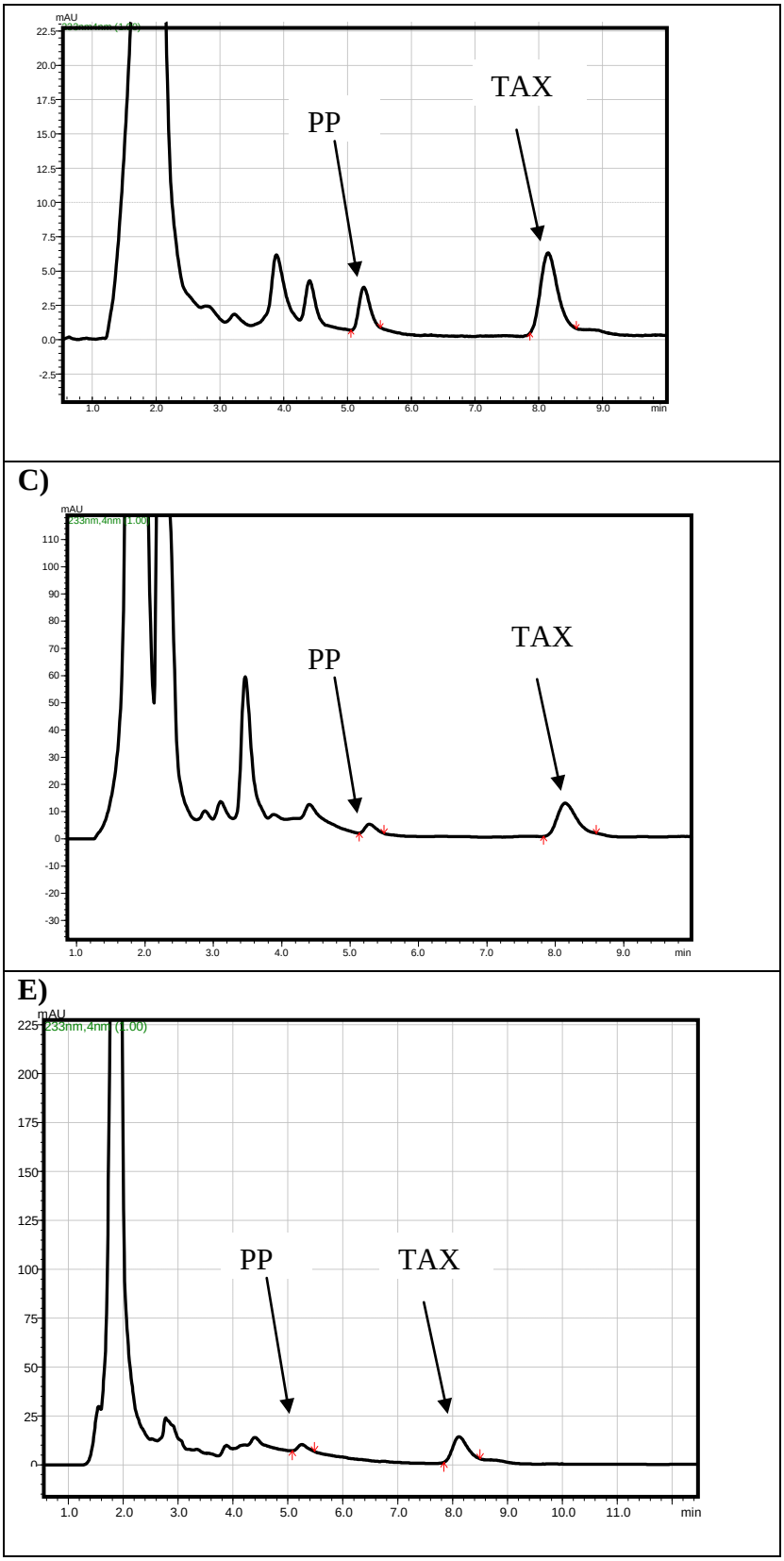


Fig. 3.7 Representative chromatograms of Paclitaxel and PP, control plasma spiked with 500 ng/mL (D) control plasma spiked with 1500 ng/mL (E) plasma sample of pharmacokinetic study of Paclitaxel (F)

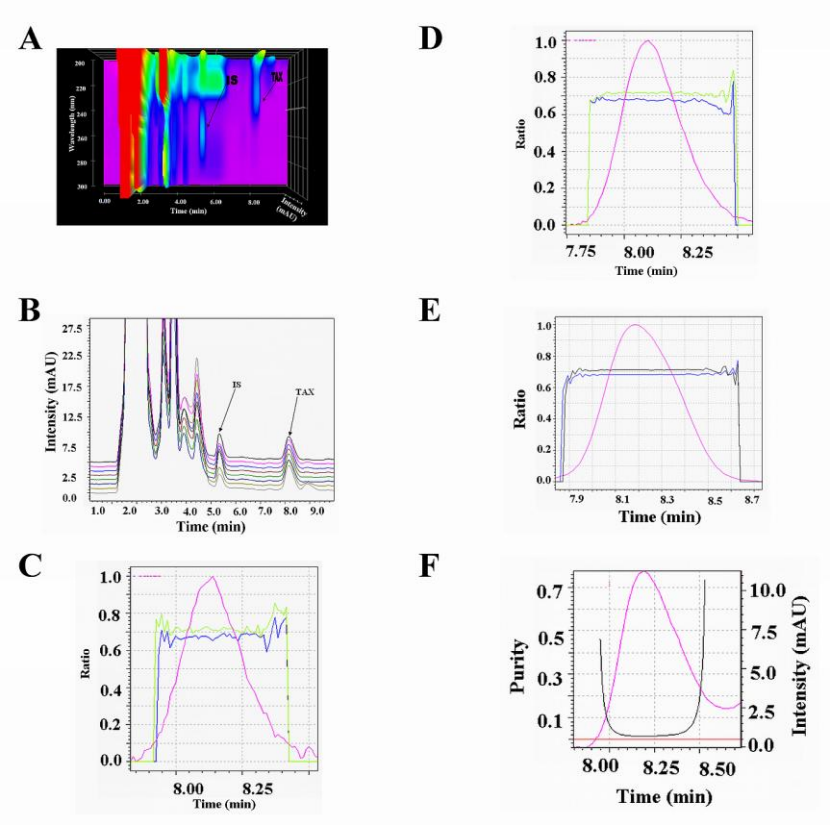


Fig. 3.8 Representative 3D chromatogram for selectivity and sensitivity, top 3D view chromatogram of 500 ng/mL (A) chromatogram of 500 ng/mL recorded at the same time at different wavelength (B) ratiograms of 10 ng/mL (C) ratiograms of 500 ng/mL (D) ratiograms of 1500 ng/mL (E) peak purity (F)

Table 3.7 Recovery of Paclitaxel and PP from micro-volume rat plasma

Concentration (ng/mL)	Mean absolute recovery (%, ± SD)[a]	% RSD
Paclitaxel		
10	100.3 ± 4.7	4.73
25	106.5 ± 7.1	7.09
50	95.3 ± 5.0	5.24
100	100.3 ± 0.6	0.62
250	99.1 ± 5.3	5.36
500	102.5 ± 3.7	3.59
750	99.7 ± 2.1	2.06
1500	100.9 ± 1.2	1.20
Average recovery (± SD)	100.6 ± 3.2	0.003
IS		
1000	91.7 ± 2.9[b]	3.19

[a] Average of six determination (n=3 on two days), [b] Average of ten determination (n = 5 on two days)

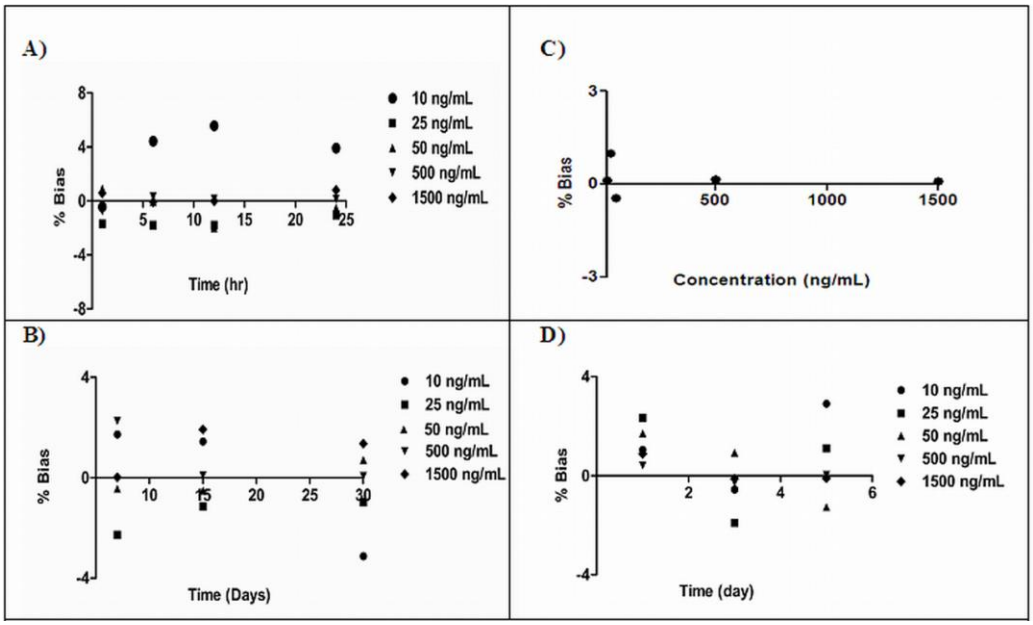


Fig. 3.9 Stability study of Paclitaxel and PP in rat plasma, Post preparative stability (A) short term stability (B) long term stability (C) freeze thaw stability (D)

3.10 Conclusion

A simple, sensitive, accurate and precise UV-spectrophometric method was developed and validated for the determination of Paclitaxel in bulk and NPDDS. The accuracy of the developed method was tested by placebo spiking and standard addition method. The selectivity of the developed method was well established at the limit of quantification. The LOD and LLOQ of the method, 0.39 µg/mL and 2.96 ng/mL respectively. A rapid, sensitive, accurate, precise and specific-stability indicating LC method was developed and validated for the estimation of Paclitaxel. The 2D view chromatogram, counter view graph, UV and IR spectrum of the degradation samples confirms the selectivity and specificity of the developed method. The LOD and LLOQ of the method, 1.18 µg/mL and 8.96 ng/mL respectively. A simple, highly repeatable and reproducible method for estimation of Paclitaxel in rat plasma, liver, kidney and spleen samples was developed and validated completely. PP was used as IS for the first time in the estimation of Paclitaxel in micro volume rat plasma. The rectangular ratiograms and purity curve demonstrates the selectivity. The validated method has been shown to be suitable for i.v.

pharmacokinetic and biodistribution study of Paclitaxel solution and NPs (10 mg/kg) in wistar rats. All the stability study results illustrates that Paclitaxel and PP were stable in plasma with less % Bias and high % recovery.

References

1. Bende G, Sivacharan K, Venugopal K, Saha RN. (2007). Development and validation of a stability indicating RP-LC method for determination of imatinib mesylate. Chromatographia, 66, 859-866.
2. Saha RN, Sajeev C, Jadhav PR, Patil SP, Srinivasan N. (2002). Determination of celecoxib in pharmaceutical formulations using UV spectrophotometry and liquid chromatography. J Pharm Biomed Anal, 28, 741-751.
3. Bende G, Sivacharan K, Snehalatha M, Ganesh M, Saha RN. (2010). Validation of an HPLC method for determination of imatinib mesylate in rat serum and its application in a pharmacokinetic study. J Chromatogr Sci, 48, 334-341.
4. Bakshi M, Singh S. (2002). Development of validated stability-indicating assay methods-critical review. J Pharm Biomed Anal, 28, 1011-1040.
5. Neue UD, Mazzeo JR. (2001). A theoretical study of the optimization of gradient at elevated temperature. J Sep Sci, 24, 921-929.
6. Nornoo AO, Chow DSL. (2008). Cremophor-free intravenous microemulsion for paclitaxel II stability, in-vitro release and pharmacokinetic. Int J Pharm, 349, 117-123.
7. Shao LK, Locke DC. (1997). Determination of paclitaxel and related taxanes in bulk drug and injectable dosage forms by reversed phase liquid chromatography. Anal Chem, 69, 2008-2016.
8. Mu Li, Feng S. (2003). PLGA/TPGS nanoparticles for controlled release of paclitaxel: effects of the emulsifier and drug loading ratio. Pharm Res, 20, 1864-1872.
9. De S, Miller DW, Robinson DH. (2005). Effect of particle size of nanospheres and microspheres on the cellular-association and cytotoxicity of paclitaxel in 4T1 cells. Pharm Res, 22, 766-775.
10. Badea L, Ciutaru D, Lazar L, Nicolescu D, Tudose A. (2004). Rapid HPLC method for the determination of paclitaxel in pharmaceutical forms without separation. J Pharm Biomed Anal, 34, 501-507.

11. Anupama MC, Deepak A, Neeraj K. (2007). HPLC method for the determination of carboplatin and paclitaxel with cremophore L in an amphiphilic polymer matrix. J Chromatogr B, 855, 211-219.
12. Sang-Hyum P, Jin-Suk C, Ho-Joon C, Byung-Hee H. (2007). Evaluation of paclitaxel rearrangement involving opening of the oxetane ring and migration of acetyl and benzoyl groups. J Pharm Biomed Anal, 43, 1141-1145.
13. Aboul-Enein HY, Serignese V. (1996). Liquid chromatographic determination of taxol and related derivatives using a new polyfluorinated reversed-phase column. Anal Chim Acta, 319, 187-190.
14. Zhang Y, Tang L, Sun L, Bao J, Song C, Huang L, Liu K, Tian Y, Tian Ge, Li Z, Sun H, Mei L. (2010). A novel paclitaxel-loaded poly (ε-caprolactone)/poloxamer 188 blend nanoparticles overcoming multidrug resistance for cancer treatment. Acta Biomater, 6, 2045-2052.
15. Pulkkinen M, Pikkarainen J, Wirth T, Tarvainen T, Haapa-aho V, Korhonen H, Seppala J, Jarvinen K. (2008). Three-step tumor targeting of paclitaxel using biotinylated PLA-PEG nanoparticles and avidin-biotin technology: formulation development and in-vitro anticancer activity. Eur J Pharm Biopharm, 70, 66-74.
16. Chakravarthi SS, De S, Miller DW, Robinson DH. (2010). Comparison of anti-tumor efficacy of paclitaxel delivery in nano- and microparticles. Int J Pharm, 383, 37-44.
17. Xu Z, Gu W, Huang J, Sui H, Zhou Z, Yang Y, Yan Z, Li Y. (2005). In-vitro and in-vivo evaluation of actively targetable nanoparticles for paclitaxel delivery. Int J Pharm, 288, 361-368.
18. Fonseca C, Simoes S, Gaspar R. (2002). Paclitaxel-loaded PLGA nanoparticles: preparation, physiochemical characterization and in-vitro anti-tumoral activity. J Control Release, 83, 273-286.
19. Yang T, Cui FD, Choi MK, Cho JW, Chung SJ, Shim CK, Kim DD. (2007). Enhanced solubility and stability of PEGyalted liposomal paclitaxel: In-vitro and in-vivo evaluation. Int J Pharm, 338, 317-326.
20. Yonemoto H, Ogino S, Nakashima MN, Wada M, Nakashima K. (2007). Determination of paclitaxel in human and rat blood samples after administration of low dose paclitaxel by HPLC-UV detection. Biomed Chromatogr, 21, 310-317.
21. Kim SC, Yu J, Lee JW, Park ES, Chi SC. (2005). Sensitive HPLC method for quantitation of paclitaxel (Genexol) in biological samples with application to preclinical pharmacokinetics and biodistribution. J Pharm Biomed Anal, 39, 170-176.

22. Li X, Choi JS. (2007). Effect of genistein on the pharmacokinetics of paclitaxel administered orally or intravenously in rats. Int J Pharm, 337, 188-193.
23. Coudore F, Authier N, Guillaume D, Beal A, Duroux E, Fialip J. (1999). High-performance liquid chromatographic determination of paclitaxel in rat serum: application to a toxicokinetic study. J Chromatogr B, 721, 317-320.
24. Wang LZ, Ho PC, Lee HS, Vaddi HK, Chan YW, Yung CY. (2003). Quantitation of paclitaxel in micro-sample rat plasam by a sensitive reversed-phase HPLC assay. J Pharm Biomed Anal, 31, 283-289.
25. Grothaus PG, Raybould TJG, Bignami GS. (1993). An enzyme immunoassay for the determination of taxol and taxanes in taxus sp.tissues and human plasma. J Immunol Methods, 158, 5-15.
26. Hempel, G. Lshmkuhl D, Krumpelmann S, Blaschke G, Boos J. (1996). Determination of paclitaxel in biological fluids by micellar electrokinetic chromatography. J Chromatogr A, 745, 173-179.
27. Mortier KA, Verstrate AG, Zhang GF, Lambert WE. (2004). Enhanced method performance due to a shorter chromatographic run-time in a liquid chromatography-tandem mass spectrometry assay for paclitaxel. J Chromatogr B, 1041, 235-238.
28. Gardner ER, Dahut W, Figg WD. (2008). Quantitative determination of total and unbound paclitaxel in human following Abraxane treatment. J Chromatogr B, 862, 213-218.
29. Vainchtein LD, Thijssen B, Stokvis E, Rosing H, Schellens JHM, Beijnen JH. (2006). A simple and sensitive assay for the quantitative analysis of paclitaxel and metabolite in human plasma using liquid chromatography/tandem mass spectrometry. Biomed Chromatogr, 20, 139-148.
30. Guo P, Ma J, Li S, Gallo JM. (2003). Determination of paclitaxel in mouse plasma and brain tissue by liquid chromatography-mass spectrometry. J Chromatogr B, 798, 79-86.
31. Kemper EM, Zandbergen AE, Cleypool C, Mos HA, Boogerd W, Beijnen JH, Tellingen OV. (2003). Increased penetration of paclitaxel into the brain by inhibition of P-Glycoprotein. Clin Cancer Res, 9, 2849-2855.
32. Fruscio R, Lissoni AA, Frapolli R, Corso S, Mangioni C, D'incalci M, Zucchetti M. (2006). Clindamycin-paclitaxel pharmacokinetic interaction in ovarian cancer patients. Cancer Chemother Pharmacol, 58, 319-325.
33. Ainley W, Paul JW. (1994). Handbook of Pharmaceutical Excipients, 2nd ed. American Pharmaceutical Association, Pharmaceutical Press, Washington, Londan, 411-414.

34. Xie J, Wang C. (2005). Self-assembled biodegradable nanoparticles developed by direct dialysis for the delivery of paclitaxel. Pharm Res, 22, 2079-2090.
35. Caporossi L, Rosa M, Pera A, Papaleo B. (2007). Simple analytical method for the determination of paclitaxel (Taxol) levels in human plasma. Chromatographia, 66, 921-924.
36. Guo W, Johnson JL, Khan S, Ahmad A, Ahmad I. (2005). Determination of paclitaxel in mouse plasma and brain tissue by liquid chromatography-mass spectrometry. Anal Biochem, 336, 213-220.
37. Gardner ER, Liau CT, Chu ZE, Figg WD, Sparreboom A. (2006). Rapid determination of paclitaxel in human plasma following the administration of Genaxol or Genetaxyl by liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom, 20, 2170-2174.

Chapter 4. Preformulation

4.1 Introduction

Preformulation studies are an investigation of physical and chemical properties of the active pharmaceutical ingredient and are important for deciding probable dosage form(s). Non-availability of these information can affect drug performance and can lead to stability problems (1-2). The formulation scientists look for the preformulation study data before design and development of any successful formulation. Paclitaxel is a well established anti-cancer drug and good amount of physico-chemical information are available in literature. In the present work, as aim is to formulate nanoparticulate drug delivery system for Paclitaxel using selected preformed polymers, some preformulation studies are required. Still now US-FDA has not framed any specific guideline for the preparation and characterization of NPs formulation. Based on the need, some important preformulation experiments were planned and performed. Study covered the bulk characterization, solubility, partition coefficient, the drug-excipient compatibility and stability of Paclitaxel as per the need (3-5).

4.2 Experimental

4.2.1 Materials and Methods

Paclitaxel was received as gift sample as mentioned in Chapter 3. Other chemicals are obtained from market and are pharmaceutical or AR grade.

4.3 Preformulation studies

4.3.1 Bulk characterization

The UV-spectrum of Paclitaxel was recorded by preparing, 26 µg/mL fresh solution in 85:15 % v/v acetonitrile-water system. The X-ray diffraction (XRD) and infra red spectrum analysis was done to characterize the powdered drug. XRD was done using RKS-400SV-R desktop X-ray powder diffractometer (Rigaku, Rigaku Corporation, Miniflex-II, Japan) and IR study was done by using fourier transform infrared spectroscopy (FTIR; IR Prestige-21, Shimadzu, Kyoto, Japan).

4.3.2 Solubility study

The saturation solubility of Paclitaxel was determined by shake flask method in buffered and un-buffered media at different selected pH conditions (pH 1.2, 2.0, 6.8, 7.4 and 11.0). The solubility of Paclitaxel in different media was determined by adding excess amount of Paclitaxel to 2 mL centrifuge tube containing 1mL of respective media and vortexed for complete dispersion of drug. All the samples were kept in shaker (MAC, Orbital Shaking Incubator, New Delhi) for 24 hr maintained at 37 ± 3°C. After 24 hr, 500 µL of sample was withdrawn and filtered using syringe filter containing 0.22 µm membrane filter. The filtered samples were diluted suitably and analyzed by the developed UV-Spectrophotometric method. All

the samples were prepared in triplicate and the average of this is represented as Paclitaxel solubility in the respective medium.

4.3.3 Partition coefficient

The partition coefficient of Paclitaxel was determined by shake flask method (5). The partition coefficient of Paclitaxel was performed in two systems, n-octanol-water and chloroform-water. Before the experiment all the phases were pre-saturated with their mutual phases for 24 hr and then the phases were separated from each other by centrifugation (CPR 24, Remi, India) at 12000 rpm for 10 min at 25°C. In a 15 mL centrifuge tube, 5.25 mL of water saturated octanol or chloroform organic phase was mixed with 7 mL of water already pre saturated with respective organic solvent. Then, this solvent mixture was placed in shaker (MAC, Orbital Shaking Incubator, New Delhi) maintained at 25 ± 3°C after adding 1.75 mL of Paclitaxel solution in octanol to the organic phase. After equilibration, all the samples were centrifuged at 12000 rpm for 10 min at 25°C to separate both the layers and Paclitaxel concentration were determined at respective layer by the developed HPLC method reported in Chapter 3. All the samples were prepared in triplicate and the result was expressed in log partition coefficient.

4.3.4 Compatibility study

a) FTIR spectroscopy

This study was carried out to check the compatibility of Paclitaxel with the selected polymers. The physical mixture of drug (Paclitaxel) and excipients (PCL, PLGA, PLA, PVA and PF 68) were prepared in 1:1 ratio and kept at room temperature (25 ± 3 ºC). FTIR was taken after 6 months of observation to monitor any incompatibility.

b) Accelerated stability test

i) Physical stability

The accelerated physical stability test of the drug in mixture with excipients was performed at two different conditions, 40 ± 2°C/75 ± 5% Relative Humidity (RH) and 60 ± 2 °C/80 ± 5% RH. Duplicates of drug and excipients mixture were taken in the amber colored bottle and these mixtures were kept in the selected storage condition. The mixtures were observed for any physical change (color and physical state) up to four weeks (4).

ii) Chemical stability

The accelerated chemical stability test of the drug in mixture with excipients was performed at two different conditions, 40 ± 2°C/75 ± 5% RH and 60 ± 2 °C/80 ± 5% RH. Triplicate of drug (1 mg) and excipients mixture were taken in the amber colored bottle and these mixtures were kept in the selected storage condition. The mixtures were estimated for Paclitaxel using HPLC method reported in Chapter 3 for any chemical change (degradation) up to four weeks (4).

4.3.5 Stability study

The stability of Paclitaxel was performed under two heads, solution state stability and solid state stability.

a) Solution state stability

The solution state stability of Paclitaxel was studied in different buffered pH solutions of pH 1.2, 3.0 4.8, 6.8, 7.4 and 11. A stock solution of 1mg/mL Paclitaxel was prepared in DMSO and from that 1 µg/mL of Paclitaxel solutions were prepared in different pH solutions for the stability analysis. The prepared samples were stored at 25 ± 3°C and at different time points (1, 2, 3, 4, 5 and 6 days) samples were take out for drug content analysis using developed HPLC method. The results were analyzed to determine order of degradation and rate constants. The $t_{90\%}$, time take to degraded 90 % of the drug was calculated to find the shelf life of Paclitaxel.

b) Solid state stability

In this study, 10 mg of Paclitaxel was weighed and transferred to three clean glass containers. These containers were stored in three separated conditions, ambient temperature, 25 ± 3°C, 60 ± 5% RH, accelerated conditions, 40 ± 2°C, 75 ± 5% RH and refrigerated condition, 5 ± 3°C for the predetermined time intervals. All the samples were analyzed for drug content at pre determined time points, 1, 2, 3, 4, 5 and 6 months. The results were analyzed to determine order of degradation, rate constants and $t_{90\%}$.

4.4 Results and Discussion

4.4.1 Bulk characterization

The UV-spectrum of Paclitaxel showed λ_{max} at 228 nm. The diffractogram (DG) showed characteristic sharp peak at 12.26° 2θ scattering angle, clearly indicated the crystallinity of Paclitaxel (1). The FTIR spectra illustrated characteristic bands of Paclitaxel. All these studies showed that the sample obtained was crystalline white powder Paclitaxel.

4.4.2 Solubility analysis

The solubility of Paclitaxel in buffer and un-buffered solution in different pH were represented in Fig. 4.1. Paclitaxel showed nearly same solubility profile in the buffered and un-buffered pH, with little higher stability in buffered media. Paclitaxel is highly lipophilic drug with solubility less than 1 µg/mL (6-8). Results of solubility of Paclitaxel in buffered and un-buffered pH solution were found to be in the range of 0.54 to 0.94 µg/mL and 0.30 to 0.73 µg/mL respectively. Paclitaxel solubility was not significantly changed when the pH was changed in both buffered and un-buffered systems, as there is no ionizing group in the structure of Paclitaxel.

4.4.3 Partition coefficient

The equilibrium partition coefficient (D) of Paclitaxel in, n-octanol-water and chloroform-water was found to be 999.10 ± 56.42 and 585.69 ± 18.14 respectively. The Log D of Paclitaxel in n-octanol-water and chloroform-water system was found to be 3.00 and 2.77 respectively (5, 8).

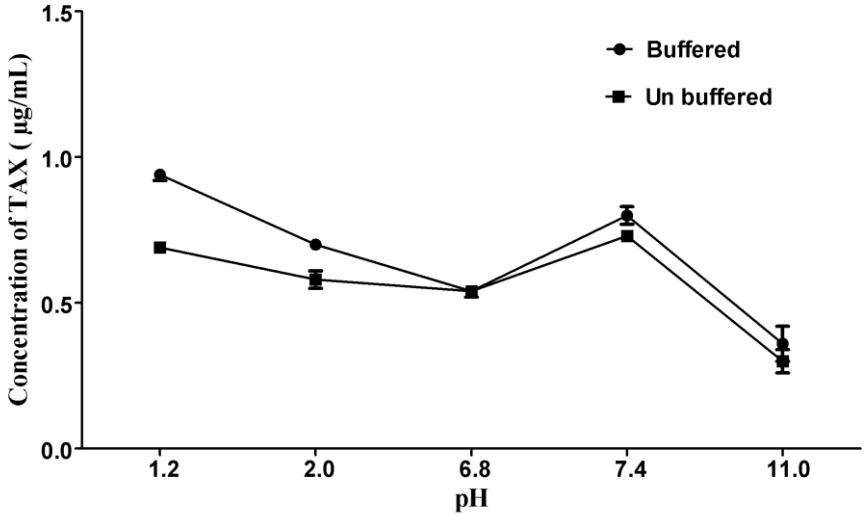


Fig. 4.1 Paclitaxel solubility in buffered and un-buffered solution of different pH 1.2, 2.0, 6.8, 7.4 and 11.0 solution (TAX-Paclitaxel)

4.4.4 Compatibility study

The FTIR study of physical mixture of drug and excipients after 6 month storage showed characteristic bands of Paclitaxel, Polymer PCL, PLGA, PLA and stabilizers, PF 68 and PVA in different proportion of mixtures. These indicate the absence of chemical interaction of Paclitaxel and polymers or stabilizers. The accelerated physical stability test at 40 ± 2°C/75 ± 5% RH of all the sample showed no characteristic change in the initial white color till 4th week (Table 4.1). However, in all samples became sticky in nature after 4th week. Though Paclitaxel has high melting point (213°C), but due to low melting point stabilizer and PCL, Paclitaxel with these excipients became sticky. The drug with polymers and stabilizer stored at 60 ± 2 °C/80 ± 5% RH became sticky on second day onwards and at the end of 1st week almost all the tested samples were in sticky nature but no major color change was observed (Table 4.2). In 4th week, all the tested samples were in melt form and it has become solid mass. However, no degradation of products or color change were observed in any sample. The accelerated chemical stability study result showed good % recovery ranging from 99.25 to 101.34. This suggested that there was no chemical interaction of Paclitaxel with the excipients mixture studied.

Table 4.1 Drug (Paclitaxel) and excipients compatibility study at accelerated storage condition (40 ± 2°C/75 ± 5% RH)

S.NO	Sample	Initial Observation	1st Week	4th Week
1	Paclitaxel + PCL	White color sample	No characteristic change	No color change, sample was sticky
2	Paclitaxel + PLGA	White color sample	No characteristic change	No color change, sample was sticky
3	Paclitaxel + PLA	White color sample	No characteristic change	No color change, sample was sticky
4	Paclitaxel + PVA	White color sample	No characteristic change	No color change, sample was sticky
5	Paclitaxel + PF 68	White color sample	No characteristic change	No color change, sample was sticky
6	PCL + PVA	White color sample	No characteristic change	No color change, sample was sticky
7	PLGA + PVA	White color sample	No characteristic change	No color change, sample was sticky
8	PLA + PVA	White color sample	No characteristic change	No color change, sample was sticky
9	PCL + PF 68	White color sample	No characteristic change	No color change, sample was sticky
10	PLGA + PF 68	White color sample	No characteristic change	No color change, sample was sticky
11	PLA + PF 68	White color sample	No characteristic change	No color change, sample was sticky
12	Paclitaxel + PCL+ PF 68 + Lactose	White color sample	No characteristic change	No color change, sample was sticky
13	Paclitaxel + PLGA + PF 68 + Lactose	White color sample	No characteristic change	No color change, sample was sticky
14	Paclitaxel + PLA + PF 68 + Lactose	White color sample	No characteristic change	No color change, sample was sticky

Table 4.2 Drug (Paclitaxel) and excipients compatibility study at accelerated storage condition (60 ± 2 °C/80 ± 5% RH)

S.NO	Sample	Initial Observation	1st Week	4th Week
1	Paclitaxel + PCL	White color sample	No color change, sample was sticky	No color change, sample was in melt form
2	Paclitaxel + PLGA	White color sample	No color change, sample was sticky	No color change, sample was in melt form
3	Paclitaxel + PLA	White color sample	No color change, sample was sticky	No color change, sample was in melt form
4	Paclitaxel + PVA	White color sample	No color change, sample was sticky	No color change, sample was in melt form
5	Paclitaxel + PF 68	White color sample	No color change, sample was sticky	No color change, sample was in melt form
6	PCL + PVA	White color sample	No color change, sample was sticky	No color change, sample was in melt form
7	PLGA + PVA	White color sample	No color change, sample was sticky	No color change, sample was in melt form
8	PLA + PVA	White color sample	No color change, sample was sticky	No color change, sample was in melt form
9	PCL + PF 68	White color sample	No color change, sample was sticky	No color change, sample was in melt form
10	PLGA + PF 68	White color sample	No color change, sample was sticky	No color change, sample was in melt form
11	PLA + PF 68	White color sample	No color change, sample was sticky	No color change, sample was in melt form
12	Paclitaxel + PCL + PF 68 + Lactose	White color sample	No color change, sample was sticky	No color change, sample was in melt form
13	Paclitaxel + PLGA + PF 68 + Lactose	White color sample	No color change, sample was sticky	No color change, sample was in melt form
14	Paclitaxel + PLA + PF 68 + Lactose	White color sample	No color change, sample was sticky	No color change, sample was in melt form

4.4.5 Stability study

a) Solution state stability

The log % remaining to degrade (RTD) versus time profile of Paclitaxel in different pH solutions gave straight line (Fig. 4.2) indicating degradation dependent on drug concentration. The regression coefficient (R^2) value was in the range of 0.9487 to 0.9899. No significant difference was observed in the degradation of Paclitaxel in between pH solution 1.2 to 7.4 (Fig. 4.3). However, in case of pH 11.00 it was observed that the Paclitaxel started degrading from the first day and almost 75 % of Paclitaxel was degraded with in six days. The degradation rate constant (K_{deg}) for all the pH solutions were, 14.00 x 10^{-2}, 13.40 x 10^{-2}, 15.61 x 10^{-2}, 11 x 10^{-2}, 11 x 10^{-2} and 20.20 x 10^{-2} $days^{-1}$ respectively. The highest K_{deg} was observed in pH 11.00 and the lowest was observed in pH 6.8 and 7.4. The $t_{90\%}$ (days) of Paclitaxel in different pH solution were, 9.1, 12.10, 10.70, 20.27, 22.87 and 5.20 days respectively. This shows that Paclitaxel is more stable in pH 6.8 and 7.4 and it is least stable in pH 11.00. The K_{deg} and $t_{90\%}$ values clearly indicated that there was no significant effect of pH up to 7.4 on the degradation of Paclitaxel, but at highly alkaline condition rapid degradation of Paclitaxel was observed.

b) Solid state stability

The log % RTD versus time profile of Paclitaxel in different temperature gave straight line (Fig. 4.3) demonstrating first order degradation with R^2 value close to one, ranging from 0.9129 to 0.9182 respectively. Paclitaxel was stable up to 6 months at all the stability conditions with Paclitaxel remaining to degrade was in the range of 99.15 to 100 % respectively. The degradation rate constant (K_{deg}) values obtained were 50 x 10^{-5}, 80 x 10^{-5} and 60 x 10^{-5} $months^{-1}$ respectively. The $t_{90\%}$ of Paclitaxel in all the three storage conditions, were 175.33 ± 11.54, 115.63 ± 14.83 and 148.76 ± 12.32 months respectively.

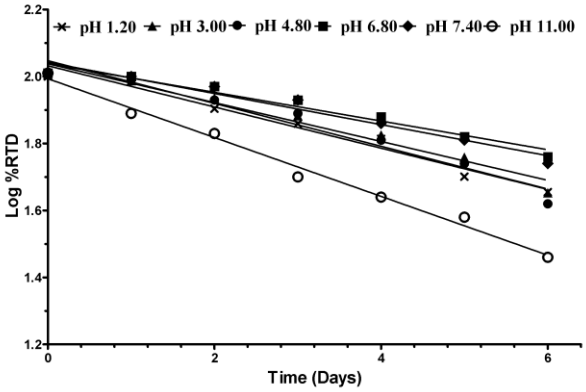


Fig. 4.2 Log % RTD vs. time graph of solution state stability of Paclitaxel at different pH

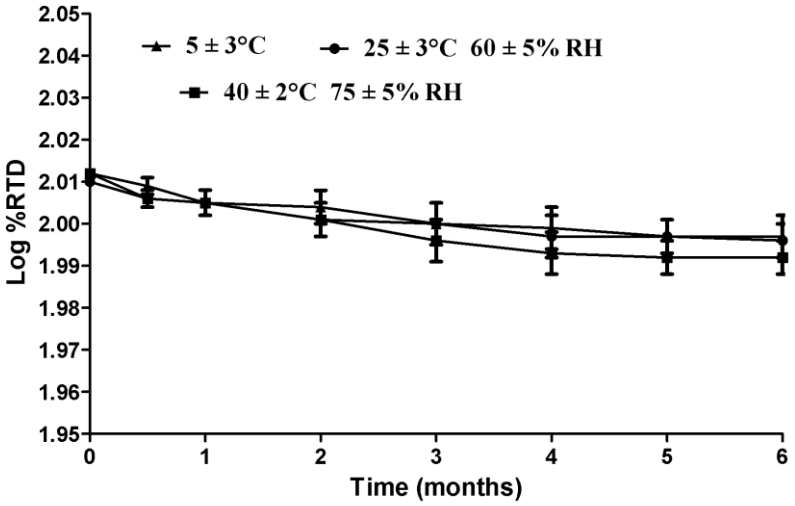


Fig. 4.3 Log % RTD vs. time graph of solid state stability of Paclitaxel at different temperature

4.6 Conclusion

The UV, FTIR and XRD analysis clearly showed that obtained Paclitaxel are crystalline nature. The solubility and partition coefficient study result showed that Paclitaxel was highly lipophilic. The solubility of Paclitaxel in buffered and un-buffered solution was less than 1µg/mL. The compatibility study, between Paclitaxel, polymer and stabilizer clearly shows that there was no chemical and physical changes (color and appearance) observed after four weeks. There was no change in peaks profile in the IR spectrum of physical mixture (Drug and Excipients) when compared to pure drug and polymer spectrum. These indicated that there was no chemical interaction between drug and excipients. The solution and solid state stability study result showed that Paclitaxel follows first order degradation kinetic and the Paclitaxel degradation was not influenced much by pH change except in highly alkaline pH.

References

1. Brittain HG. Profiles of drug substances, excipients, and related methodology. ln: Jauhari S, Singh S, Dash AK, editors. Paclitaxel. UK: Academic Press; 2009. 299-344.
2. J.T. Carstensen. (1998). Pharmaceutical Preformulation. 1st ed. Technomic Publishing Company, Lancaster, USA, 1-306.
3. P.J. Sinko. (2006). Martin's Physical Pharmacy and Pharmaceutical Sciences: Physical Chemical and Biopharmaceutical Principles in the Pharmaceutical Sciences. 1st ed. Lippincott Williams & Wilkins, Philadelphia, USA, 82-106.
4. Vasanthakumar S, Vijayaragavan C. (2008). Immediate release tablets of telmisartan using superdisintegrant- formulation, evaluation and stability studies, Chem Pharm Bull, 56, 575-577.

5. Bende G. Design, development and pharmacokinetic studies of nanoparticulate drug delivery systems of imatinib mesylate, Birla Institute of Technology and Science, Pilani, India, Ph. D. Thesis, 2008, 1-258.
6. Panchagnula R. (1998). Pharmaceutical aspects of paclitaxel. Int J Pharm, 172, 1-15.
7. Singla A, Garg A, and Aggarwal D. (2002). Paclitaxel and its formulations. Int J Pharm, 235, 179-192.
8. Shapira A, Assaraf YG, Epstein D, Livney YD. (2010). Beta-casein nanoparticles as an oral delivery system for chemotherapeutic drugs: Impact of drug structure and properties on co-ssembly. Pharm Res, 27, 2175-2186.

Chapter 5.Formulation Design, Development and Characterization of NPDDS

5.1 Introduction

Design of dosage form is very essential for delivering a drug to achieve effective therapy. Over the years continuous progress is happening in delivery systems. The conventional dosage forms have various shortcomings in therapy. Most important one of these drawbacks is nonselective distribution of drugs, more so serious in cancer chemotheraphy. Paclitaxel is currently marketed as non-aqueous single-dose i.v solution (Taxol®, Bristol-Myers Squibb Co, USA in 5 mL pack) containing 6 mg/mL Paclitaxel in 1:1 v/v ratio of Cremophore EL (polyethoxylated castor oil) and dehydrated alcohol. Cremophore EL may produce fatal anaphylactic hypersensitivity reactions, hyperlipidaemia, nephrotoxicity, neurotoxicity, cardiotoxicity and hypotension at the dosage used in the cancer patients (1-6).

Progress in nanoscience and nanotechnology laid foundation for nanotherapy based approach for cancer drug delivery for improved therapy and quality of life. In this project attempts have been made to prepare and characterize Paclitaxel loaded PLGA, PCL and PLA NPs for better therapy. As an effort to formulate more efficient Paclitaxel NPs for systemic administration, this research work is an endeavour to optmize the amount of polymer/stabilizer, concentration of stabilizer and amount of Paclitaxel required to get the ideal NPs using three different biodegradable and biocompatible polymers. The ideal particle size, PCL, PLGA and PLA NPs were prepared and selected for the in-vitro and in-vivo study.

5.2 Experimental

5.2.1 Materials and Methods

Paclitaxel was received as gift sample as mentioned in Chapter 3. Polyvinyl alcohol (PVA), (98 % hydrolyzed, molecular weight 13000-23000) were procured from Sigma-Aldrich chemicals, Bangalore, India. Other chemicals are obtained from market and are pharmaceutical and AR grade.

5.3 Preparation of PNPs

Paclitaxel loaded PNPs were prepared by, interfacial deposition (nanoprecipitation) and solvent evaporation method (7, 8). These methods were modified according to the present requirement. The PCL and PLA NPs were prepared by nanoprecipitation and PLGA NPs were prepared by solvent evaporation method. In case of nanoprecipitation method, different ratio of Paclitaxel and polymer were dissolved in acetone (5 mL) with mild heating and the loss of solvent was adjusted finally. The aqueous phase was prepared by adding different amount (0.25, 0.5, 0.75 and 1 % w/v) of stabilizer (PVA/PF 68) to triple distal water (20 mL). In case of solvent evaporation method, dichloromethane was used as organic phase to dissolve Paclitaxel and polymer. The organic phase was poured into aqueous phase with ultra-sonication treatment using a microtip probe sonicator (Microson, Misonix, USA) under controlled temperature

(cooling bath maintained at 5°C ± 1.00) with intensity 15 W for 15 min. Once the organic phase is added to aqueous phase, the milky nanodispersion was formed instantaneously. The formed nanodispersion was continuously stirred for 3 hr using magnetic stirrer (Tarsons, SPINOT digital magnetic stirrer) to evaporate the organic solvent and then the dispersion was subjected to rotavapor (Buchi, Switzerland) at reduced pressure at 40°C for 2 hr to reduce the volume, 10 mL. The nanodispersion was filtered through syringe filter holder (Axiva, India) with 0.44 µm filter membrane (Millipore Co., USA) to remove the free Paclitaxel and aggregates. The entire nanodispersion was centrifuged at 14,000 rpm at 15°C for 30 min (Cooling Compufuge, Remi, Mumbai, India). The NPs got settled and supernatant was analyzed for free drug content and the sediment NPs was freeze-dried. Before freeze-drying, pre-freezing of PNPs was done at –20°C for 18 hr, then the flasks were connected to freeze-drier (Maxi Dry Lyo, Heto, Germany) under vacuum (1 mbar, –110°C). Freeze-drying was continued until free-flowing PNPs powder was obtained.

5.3.1 Effect of formulation and process variables

The influences of formulation and process variables, polymer amount, concentration of stabilizer and Paclitaxel amount on the size, polydispersity index (PDI)**,** zeta potential (ZP), encapsulation efficiency (EE), drug content (DC), in-vitro dissolution were studied extensively. In addition, the effect of stabilizer (PVA/PF 68) and polymers (PCL, PLGA and PLA) on the of NPs were also studied comprehensively. The effect of amount of polymer in NPs formation and characterization was studied at 1, 3, 5, 10, 20, 40, 50, 75, 100, 125, 150, 175 and 200 mg for all the three polymers, PCL (PCL/F68/01 to PCL/F68/13), PLGA (PLGA/F68/01 to PLGA/F68/13) and PLA (PLA/F68/01 to PLA/F68/13). The PCL (PCL/F68/14 to PCL/F68/17 and PCL/PVA/23 to PCL/PVA/26), PLGA (PLGA/F68/14 to PLGA/F68/17 and PLGA/PVA/23 to PLGA/PVA/26) and PLA (PLA/F68/14 to PLA/F68/17 and PLA/PVA/23 to PLA/PVA/26) NPs were prepared using two stabilizers PF 68 and PVA at four different concentrations, 0.25, 0.5, 0.75 and 1 % w/v. The effect of drug amount (1, 2, 3, 4 and 5 mg) in the preparation and characters of NPs using two stabilizers PF 68 and PVA, was studied in all the polymers, PCL (PCL/F68/18 toPCL/F68/22 and PCL/PVA/27 to PCL/PVA/31), PLGA (PLGA/F68/18 to PLGA/F68/31 and PLGA/PVA/27 to PLGA/PVA/31) and PLA (PLA/F68/18 to PLA/F68/22 and PLA/PVA/27 to PLA/PVA/31).

5.4 Characterization of PNPs

5.4.1 Determination of Paclitaxel in PNPs

The EE of the prepared PNPs was determined by direct method. Sediment formed after centrifugation of nanodispersion was digested (30 min) with acetonitrile through sonication (6.5 L Toshibha Laboratory testing instruments, Delhi, India), then suitably diluted with

acetonitrile:water (50:50 % v/v) for analysis. The DC in PNPs was determined by taking required amount of freeze-dried PNPs/nanodispersion and digested (30 min) with acetonitrile through sonication. The obtained solution was filtered through 0.44 µm membrane filter and the DC was determined using developed HPLC method. The EE and DC were calculated using standard equation (1).

5.4.2 Surface properties of PNPs

The prepared PNPs were characterized for the size, distribution and PDI by photon correlation spectroscopy using a Zetasizer, Nanoseries (Nano-ZS, Malvern, UK). The surface charge/ZP was measured in water (pH 6.2-7.4) with a Zetasizer, Nanoseries at 25°C. PNPs morphology was examined by scanning electron microscopy (SEM, JSM-7600F), transmission electron microscopy (TEM, Philips, CM200, Netherlands) and atomic force microscopy (AFM, Nanoscope II, USA).

5.5 In-vitro dissolution study

To study the release behaviour of PNPs, three millilitre of nanodispersion was spiked into a dialysis bag (Spectrapor, molecular weight cut off: 12,500 Da, USA) which is then sealed and dropped into a beaker containing 200 mL of phosphate buffer (pH 7.4) with 0.5 % (w/v) Tween 80. The whole experiment set-up was placed in a shaker maintained at temperature 37°C ± 2 with continuous horizontal shaking at 100 rpm. Samples were withdrawn from the beaker at predetermined time points, with spontaneous replacement of fresh buffer media. Paclitaxel release from the PNPs into the medium was estimated by developed HPLC method mentioned in Chapter 3. The obtained dissolution data were fitted to various kinetic equations, Zero order, First order, Higuchi, Korsmeyer-Peppas, Hixson-Crowell and Baker-Lonsdate model to find the order and mechanism of Paclitaxel release from the PNPs (9).

Zero order kinetic equations

$$F = k_0 \times t \quad (1)$$

First order kinetic equations

$$F = 100\left(1 - e^{-kt}\right) \quad (2)$$

Higuchi square-root equation

$$F = k_H t^{0.5} \quad (3)$$

Korsmeyer-Peppas equation

$$F = k_{kp} t^{n} \quad (4)$$

Hixson-Crowell equation

$$F = 100\left[1 - \left(1 - k_{hc} t\right)^3\right] \quad (5)$$

Baker-Lonsdate (BK) equation

$$BL = 3/2\left[1-(1-F/100)^{2/3}\right]-F/100 = k_{BL}t, k_{BL} = \left[3\times D\times C_s/(r_0^{\,2}\times C_0)\right] \qquad (6)$$

Where, F is the percentage drug released in time t, k_0 (µg/h) is the zero-order release constant, k (µg/h) is the first-order release constant, k_H (µg/$h^{-0.5}$) is the Higuchi release constant, k_{KP} (µg/h^{-n}) is the release constant incorporating structural and geometric characteristics of the drug-dosage form; n is the diffusional exponent indicating the drug-release mechanism, in case of non-Fickian release, n falls between 0.5 and 1.0 (0.5 < n < 1.0), while for Fickian diffusion n = 0.5, for zero order release (case II transport) n = 1, and for supercase II transport, n > 1. The values of n as estimated by linear regression of F vs. log (t). k_{HC} (µg/h^{-3}) is the release constant in Hixson–Crowell model, k_{BL} (µg/h), is the combined constant in Baker–Lonsdale model, D is the diffusion coefficient, Cs is the saturation solubility, r_0 is the initial radius for a sphere or cylinder or the half-thickness for a slab, C_0 is the initial drug loading in the matrix.

The in-vitro dissolution data was used to find, area under the dissolution curve (AUDC), area between the drug dissolution curve and its asymptote (ABC), mean dissolution time (MDT), variance of dissolution time (VDT), relative dispersion of dissolution time (RD) and dissolution efficiency (DE). All the parameters were calculated using a model-independent nonparametric method based on the linear trapezoidal rule, using standard formula (9).

5.6 Stability study

US-FDA has not framed any specific guidelines for NPDDS, production, characterization, handling and use. There is no protocol and limits available from any international bodies to conduct stability studies of these formulations. In the present work, the stability study was performed by keeping the NPs samples in three different conditions, room temperature (15 ± 5°C), refrigerator (5 ± 2°C) and at 37 ± 5 °C over a period of 4 months. The NPs were evaluated at 0, 2 and 4 months for there size, PDI, ZP, DC and in-vitro dissolution. In addition any change in physical appearances was observed and the samples were characterized for their shape and structure using AFM.

5.7 Result and Discussion

5.7.1 Preparation of NPs

Based on the initial experiment, organic to aqueous phase ratio, 1:4 was fixed constant throughout the study when other formulation parameters were changed. Paclitaxel loaded PCL and PLA NPs were prepared successfully using simple and fast nanoprecipitation method which is the most reproducible and economical method among all other methods. The nanoprecipitation method produces stable, small and narrow size NPs without using any toxic chlorinated solvents. Paclitaxel loaded PLGA NPs were prepared by solvent evaporation

method which is an ideal method to incorporate lipophilic drugs and it was the first method developed to prepare NPs.

In the nanoprecipitation method when acetone solution of polymers and drug was added to the aqueous phase, there was rapid gradient-driven diffusion of amphiphilic acetone across the interface, which creates a kind of instability at interface and there was marked decrease in the interfacial tension which resulted in the spontaneous emulsification of the acetone solution in the form of nanodroplets. The formed nanodroplets which contain the dissolved polymer and drug (PCL and PLA) will aggregate as NPs, because of the spontaneous diffusion of acetone and presences of nonsolvent medium (water) to the dissolved polymer. This initial precipitation of polymer forms polymeric nano matrix, further evaporation of the surface solvent by stirring or rotavapor resulted in solidification of the NPs formed (1). In nanoprecipitation method, acetone was selected because it solubilized Paclitaxel and PCL and PLA effectively and it took very less time to evaporate acetone during hardening of NPs, which is a very critical step in NPs preparation (1). In case of emulsion and solvent evaporation method dichloromethane was selected because it has very low water solubility of 2 % w/v when compare to other solvents like ethyl acetate (8.7 % w/v) which is used very often (10, 11).

To get high value of drug encapsulation, the challenging issue in the NPs preparation protocol is to avoid the diffusion of drug along with the solvent to the other phase. The role of surfactant or stabilizer (PVA/PF 68) in nanoprecipitation process is it lowers interfacial energy and helps in the rapid diffusion of acetone to the aqueous phase. The above instantaneous process controls the size of polymer precipitation into NPs. Hence the finest amount of stabilizer in the formula will result in instant and reproducible NPs formation with low PDI with high drug loading capacity (12). Recently in our group, etoposide loaded PLGA NPs wee prepared with very small size, 105.1 ± 2.38 nm with high EE, 78.99 ± 1.04 % by nanoprecipitation technique and prepared PCL NPs were prepared by emulsification and solvent evaporation method using PF 68 as stabilizer with 257 ± 3.96 nm and the EE was 80.15 ± 1.01% respectively (7).

5.7.2 Physiochemical characterization of PNPs

a) Surface properties of PNPs

The detail morphological properties of the prepared PNPs were investigated and presented using the sophisticated microscopic techniques, SEM (Fig. 5.1), TEM (Fig. 5.2) and AFM (Fig. 5.3). The SEM image shows that all the prepared PNPs were spherical in shape with homogeneous solid matrix structure with no evidences of aggregation and crystals of Paclitaxel on the surface. From the TEM image, it was evident that the prepared NPs were in fine globular profile and close view of a single NP (Fig. 5.2) clearly showed the various degree of smooth structure without any amorphous arrangement. Because of small size of the NPs the

close investigation of single particle was done using AFM which gave clear 3D morphological images (Fig. 5.3). The 3D view of multiple particles showed smooth surface, but the single particles showed some roughness on their surface which is physical evidence for diffusion and matrix erosion drug release mechanism (7, 8).

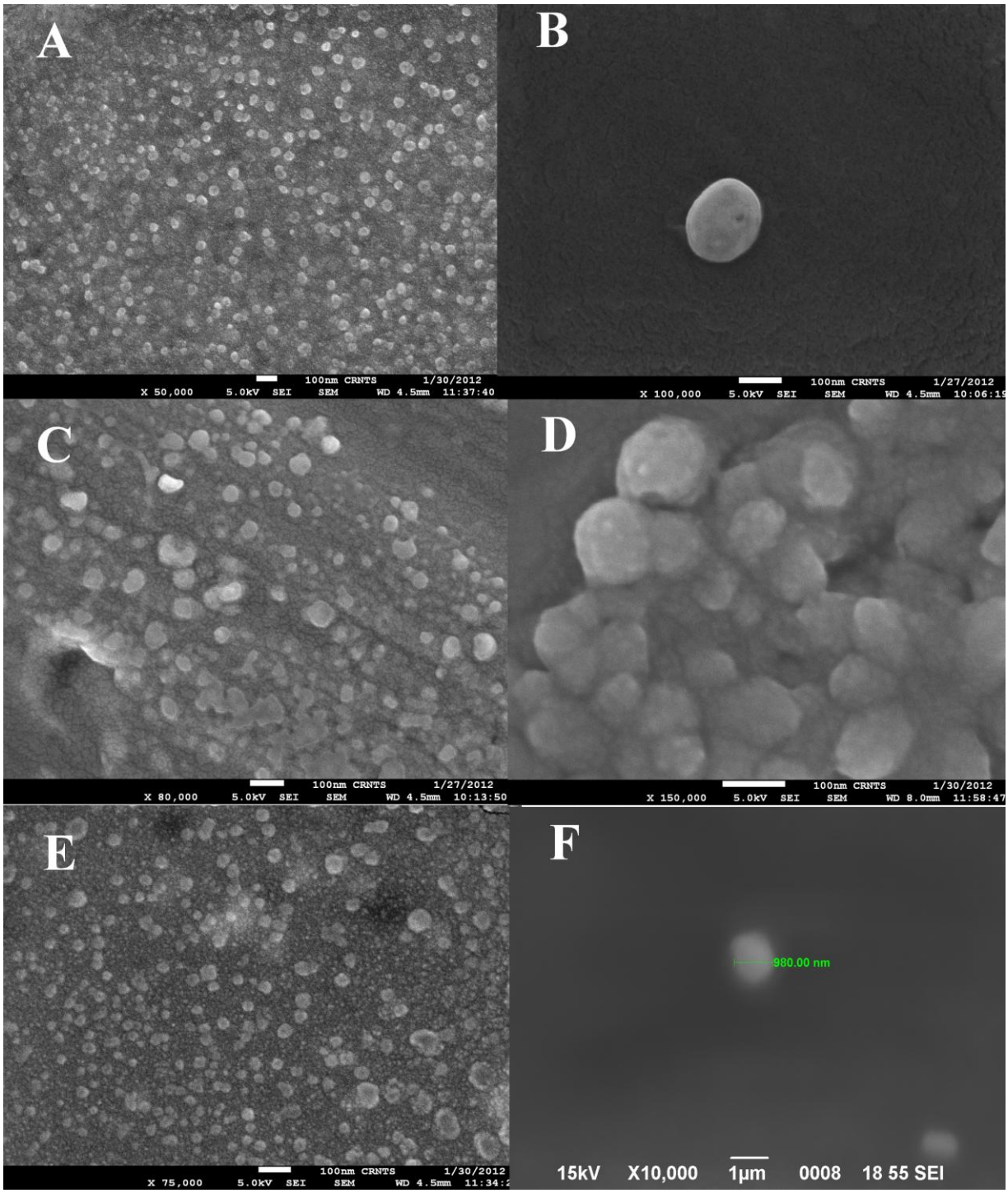


Fig. 5.1 Characterization of Paclitaxel loaded PNPs by SEM, cluster and single PCL NPs (A and B), cluster PLGA NPs (C and D) and cluster and single PLA NPs (E and F)

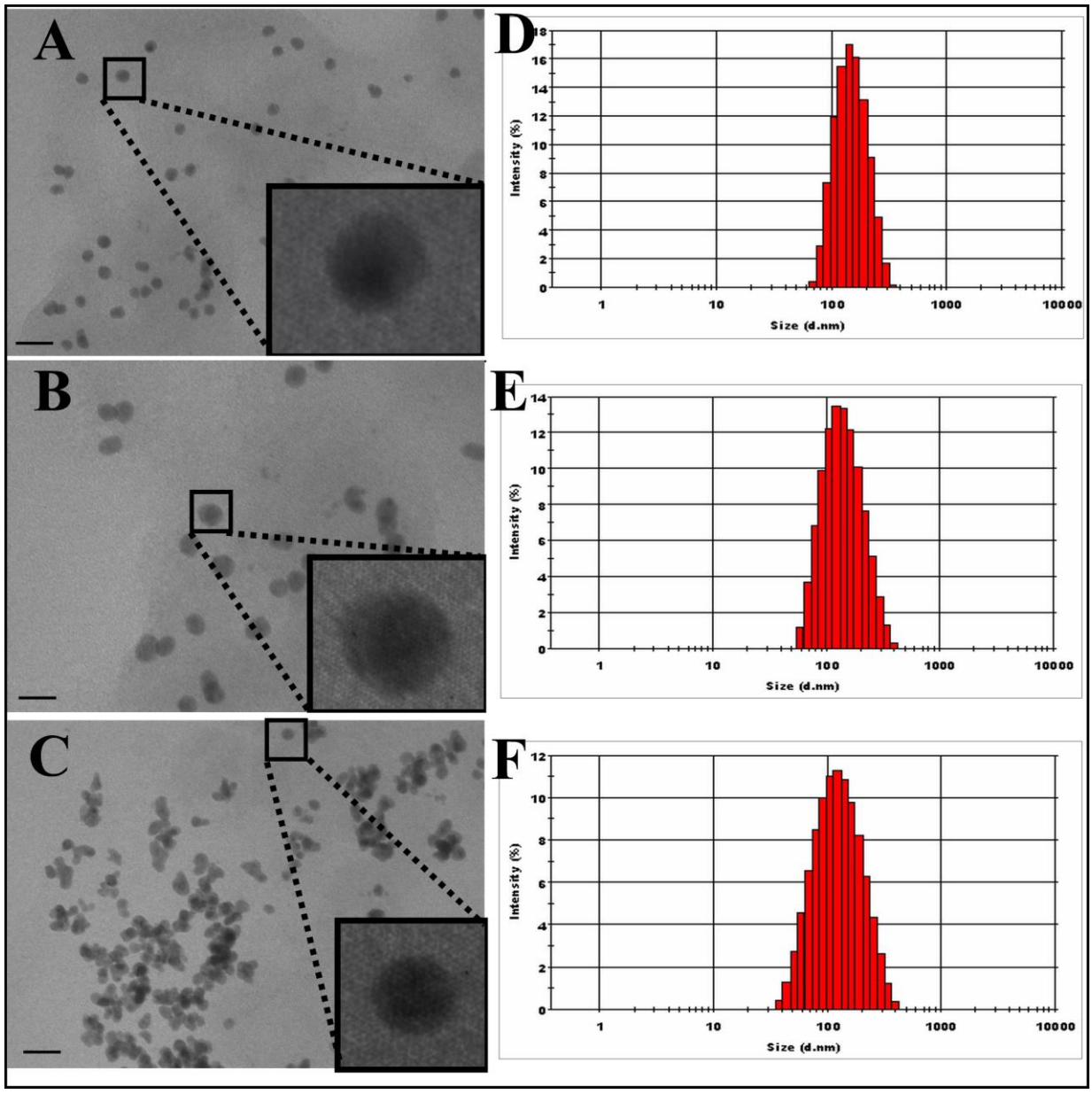


Fig. 5.2 Characterization of Paclitaxel loaded PNPs by TEM, PCL NPs (A), PLGA NPs (B) PLA NPs (C), particle size distribution of PCL NPs (D), PLGA NPs (E) and PLA NPs (F), bar represents 100 nm

5.7.3 Influences of polymer amount on NPs characterization

a) PCL amount vs. NPs characterization

The effect of polymer, PCL, PLGA and PLA amount on the mean particle size, DC and EE were shown in Fig. 5.4. In PCL NPs, when the polymer amount is increased from 1 (PCL/F68/01) to 3 (PCL/F68/03) mg, there was no significant increase in the size but the PDI was increased (0.04 to 0.12) considerably (Fig. 5.5). There was no linear (r^2 = 0.7792) increase in particle size when the polymer amount was increased from 1to 200 mg for 1mg of Paclitaxel with 0.5 % stabilizer. When the PCL amount was increased from 5 to 40 mg (Table 5.1), there was significant increase in particle size 135.32 ± 4.99 nm, 157.63 ± 2.34 nm, 174.02 ± 9.34 nm

and 187.32 ± 1.35 nm with increase of polymer amount. There was effect on particle size when amount of polymer increased beyond 50 mg. In case of 50 mg of PCL, the particle size observed was 191.04 ± 5.63 nm, and when the polymer amount is doubled to 100 mg (PCL/F68/09), the particle size was only, 203.33 ± 14.01 nm (Fig. 5.5).

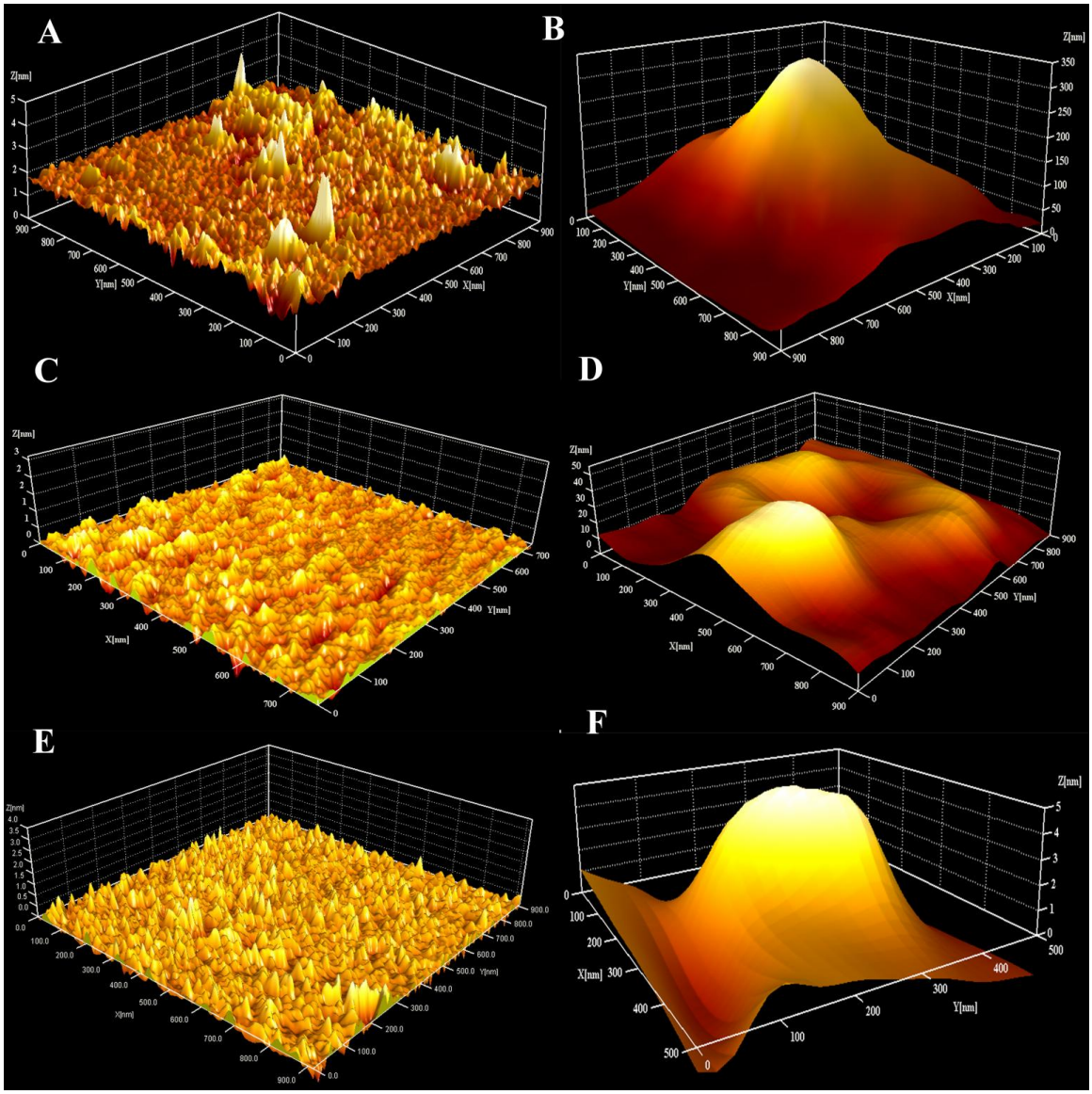


Fig. 5.3 Characterization of Paclitaxel loaded PNPs by AFM, cluster and single PCL NPs (A and B), cluster PLGA NPs (C and D) and cluster and single PLA NPs (E and F)

Hence for formulation PCL/F68/07 to PCL/F68/13 (50 to 200 mg) there was no significant increase in the particle size (Table 5.1). The SD of formulation PCL/F68/07 to PCL/F68/13 is very high (Fig. 5.4) indicating the particle size repeatability is very poor (7, 10). To get polydisperse nanosuspension the PDI should be less than one. The influences of polymer amount on PDI of the formulations PCL/F68/01 to PCL/F68/13 was represented in Table 5.1 and Fig. 5.5. In the present work, the PDI was less than 0.23 which shows the narrow size distribution. The EE of the PCL NPs with varying amount of polymer was represented in Table

5.1. It can be observed that after 20 mg (PCL/F68/05) of addition of PCL to the organic phase, there was no significant increase in the particle size and EE, in fact it decreases the EE (Table 5.1). Hence addition of more than 20 mg is surplus and it's going to be settled during the purification processing and hence it will decrease the EE (Fig. 5.4). If the EE is low then there is need for more volume of nanosuspension to be administered, which leads unnecessary systemic exposure of excipients. The EE of PCL NPs was high when compared to PLA NPs (Fig. 5.4) even though both the NPs were prepared by nanoprecipitation method. The possible reason for this variation could be difference in the interaction of Paclitaxel and polymer when they are solubilized in acetone (12).

Zhang et al. (13) prepared Paclitaxel loaded PCL NPs by nanoprecipitation method using 1:100 drug polymer ratio with 2 % PVA stabilizer. They reported particle size 272.67 ± 4.44 nm, PDI 0.30, DL, 9.98 % and EE 71.49 %. In the present method, evaluation of optimized PCL NPs (PCL/F68/05) with 1:20 drug polymer ratio shows particle size of 174.02 ± 9.34 nm, DC of 4.60 ± 0.02 % with high EE, 96.40 ± 0.13 % and % recovery of NPs, 99.53 ± 0.39. Hence it was clear that, the prepared Paclitaxel loaded PCL NPs were efficient and cost effective when compared to the earlier reported method, where they used five times of polymer to get low EE and higher particle size. During our optimization experiment, formulation PCL/F68/09 whose PCL amount was 100 mg with stabilizer concentration 0.5 % shows particle size, 203.33 ± 14.01 nm with EE 84.37 ± 0.50 % and DC 1.14 ± 0.02 %, this result is also better than Zhang et al (13).

b) PLGA amount vs. NPs characterization

In case of PLGA NPs, prepared by solvent evaporation method, when the polymer amount was increased form 1 (PLGA/F68/01) to 3 mg (PLGA/F68/03), as like PCL NPs, there was no significant increase in size, 72.56 ± 1.21 nm and 76.63 ± 0.89 nm respectively (Table 5.2). In case of formulation PLGA/F68/03 to PLGA/F68/13, where the polymer amount was increased from 5 to 200 mg, there were increment in particle size and PDI with low SD (Fig. 5.4 and 5.5). It was observed that there was linear (r^2 = 0.9342) increase in particle size when the polymer amount is increased from 1 to 200 mg. The particle size of formulations PLGA/F68/03 to PLGA/F68/13 was in the range of 84.12 to 188.45 nm respectively (Table 5.2, Fig. 5.4). The influences of polymer amount on PDI of the prepare PLGA NPs was represented in Fig. 5.5. The particle size analysis results indicate that, to prepare NPs of a any size, by solvent evaporation method, more amount of PLGA is required that PCL. In case of PCL NPs, 20 mg PCL was sufficient to get NPs of particle size 174.02 nm with low PDI (0.09 ± 0.02) and high EE (96.40 ± 0.13 %) with good % recovery of NPs (99.53 ± 0.39). For NPs of same size and characters PLGA required is 100 mg. On analysis of effect of polymer amount on EE, indicate

that EE is increased with increase in amount of PLGA up to 100 mg. Beyond which EE found to decrease, Fig. 5.4 and 5.5. Sinjan et al (14) prepared PLGA NPs by solvent evaporation method using 1.5 % PVA and found Paclitaxel crystals on the surface indicating incomplete entrapment and encapsulation.

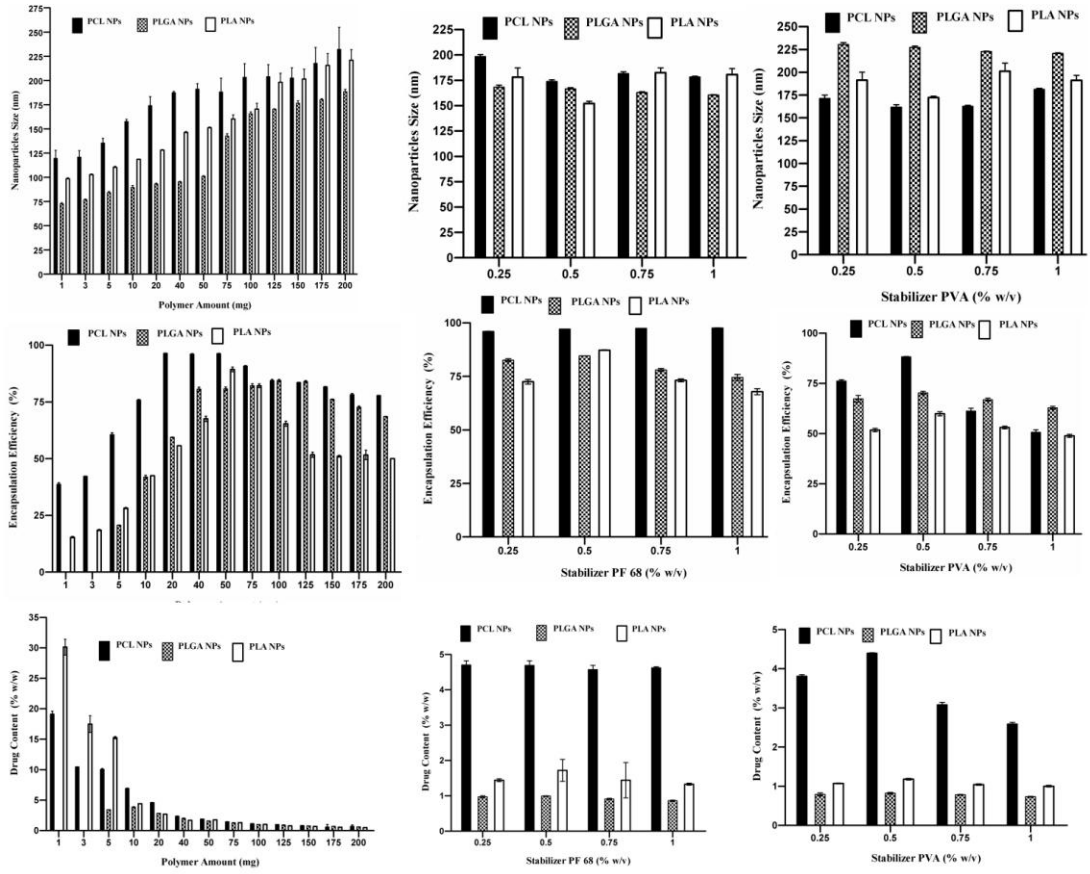


Fig. 5.4 Influences of polymer amount (A, D and G), PF 68 amount (B, E and H) and PVA amount (C, F and I) on nanoparticle size, EE and DC of Paclitaxel loaded PNPs

However, in present work no such Paclitaxel crystals were found on surface of NPs and surface were smooth. Amount of surfactant (PF 68) was also required in much lesser amount (0.5 %) to get NPs. Similarly, in another study by Chakravarthi et al. (15) produced PLGA NPs by solvent evaporation method using 90 mg of PLGA and obtained larger average particle size (315 nm) where as present method produces NPs of smaller size nanoparticles.

c) PLA amount vs. NPs characterization

In PLA NPs formulations PLA/F68/01 to PLA/F68/13, increase in polymer amount (1 to 200 mg) for 1 mg drug, was found to have linear relationship (Fig. 5.4) with increase in particle size (r^2 = 0.9636) (Table 5.3). The PDI of the formulations PLA/F68/01 to PLA/F68/13 was less than one, which suggests the homogeneous polydisperse NPs. As like other two polymers, PLA also decreased EE beyond 50 mg of polymer (Fig. 5.4). This confirmed that the polymer

amount is the critical parameters in the formulation of polymeric NPs. Using beyond 50 mg of polymer, there were decrease in EE and % recovery (Fig. 5.4 and 5.5).

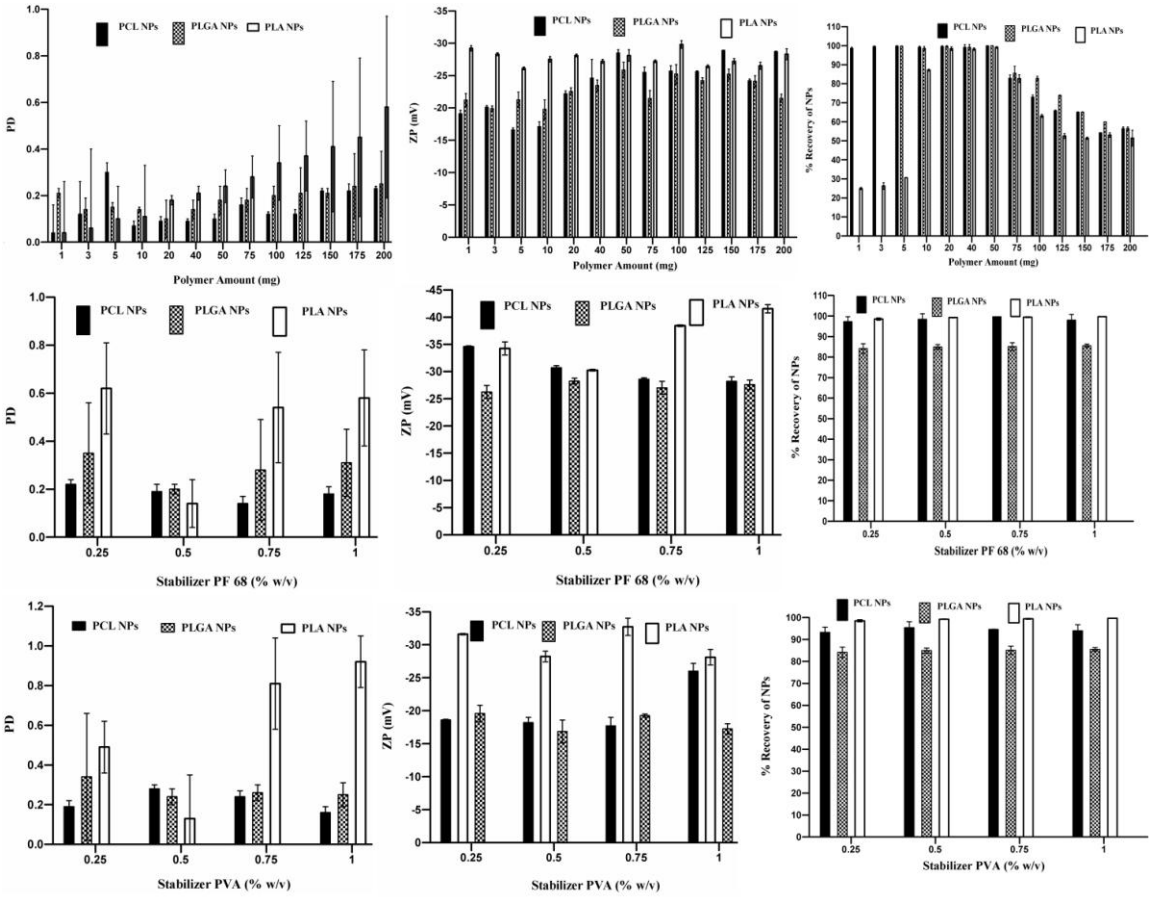


Fig. 5.5 Influences of polymer (A, B and C), PF 68 (D, E and F) and PVA amount (G, H and I) on PDI, ZP and % recovery of NPs of Paclitaxel loaded PNPs

The particle size of PLA/F68/07 formation was 151.23 ± 0.73 nm whose PLA amount was 50 mg, but when the polymer amount was doubled to 100 mg, the obtained particle size was 170.42 ± 6.11 nm. The EE of the formulation PLA/F68/07 was 89.32 ± 1.03 %, but it was decreased to 65.38 ± 1.13 % when the polymer amount was doubled. In case of formulation PLA/F68/13, where the polymer amount was 200 mg, there was decrease in EE, 50.05 ± 0.08 % and the particle size, 220.93 ± 11.05 nm. This once again showed that addition of more than 50 mg PLA will be surplus and it will increase the cost of formulation unnecessarily. The DC of prepared formulations was represented in Table 5.3. Already we have showed that, increase in particle size when there was increase in polymer amount in the etoposide loaded polymeric NPs and few other authors were reported this effect in the biodegradable polymers (7, 10). In all the polymers, general observation is that, EE increased with the increase in polymer amount up to a certain limit above which EE decreased (Fig. 5.4). This may be due to insufficient stabilizer with respect increased polymer, which led to agglomeration of NPs and settling at the bottom as agglomerate (Fig. 5.5).

Table 5.1 Composition and characterization of Paclitaxel loaded PCL NPs with variable concentration of polymer PCL*

Batch Code	Drug (mg)	Polymer (mg)	PF 68 (%w/v)	Mean Size (nm ±SD)	Distribution (d. nm)	[a]PDI ±SD	[b]ZP ±SD	[c]EE (%)	[d]DC (% w/w)
PCL/F68/01	1	1	0.5	119.51 ± 8.32	35-250	0.04 ± 0.12	-19.13 ± 0.52	38.68 ± 0.74	19.14 ± 0.47
PCL/F68/02	1	3	0.5	120.83 ± 6.73	35-220	0.12 ± 0.14	-20.14 ± 0.24	42.20 ± 0.08	10.46 ± 0.03
PCL/F68/03	1	5	0.5	135.32 ± 4.99	68–255	0.30 ± 0.04	-16.63 ± 0.31	60.54 ± 0.91	10.03 ± 0.16
PCL/F68/04	1	10	0.5	157.63 ± 2.34	68–342	0.07 ± 0.02	-17.12 ± 0.72	75.78 ± 0.37	6.91 ± 0.06
PCL/F68/05	1	20	0.5	174.02 ± 1.34	79–396	0.09 ± 0.02	-22.22 ± 0.42	96.40 ± 0.13	4.60 ± 0.02
PCL/F68/06	1	40	0.5	187.32 ± 1.35	91-459	0.09 ± 0.01	-24.62 ± 2.89	96.02 ± 0.23	2.36 ± 0.04
PCL/F68/07	1	50	0.5	191.04 ± 5.63	91-459	0.10 ± 0.02	-28.52 ± 0.49	96.25 ± 0.20	1.89 ± 0.02
PCL/F68/08	1	75	0.5	188.13 ± 14.29	68-825	0.16 ± 0.03	-25.54 ± 0.81	90.75 ± 0.30	1.44 ± 0.03
PCL/F68/09	1	100	0.5	203.33 ± 14.01	68-531	0.12 ± 0.01	-25.74 ± 0.81	84.37 ± 0.50	1.14 ± 0.02
PCL/F68/10	1	125	0.5	203.90 ± 12.44	68-615	0.12 ± 0.02	-25.63 ± 0.12	83.55 ± 0.09	1.01 ± 0.01
PCL/F68/11	1	150	0.5	202.52 ± 10.59	68-615	0.22 ± 0.01	-28.92 ± 0.01	81.64 ± 0.18	0.83 ± 0.02
PCL/F68/12	1	175	0.5	217.73 ± 16.43	58-955	0.22 ± 0.03	-24.24 ± 0.25	78.17 ± 0.54	0.57 ± 0.43
PCL/F68/13	1	200	0.5	232.13 ± 22.85	59-825	0.23 ± 0.01	-28.72 ± 0.06	77.82 ± 0.08	0.69 ± 0.25

*Each data represents the average and standard deviation of three independent determinations, [a]Polydispersity index, [b]Zeta potential, [c]Encapsulation Efficiency, [d]Drug Content

Table 5.2 Composition and characterization of Paclitaxel loaded PLGA NPs with variable concentration of polymer PLGA*

Batch Code	Drug (mg)	Polymer (mg)	PF 68 (% w/v)	Mean Size (nm ±SD)	Distribution (d. nm)	[a]PDI ±SD	[b]ZP ±SD	[c]EE (%)	[d]DC (% w/w)
PLGA/F68/01	1	1	0.5	72.56 ± 1.21	40-250	0.21 ± 0.02	-21.21 ± 1.01	-	-
PLGA/F68/02	1	3	0.5	76.63 ± 0.89	50-225	0.14 ± 0.05	-19.89 ± 0.41	-	-
PLGA/F68/03	1	5	0.5	84.12 ± 1.24	45-225	0.15 ± 0.02	-21.24 ± 1.20	20.67 ± 0.07	3.43 ± 0.01
PLGA/F68/04	1	10	0.5	89.41 ± 2.26	50-250	0.14 ± 0.01	-19.78 ± 1.45	41.84 ± 0.82	3.84 ± 0.10
PLGA/F68/05	1	20	0.5	92.96 ± 1.01	45-275	0.10 ± 0.08	-22.52 ± 0.58	59.38 ± 0.13	2.83 ± 0.02
PLGA/F68/06	1	40	0.5	95.22 ± 0.56	50-250	0.14 ± 0.04	-23.45 ± 0.85	80.60 ± 0.89	1.98 ± 0.10
PLGA/F68/07	1	50	0.5	100.85 ± 0.85	50-300	0.18 ± 0.06	-25.85 ± 1.20	80.76 ± 0.83	1.58 ± 0.02
PLGA/F68/08	1	75	0.5	142.52 ± 2.56	42-275	0.18 ± 0.05	-21.45 ± 1.23	82.11 ± 0.86	1.26 ± 0.05
PLGA/F68/09	1	100	0.5	165.52 ± 2.10	50-300	0.20 ± 0.04	-25.23 ± 1.45	84.37 ± 0.50	1.01 ± 0.01
PLGA/F68/10	1	125	0.5	170.29 ± 0.45	58-325	0.21 ± 0.11	-24.21 ± 0.45	83.92 ± 0.44	0.90 ± 0.05
PLGA/F68/11	1	150	0.5	176.44 ± 2.56	60-400	0.21± 0.12	-25.21 ± 0.78	76.09 ± 0.18	0.77 ± 0.01
PLGA/F68/12	1	175	0.5	180.11 ± 1.05	42-325	0.24 ± 0.14	-24.12 ± 0.85	72.62 ± 0.54	0.69 ± 0.01
PLGA/F68/13	1	200	0.5	188.45 ± 2.45	40-350	0.25 ± 0.14	-21.48 ± 0.65	68.56 ± 0.08	0.60 ± 0.02

*Each data represents the average and standard deviation of three independent determinations, [a]Polydispersity index, [b]Zeta potential, [c]Encapsulation Efficiency, [d]Drug Content

Table 5.3 Composition and characterization of Paclitaxel loaded PLA NPs with variable concentration of polymer PLA*

Batch Code	Drug (mg)	Polymer (mg)	PF 68 (% w/v)	Mean Size (nm ±SD)	Distribution (d. nm)	[a]PDI ±SD	[b]ZP ±SD	[c]EE (%)	[d]DC (% w/w)
PLA/F68/01	1	1	0.5	98.42 ± 1.02	40-450	0.04 ± 0.22	-29.2 ± 0.42	15.37 ± 0.34	30.13 ± 1.30
PLA/F68/02	1	3	0.5	102.82 ± 0.73	45-390	0.06 ± 0.34	-28.3 ± 0.24	18.52 ± 0.27	17.49 ± 1.37
PLA/F68/03	1	5	0.5	110.31 ± 0.99	55–450	0.10 ± 0.14	-26.1 ± 0.21	28.19 ± 0.29	15.25 ± 0.18
PLA/F68/04	1	10	0.5	118.63 ± 0.24	65–500	0.11 ± 0.22	-27.5 ± 0.42	42.47 ± 0.10	4.41 ± 0.02
PLA/F68/05	1	20	0.5	128.14 ± 0.48	80–450	0.18 ± 0.02	-28.1 ± 0.22	55.70 ± 0.14	2.69 ± 0.02
PLA/F68/06	1	40	0.5	146.33 ± 1.01	90-525	0.21 ± 0.03	-27.2 ± 0.29	67.58 ± 1.18	1.68 ± 0.02
PLA/F68/07	1	50	0.5	151.23 ± 0.73	90-450	0.24 ± 0.07	-28.1 ± 0.89	89.32 ± 1.03	1.77 ± 0.02
PLA/F68/08	1	75	0.5	160.23 ± 4.29	55-500	0.28 ± 0.09	-27.2 ± 0.21	82.11 ± 0.86	1.31 ± 0.04
PLA/F68/09	1	100	0.5	170.42 ± 6.11	60-575	0.34 ± 0.16	-29.8 ± 0.61	65.38 ± 1.13	1.03 ± 0.03
PLA/F68/10	1	125	0.5	198.23 ± 9.14	65-600	0.37 ± 0.15	-26.4 ± 0.22	51.70 ± 1.14	0.78 ± 0.04
PLA/F68/11	1	150	0.5	201.52 ± 10.19	65-650	0.41 ± 0.28	-27.2 ± 0.41	51.05 ±0.42	0.66 ± 0.01
PLA/F68/12	1	175	0.5	215.63 ± 12.13	50-750	0.45 ± 0.34	-26.5 ± 0.55	51.64 ± 2.07	0.55 ± 0.02
PLA/F68/13	1	200	0.5	220.93 ± 11.05	55-700	0.58 ± 0.39	-28.3 ± 0.86	50.05 ± 0.08	0.49 ± 0.04

*Each data represents the average and standard deviation of three independent determinations, [a]Polydispersity index, [b]Zeta potential, [c]Encapsulation Efficiency, [d]Drug Content

Table 5.4 Composition and characterization of Paclitaxel loaded and blank PCL NPs with variable concentration of stabilizers

Batch Code	Drug (mg)	Polymer (mg)	Stabilizer (% w/v)		Mean Size (nm ±SD)	Distribution (d. nm)	[a]PDI ±SD	[b]ZP ±SD	[c]EE (%)	[d]DC (% w/w)
			PF 68	PVA						
PCL/F68/14	1	20	0.25	-	198.32 ± 2.05	91–459	0.22 ± 0.02	-34.62 ± 0.10	96.04 ± 0.02	4.70 ± 0.12
PCL/F68/15	1	20	0.5	-	173.81 ± 1.64	91–342	0.19 ± 0.03	-30.72 ± 0.36	97.09 ± 0.01	4.69 ± 0.13
PCL/F68/16	1	20	0.75	-	181.72 ± 1.82	79–459	0.14 ± 0.03	-28.62 ± 0.25	97.45 ± 0.01	4.57 ± 0.12
PCL/F68/17	1	20	1	-	178.22 ± 0.84	79–397	0.18 ± 0.03	-28.24 ± 0.85	97.64 ± 0.01	4.62 ± 0.03
PCL/F68/18	1	20	0.5	-	184.03 ± 0.34	80–400	0.07 ± 0.02	-28.21 ± 0.42	95.38 ± 0.20	4.84 ± 0.04
PCL/F68/19	2	20	0.5	-	182.24 ± 1.35	90-550	0.28 ± 0.03	-27.31 ± 0.34	96.07 ± 0.18	8.91 ± 0.14
PCL/F68/20	3	20	0.5	-	185.42 ± 0.67	90-500	0.26 ± 0.01	-28.42 ± 0.27	97.05 ± 1.34	12.87 ± 0.06
PCL/F68/21	4	20	0.5	-	184.51 ± 0.32	82-550	0.54 ± 0.41	-30.23 ± 0.87	97.26 ± 1.78	16.61 ± 0.59
PCL/F68/22	5	20	0.5	-	186.42 ± 1.01	90-450	0.45 ± 0.61	-30.83 ± 0.69	98.2 5 ± 0.02	19.99 ± 0.24
PCL/PVA/23	1	20	-	0.25	171.31 ± 3.60	91–342	0.19 ± 0.03	-18.64 ± 0.10	76.17 ± 0.69	3.81 ± 0.04
PCL/PVA/24	1	20	-	0.5	161.71 ± 2.78	79–295	0.28 ± 0.02	-18.21 ± 0.80	88.19 ± 0.24	4.40 ± 0.01
PCL/PVA/25	1	20	-	0.75	162.72 ± 1.02	79–342	0.24 ± 0.03	-17.72 ± 1.30	61.24 ± 1.40	3.08 ± 0.06
PCL/PVA/26	1	20	-	1	181.24 ± 1.30	91–396	0.16 ± 0.03	-26.03 ± 1.17	50.64 ± 1.30	2.59 ± 0.04
PCL/PVA/27	1	20	-	0.5	163.13 ± 1.08	91-350	0.09 ±0.51	-20.12± 1.10	86.70 ± 0.02	4.40 ± 0.03
PCL/PVA/28	2	20	-	0.5	165.62 ± 1.09	91-350	0.13 ±0.25	-19.56± 0.95	86.71 ± 0.02	8.04 ± 0.13
PCL/PVA/29	3	20	-	0.5	161.82 ± 2.05	91-350	0.42 ±0.65	-18.45± 1.32	89.46 ± 0.02	11.86 ± 0.11
PCL/PVA/30	4	20	-	0.5	166.52 ± 1.06	91-350	0.06 ±0.89	-22.52± 0.56	90.06 ± 0.02	15.38 ± 0.28
PCL/PVA/31	5	20	-	0.5	166.12 ± 2.34	80-325	0.08 ±0.54	-20.12± 0.75	96.83 ± 0.01	19.70 ± 0.23
BPCL/F68/01	-	50	0.25	-	181.42 ± 2.21	90-500	0.11 ± 0.03	-41.02 ± 4.11	-	-
BPCL/F68/02	-	50	0.50	-	147.42 ± 3.65	70-350	0.15 ± 0.03	-29.02 ± 1.65	-	-
BPCL/F68/03	-	50	0.75	-	205.61 ± 2.32	50-450	0.21 ± 0.03	-27.13 ± 1.45	-	-
BPCL/F68/04	-	50	1	-	197.61 ± 3.08	50-400	0.14± 0.03	-28.33 ± 1.27	-	-

[*]Each data represents the average and standard deviation of three independent determinations, [a]Polydispersity index, [b]Zeta potential, [c]Encapsulation Efficiency, [d]Drug Content

5.7.4 Influences of stabilizer (PF 68/PVA) and its concentration on NPs characterization

The amount of surfactant in the formulation of NPs play very vital role in determining the size and EE. Care should be taken that only the required amount of stabilizer was added in the formulation because at higher concentrations these surfactants produce potential toxicity. It has been reported that at higher concentration of surfactants are capable of disrupting the biological membrane and it can interact with some body proteins (10, 11). Hence addition of optimal concentration of surfactant is critical to get not only stable, ideal size and good EE, but also to get safe NPs formulation. The polydisperse NPs with small particles size enables i.v administration without any irritant reaction at the sit of injection and there will not be any risk of embolic.

The non-ionic emulsifier PF 68 and PVA offers steric stabilization, which avoids the aggregation of the fine particles in the prepared colloidal system, which results in smaller particles and narrow size distribution (7). In the present study when the surfactants, PF 68 or PVA concentration was increased (Table 5.4, 5.5 and 5.6), keeping the other component constant, the particle size of the respective PCL, PLGA and PLA NPs decreased and after certain concentration of stabilizer the size was increasing (Fig. 5.4). This happens because at the lower concentration (<0.5 % w/v), the surfactant amount was not sufficient to stabilize the instantaneously formed NPs, which results the formed NPs to come closer due to their attractive force and to form bigger nanoparticles or aggregates. This aggregation continues still the total surface area of the NPs was decreased to a point (the particle size increases) where the amount of available surfactant was sufficient to produce a stable system which results in the lager particles, which may be in manometer or micrometer (1, 7, 8).

In case of PCL and PLA NPs when the stabilizer PF 68/PVA concentration was increased from 0.25 to 1 %, the particle size decreased at 0.5 % when compared to 0.25 % level, but the particle size was increased at 0.75 % level (Table 5.4 and 5.6). The expected reason for increase in particle size when stabilizer concentration is increased could be that the surplus stabilizer within the formulation was incorporated into a multilayer arrangement surrounding the nanoparticles, which eventually results in increased particle size. This excess surfactant on the particles surface not only play role in size, it also affects the Paclitaxel release from the NPs, which is explained in the later section. Hence, at the optimum stabilizer concentration in our case it was 0.5 % for both PF 68 and PVA resulted in small size, narrow distribution particles with low PDI (Fig. 5.5) and high EE (Fig. 5.4). However, in case of PLGA NPs increase in stabilizer concentration is same range produced marginal decrease in size.

In case of PLGA NPs when the PF 68/PVA concentration was increased from 0.25 to 1 % (PLGA/F68/14 to PLGA/F68/17 and PLGA/PVA/23 to PLGA/PVA/26), there was decrease in

EE (Table 5.5). In PLGA NPs formulation PLGA/F68/14 to PLGA/F68/17 and PLGA/PVA/23 to PLGA/PVA/26, when the stabilizers PF 68/PVA concentration was increased, the EE decreases from, 87.26 to 48.92 % respectively (Table 5.4). But in case of PCL NPs when the stabilizer PF 68 concentration was increased (PCL/F68/14 to PCL/F68/17) there was no increase as observed from value of EE (%), 96.04 ± 0.02, 97.09 ± 0.01, 97.45 ± 0.01 and 97.64 ± 0.01 (Fig. 5.4). In case of PVA there was increase of EE (%) from 76.17 ± 0.69 to 88.19 ± 0.24 for increase in stabilizer from 0.25 % to 0.5 %. However further increase in stabilizer decrease EE (%) as found 61.24 ± 1.40 for 0.75 % and 50.64 ± 1.30 for 1.0 %.

The reasons for the decrease in EE, when the stabilizer concentration was increased could be diffusion of Paclitaxel into the medium during the particle formulation and the Paclitaxel bind to the surplus surfactant and get precipitated during the centrifugation step. In addition, when the concentration of surfactant was increased the particle size of NPs decreased due to increase of surface area per unit volume, probably because of Paclitaxel diffused out into the medium (7, 10, 11). As the Paclitaxel amount in organic phase was increased from 1 to 5 mg (Table 5.4, 5.5 and 5.6) there was no significant change in the particle size and its distribution. This showed that the organic phase volume was sufficient to disperse 5 mg of Paclitaxel and for the mutual dispersion of the phases. There was increase in the PDI and marginal increase in EE and LE. Paclitaxel crystals were not observed in the NPs formulation during SEM, TEM and AFM characterization which confirms the encapsulation of Paclitaxel in polymer matrix.

5.7.5 Influences of polymer amount, stabilizer and its concentration on ZP

The properties of any colloidal system especially stability is affected by the presences of charge on the surface of the particles. The ZP governs the degree of repulsion between adjacent, similarly charged and dispersed particles. If the ZP is reduced below the stable value the attractive forces exceed the repulsive forces and the NPs get aggregated and it leads to unstable formulation. In general for a physically stable nanosuspension, the minimum ZP value should be within or around -30 mV to + 30 mV. However, few reported stability ever below -30 mV or above + 30 mV (1). It was known that the ZP value can be in negative or positive, the only condition was it should he high enough to get stable nanosuspension. The larger value of ZP indicates higher repulsive forces between the inter-particles and therefore decreases the particles aggregation and increase the shelf life of the prepared NPs.

Table 5.5 Composition and characterization of Paclitaxel loaded and blank PLGA NPs with variable concentration of stabilizers

Batch Code	Drug (mg)	Polymer (mg)	Stabilizer (% w/v)		Mean Size (nm ±SD)	Distribution (d. nm)	[a]PDI ±SD	[b]ZP ±SD	[c]EE (%)	[d]DC (% w/w)
			PF 68	PVA						
PLGA/F68/14	1	100	0.25	-	168.21 ± 2.10	40-350	0.35 ± 0.21	-16.25 ± 1.21	82.53 ± 0.66	0.97 ± 0.03
PLGA/F68/15	1	100	0.5	-	166.56 ± 1.20	40-365	0.20 ± 0.02	-18.24 ± 0.52	84.60 ± 0.01	0.99 ± 0.01
PLGA/F68/16	1	100	0.75	-	162.89 ± 0.85	50-285	0.28 ± 0.21	-20.01 ± 1.20	78.01 ± 0.70	0.91 ± 0.02
PLGA/F68/17	1	100	1	-	160.52 ± 0.41	40-300	0.31 ± 0.14	-17.58 ± 0.85	74.50 ± 1.45	0.86 ± 0.02
PLGA/F68/18	1	100	0.5	-	169.12 ± 1.01	55-275	0.18 ± 0.08	-20.01 ± 0.56	84.06 ± 1.72	0.99 ± 0.03
PLGA/F68/19	2	100	0.5	-	172.54 ± 0.75	45-350	0.19 ± 0.16	-19.25 ± 1.24	88.15 ± 0.63	2.08 ± 0.02
PLGA/F68/20	3	100	0.5	-	178.52 ± 0.71	40-350	0.21 ± 0.24	-15.24 ±1.56	88.31 ± 1.68	3.11 ± 0.06
PLGA/F68/21	4	100	0.5	-	180.12 ± 1.02	50-325	0.23 ± 0.41	-18.45 ± 0.85	90.89 ± 1.43	4.23 ± 0.09
PLGA/F68/22	5	100	0.5	-	184.41 ± 0.76	30-300	0.23 ± 0.04	-20.14 ± 0.96	90.65 ± 1.62	5.24 ± 0.07
PLGA/PVA/23	1	100	-	0.25	230.41 ± 2.11	55-325	0.34 ± 0.32	-19.58 ± 1.25	67.32 ± 1.66	0.79 ± 0.04
PLGA/PVA/24	1	100	-	0.5	227.23 ± 1.47	48-275	0.24 ± 0.04	-16.85 ±1.74	70.25 ± 0.81	0.82 ± 0.02
PLGA/PVA/25	1	100	-	0.75	222.57 ± 0.54	45-325	0.26 ± 0.04	-19.25 ± 0.25	66.90 ± 0.70	0.78 ± 0.01
PLGA/PVA/26	1	100	-	1	220.54 ± 0.65	40-300	0.25 ± 0.06	-17.25 ± 0.78	62.80 ± 0.76	0.73 ± 0.01
PLGA/PVA/27	1	100	-	0.5	229.32 ± 0.85	45-325	0.14 ±- 0.05	-20.01 ± 1.45	69.19 ± 1.54	0.81 ± 0.04
PLGA/PVA/28	2	100	-	0.5	234.85 ± 0.65	50-300	0.18 ± 0.08	-20.31 ± 1.42	71.69 ± 1.18	1.67 ± 0.01
PLGA/PVA/29	3	100	-	0.5	238.96 ± 0.85	40-300	0.19 ± 0.14	-19.74 ± 0.54	73.91 ± 2.88	2.58 ± 0.16
PLGA/PVA/30	4	100	-	0.5	240.74 ± 0.79	50-325	0.13 ± 0.14	-19.25 ± 0.62	73.40 ± 1.64	3.40 ± 0.05
PLGA/PVA/31	5	100	-	0.5	245.47 ± 1.02	40-325	0.15 ± 0.05	-18.23 ± 0.95	70.24 ± 0.80	4.14 ± 0.03
BPLGA/F68/01	-	50	0.25		109.4 ± 2.85	40-250	0.18 ± 0.23	-34.04 ± 1.21	-	-
BPLGA/F68/02	-	50	0.50		99.1 ± 1.11	70-350	0.11 ± 0.13	-24.51 ± 1.25	-	-
BPLGA/F68/03	-	50	0.75		86.3 ± 2.54	50-350	0.31 ± 0.04	-24.14 ± 1.05	-	-
BPLGA/F68/04	-	50	1		77.6 ± 3.25	50-240	0.18 ± 0.09	-22.32 ± 0.11	-	-

[*]Each data represents the average and standard deviation of three independent determinations, [a]Polydispersity index, [b]Zeta potential, [c]Encapsulation Efficiency, [d]Drug Content

Table 5.6 Composition and characterization of Paclitaxel loaded and blank PLA NPs with variable concentration of stabilizers

Batch Code	Drug (mg)	Polymer (mg)	Stabilizer (% w/v)		Mean Size (nm ±SD)	Distribution (d. nm)	[a]PDI ±SD	[b]ZP ±SD	[c]EE (%)	[d]DC (% w/w)
			PF 68	PVA						
PLA/F68/14	1	50	0.25	-	178.12 ± 9.12	90–760	0.62 ± 0.19	-34.23 ± 1.20	72.50 ± 1.11	1.44 ± 0.04
PLA/F68/15	1	50	0.5	-	152.13± 2.14	95–455	0.14 ± 0.10	-30.24 ± 0.16	87.26 ± 0.13	1.72 ± 0.31
PLA/F68/16	1	50	0.75	-	182.61 ± 4.72	99–559	0.54 ± 0.23	-38.42 ± 0.15	73.15 ± 0.69	1.44 ± 0.50
PLA/F68/17	1	50	1	-	180.72 ± 5.84	80–350	0.58 ± 0.20	-41.53 ± 0.75	67.90 ± 1.42	1.33 ± 0.03
PLA/F68/18	1	50	0.5	-	151.52 ± 1.24	80–400	0.17 ± 0.22	-28.42 ± 0.42	88.34 ± 1.77	1.76 ± 0.03
PLA/F68/19	2	50	0.5	-	152.23 ± 0.35	90-550	0.23 ± 0.05	-29.61 ± 0.54	87.12 ± 2.27	3.40 ± 0.10
PLA/F68/20	3	50	0.5	-	155.41 ± 2.67	90-500	0.36 ± 0.06	-29.61 ± 0.87	65.50 ± 3.39	3.72 ± 0.20
PLA/F68/21	4	50	0.5	-	158.52 ± 1.42	82-550	0.54 ± 0.43	-30.21 ± 0.77	50.70 ± 2.41	3.76 ± 0.20
PLA/F68/22	5	50	0.5	-	160.43 ± 1.45	90-450	0.48 ± 0.62	-30.82 ± 0.49	28.90 ± 0.32	2.63 ± 0.03
PLA/PVA/23	1	50	-	0.25	191.33 ± 8.72	91–840	0.49 ± 0.13	-31.61 ± 0.10	51.80 ± 0.86	1.07 ± 0.01
PLA/PVA/24	1	50	-	0.5	172.32 ± 1.38	80–595	0.13 ± 0.22	-28.22 ± 0.80	59.90 ± 1.00	1.18 ± 0.02
PLA/PVA/25	1	50	-	0.75	201.11 ± 9.02	80–542	0.81 ± 0.23	-32.72 ± 1.30	53.02 ± 0.70	1.04 ± 0.02
PLA/PVA/26	1	50	-	1	191.23 ± 5.30	90–596	0.92 ± 0.13	-28.11 ± 1.17	48.92 ± 0.76	1.00 ± 0.02
PLA/PVA/27	1	50	-	0.5	173.11 ± 0.08	90-550	0.39 ± 0.51	-28.42 ± 1.08	62.50 ± 0.46	1.24 ± 0.02
PLA/PVA/28	2	50	-	0.5	178.44 ± 0.09	90-550	0.41 ± 0.25	-29.22 ± 1.15	62.50 ± 0.58	2.42 ± 0.03
PLA/PVA/29	3	50	-	0.5	182.62 ± 1.05	90-450	0.42 ± 0.65	-27.15 ± 0.31	53.34 ± 1.55	3.03 ± 0.10
PLA/PVA/30	4	50	-	0.5	186.41 ± 2.06	90-450	0.56 ± 0.89	-26.52 ± 0.87	43.20 ± 0.66	3.20 ± 0.05
PLA/PVA/31	5	50	-	0.5	189.22 ± 1.34	80-525	0.58 ± 0.54	-29.26 ± 0.34	27.20 ± 1.05	2.48 ± 0.10
PLA/F68/01	-	50	0.25	-	176.12 ± 2.19	90-600	0.41 ± 0.13	-31.0 ± 1.01	-	-
PLA/F68/02	-	50	0.50	-	149.82 ± 1.25	70-450	0.27 ± 0.23	-29.0 ± 0.21	-	-
PLA/F68/03	-	50	0.75	-	182.62 ± 2.01	50-450	0.29 ± 0.11	-27.1 ± 0.34	-	-
PLA/F68/04	-	50	1	-	179.63 ± 1.11	50-500	0.30 ± 0.21	-28.3 ± 0.23	-	-

[*]Each data represents the average and standard deviation of three independent determinations, [a]Polydispersity index, [b]Zeta potential, [c]Encapsulation Efficiency, [d]Drug Content

The influences of polymer amount, nature of stabilizer and its concentration (Table 5.1 to 5.6) on ZP of the prepared NPs formulations are give in Fig. 5.5. In our study, both the stabilizers PF 68 and PVA imparted negative ZP value; this was due to the presences of terminal carboxylic groups and ionization of groups on the side chain of polymer and surfactant. PF 68 adsorb strongly onto the surface of hydrophobic particles (e.g. PLGA) through their centre block hydrophobic poly (propyl oxide), which leaves the hydrophilic poly (ethyl oxide) side arms in movable state and which extent outwards from the particle surface (10, 13). This hydrophilic tails on each particle surface results in the repulsion effect and provides stability to the system through the steric stabilization with enthalpic and entropic contributions. In case of the PVA, the hydroxyl group in the side chain resulted in the negative ZP value and it was totally a hydrophilic polymer which formed covalent linkage with the polymer through the hydroxyl group and hence it remained attached to the particles surface by forming multipoint linkage (12). In the present NPs preparation method, when the polymer amount was increased, the ZP ranged from, -16.63 to -28.92 for PCL NPs, -19.78 to -25.23 for PLGA NPs and -26.1 to -29.8 for PLA NPs (Table 5.1 to 5.3).

In case of PCL and PLA NPs, stabilizer PF 68 produced higher ZP (ranged from 28.24 to -34.62 and -30.24 to -41.53) than PVA which produced ZP ranged for -17.72 to -26.03 and -28.11 to -32.72. In case of PLGA NPs, stabilizer PF 68 and PVA produced stable NPs with same ZP value (Table 5.4 to 5.6).

5.7.6 Influences of polymer and stabilizer amount on % recovery of NPs

The influences of polymer amount on % recovery of NPs were represented in Fig. 5.5. In PCL and PLGA NPs, the % recovery of NPs was almost 100 % (99.79 and 99.74) in increase in polymer from 5 to 50 mg (Fig. 5.5). But, when the polymer amount was increased from 75 to 200 mg there was significant decrease in % recovery of NPs. In case of PLA NPs, the % recovery was very less (24.91 to 30.56) for the initial batches when the polymer amount is increased from 1 to 5 mg, the reason could be small particle size, which was not getting settled during the centrifugation process. Once the PLA amount was increased from 10 to 50 mg, the % recovery was almost 100 %, but after 75 mg and up to 200 mg the % recovery decreases significantly from 82.68 to 51.44. This showed the formation of agglomerates or larger particles which was getting settled during purification step.

The reason for decrease in % recovery of NPs when the polymer amount was increased could be due to increase in viscosity of the dispersed phase, which defend against the shear forces during dispersability of the polymer into the aqueous phase to form nanodroplets. This resistance in dispersability leads to bigger particles and agglomerates which was getting settled during purification step. One more possibility, the stabilizer in the aqueous phase was

insufficient to coat the particles formed when the polymer amount was increased above 100 mg, which resulted in aggregation of formed NPs to a bigger particles and which get settled during purification (1, 7). The ideal NPs formulation was one which uses less amount of excipients and hence less exposure of foreign material to the human body.

5.8 In-vitro dissolution

5.8.1 Influences of polymer amount on in-vitro drug release and it's kinetic

Apart from helping in formulation of NPs and stabilization, Polymers also help to extend drug release for longer duration. In view of the fact, cytotoxicity of Paclitaxel depends on high AUC and exposure time rather than level of concentration and as Paclitaxel acts in the M phase of the cell, it is necessary to have sufficient exposure to show its pharmacological action (3). In-vitro cumulative percent release profile of PNPs prepared with different polymer and stabilizer amount were represented in Fig. 5.6 to 5.15. It was observed that pure Paclitaxel got dissoluted completely with in one hour and the PNPs showed biphasic release pattern, with control release varied from 42 to 48 hr for most of the formulations. However, formulations PCL/F68/09 and PCL/F68/13 produced release of only 60 % till 48 hr.

The drug release from PNPs is a complex process, dependent on many factors like nature of drug, polymer degradation rate, water permeability and drug-polymer matrix interaction (7). As the amount of polymer was increased (Fig. 5.6, 5.9, 5.12 and 5.15) in formulation, the time to release 50 % of drug ($T_{50\%}$) was found to increase considerably (Table 5.7). The release profile and value of $T_{50\%}$ indicate that PCL produced more extended release than PLGA and PLA due to more hydrophobic character of PCL. Maximum value of $T_{50\%}$ was found to be 30 hr in PCL NPs and 10 hr for PLA NPs. However between PLGA and PLA, drug release was marginally faster in PLA NPs.

Chakravarthi et al. (15) prepared Paclitaxel loaded PLGA NPs whose in-vitro dissolution study results showed that the Paclitaxel was completely released within 24 hr with high burst release effect. In close view of Table 5.7, the PLGA NPs prepared in this work showed more extensive release and varying release rate depending on polymer proportion, stabilizer and its percentage and $T_{50\%}$ varied from 5 hr to 15.4 hr. Better control of release may be due to better encapsulation of PNPs.

For better understanding the release process, the dissolution data were fitted to different models (9). All NPs produced with 3 polymer and PF 68 stabilizer, followed Baker-Lonsdale model (Table 5.7). PCL based NPs prepared with PVA followed Higuchi model. PLGA and PLA polymer NPs with PVA variable formulations follows Korsmeyer-Peppas model.

The dissolution data of formulations following Higuchi model suggested that the release of Paclitaxel is mainly controlled by diffusion. In case of Korsmeyer-Peppas model, some dissolution data follows Fickian diffusion (n=0.4 to 0.45) and some data followed non- Fickian diffusion (0.5 to 0.62) process, known as anomalous transport. The fitting of best model to the dissolution data was selected through three important parameters, R^2, Akaike Information Criterion (AIC) and Model Selection Criterion (MSC). In general, R^2 value close to one, model with low AIC value and MSC value more than 2 to 3 are considered for deciding best model for the analyzed in-vitro dissolution data of the prepared PNPs (9). The R^2, AIC and MSC value best fit model of PNPs were represented in Table 5.7.

5.8.2 Influences of stabilizer on in-vitro drug release and it's kinetic

The influences of stabilizer PF 68 and PVA in drug release was represented in Fig. 5.7, 5.10 and 5.13 and Fig. 5.8, 5.11 and 5.14 respectively. Among the two stabilizers, presences of PVA extended drug release more than formulation with PF 68 (Table 5.7).

As PVA is more hydrophobic it extended the release more than PF 68. Further also higher molecular size and swelling character of PVA extended the release more. PVA coated NPs, when came in contact with dissolution medium it formed hydrogel which could have acted as barrier for diffusion release of Paclitaxel from polymeric NPs (12, 18-19). As stabilizer PF 68 is hydrophilic in nature and it could not extend the release as observed in case of PVA (Table 5.7). Fitting of dissolution data of PF 68 and PVA to different models of all the polymers were represented in Table 5.7 respectively.

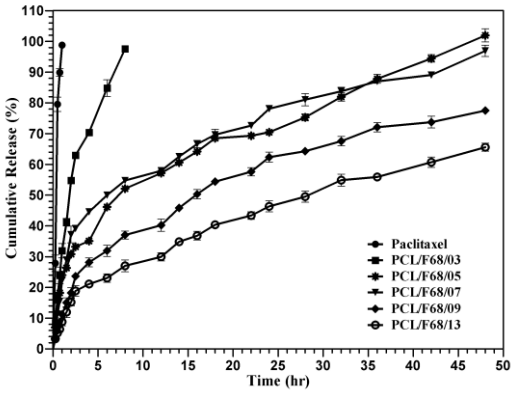


Fig. 5.6 Influences of polymer amount on in-vitro dissolution profile of Paclitaxel loaded PCL NPs

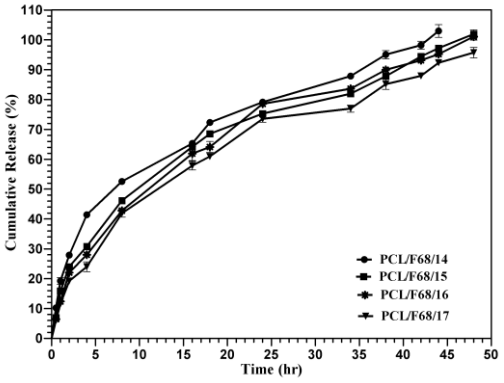


Fig. 5.7 Influences of PF 68 amount on in-vitro dissolution profile of Paclitaxel loaded PCL NPs

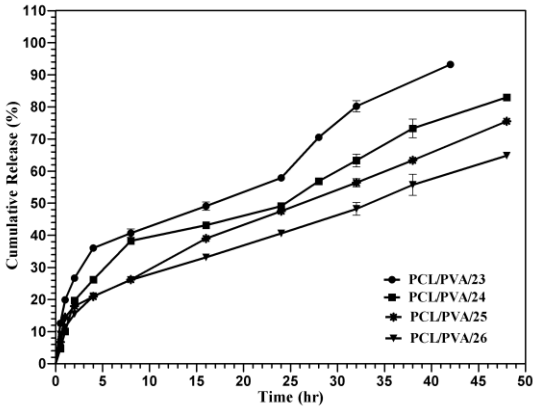


Fig. 5.8 Influences of PVA amount on in-vitro dissolution profile of Paclitaxel loaded PCL NPs

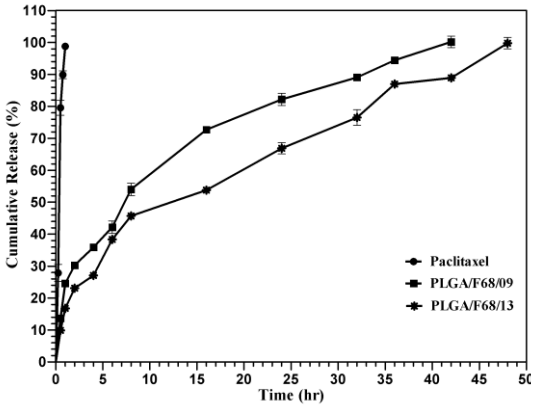


Fig. 5.9 Influences of polymer amount on in-vitro dissolution profile of Paclitaxel loaded PLGA NPs

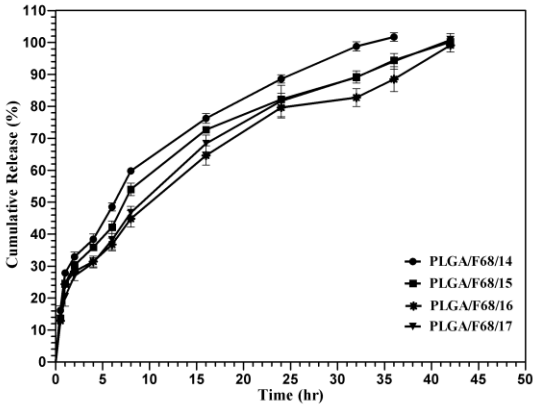


Fig.5.10 Influences of PF 68 amount on in-vitro dissolution profile of Paclitaxel loaded PLGA NPs

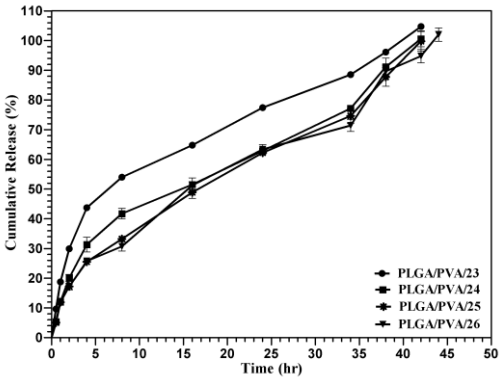


Fig. 5.11 Influences of PVA amount on in-vitro dissolution profile of Paclitaxel loaded PLGA NPs

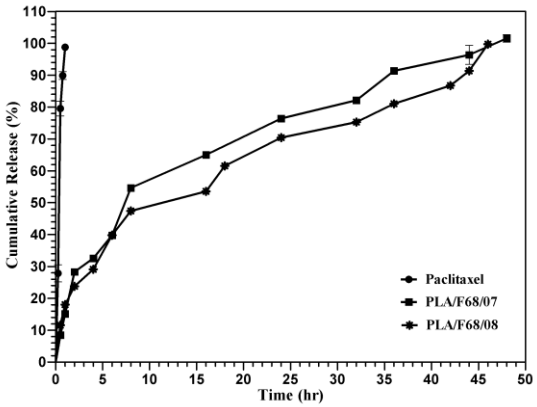


Fig. 5.12 Influences of polymer amount on in-vitro dissolution profile of Paclitaxel loaded PLA NPs

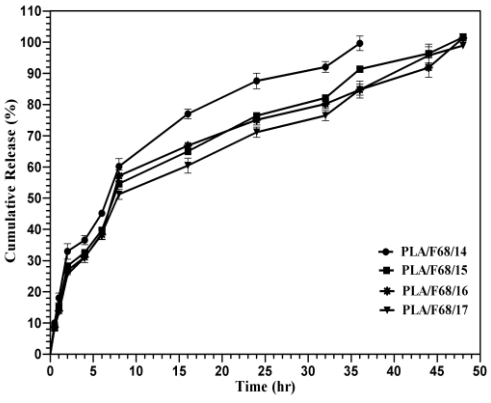


Fig. 5.13 Influences of PF 68 amount on in-vitro dissolution profile of Paclitaxel loaded PLA NPs

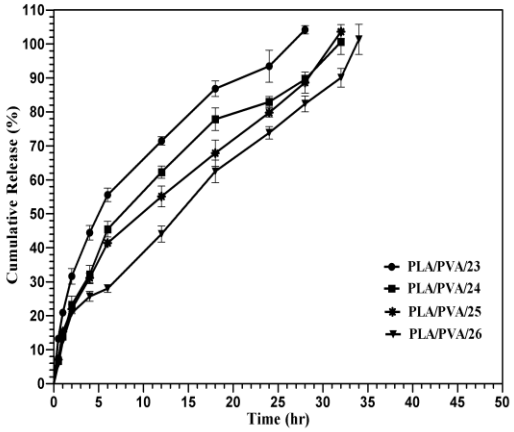


Fig. 5.14 Influences of PVA amount on in-vitro dissolution profile of Paclitaxel loaded PLA NPs

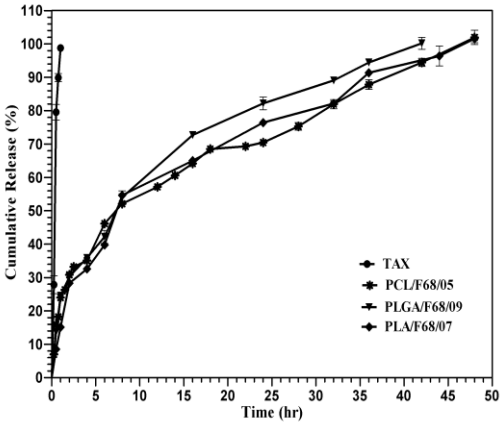


Fig.5.15 Influences of polymer amount on in-vitro dissolution profile of Paclitaxel loaded PNPs

5.9 Stability study

The stability study result showed that all the prepared NPs, were stable up to 4 months at 5 ± 2°C and 15 ± 5°C. All the NPs stored at 37 ± 5 °C for 4 months showed ssome increase in size, due to aggregation of particles (Table 5.8). The AFM analysis showed (Fig. 5.16) no change of surface character of stored NPs. The in-vitro dissolution study of all stored formulations confirmed no change in release profile. The stability study result confirmed that the prepared PNPs were stable in all the three temperatures tested.

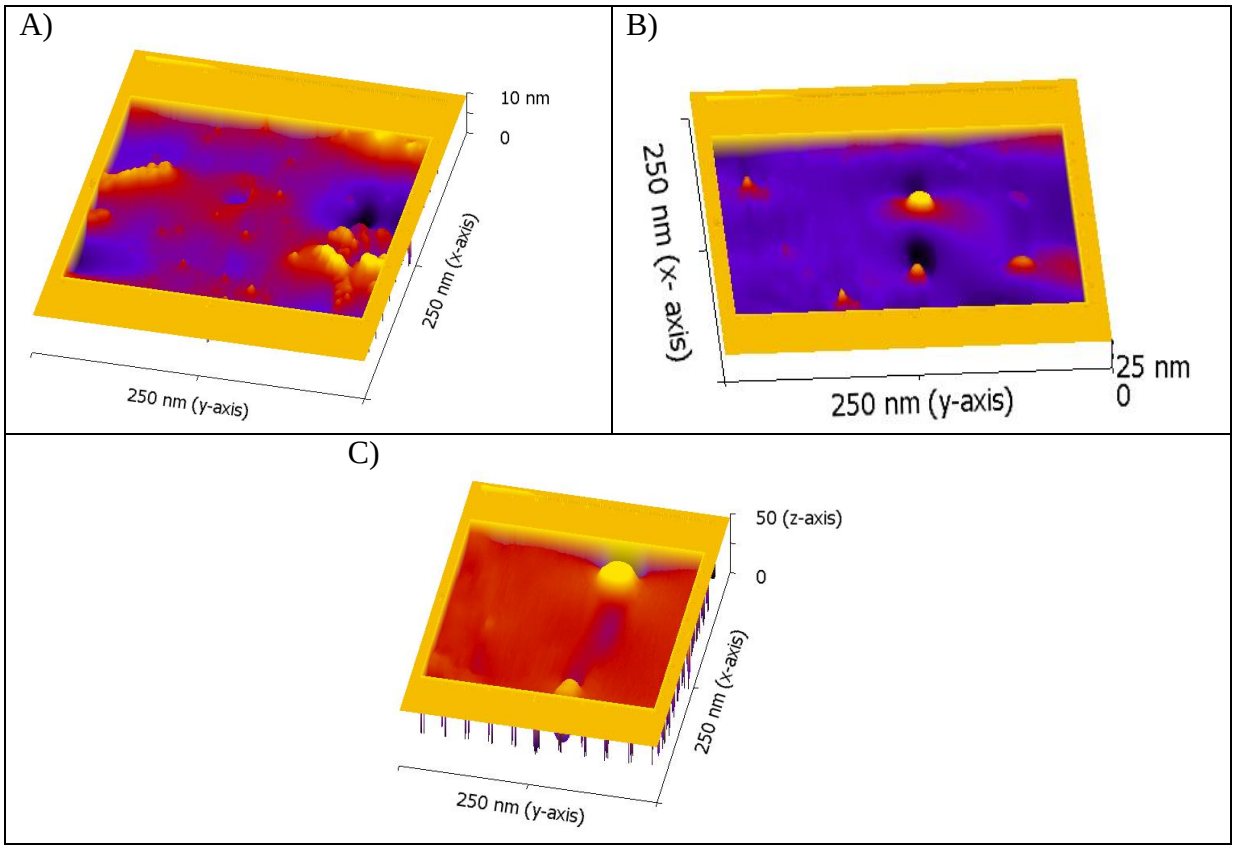


Fig. 5.16 Stability study results, PCL NPs AFM after 4 month storage (A) PLGA NPs AFM after 4 month storage(B) PLA NPs AFM after 4 month storage (C)

Table 5.7 Best fitting of in-vitro release data using mathematical modeling

S.NO	Batch	R^2	AIC	MSC	n^z	$^g T_{50\%}$ (hr)
1	PCL/F68/03	0.9199	66.60	2.32	-	1.80
2	PCL/F68/05	0.9821	101.00	3.92	-	8.11
3	PCL/F68/07	0.9755	126.20	3.92	-	7.45
4	PCL/F68/09	0.9903	101.64	4.54	-	16.02
5	PCL/F68/13	0.9886	95.40	4.40	-	29.70
6	PCL/F68/14	0.9867	50.60	4.13	-	6.94
7	PCL/F68/15	0.9821	66.00	3.90	-	8.40
8	PCL/F68/16	0.9730	71.70	3.50	-	8.72
9	PCL/F68/17	0.9740	70.10	3.50	-	10.40
10	PCL/PVA/23	0.9481	60.03	2.80	-	13.23
11	PCL/PVA/24	0.9740	58.54	3.50	-	19.20
12	PCL/PVA/25	0.9843	45.10	4.00	-	23.70
13	PCL/PVA/26	0.9780	45.02	3.62	-	31.23
14	PLGA/F68/09	0.9886	53.40	4.30	-	6.54
15	PLGA/F68/13	0.9725	69.82	3.43	-	10.00
16	PLGA/F68/14	0.9845	50.80	4.00	-	5.23
17	PLGA/F68/15	0.9886	53.40	4.30	-	6.54
18	PLGA/F68/16	0.9731	62.00	3.50	-	8.13
19	PLGA/F68/17	0.9734	62.52	3.60	-	7.32
20	PLGA/PVA/23	0.9861	53.30	3.90	0.417	7.84
21	PLGA/PVA/24	0.9774	58.00	3.40	0.542	13.22
22	PLGA/PVA/25	0.9847	54.00	3.81	0.611	15.40
23	PLGA/PVA/26	0.9828	62.82	3.72	0.620	15.40
24	PLA/F68/07	0.9820	66.00	4.00	-	7.80
25	PLA/F68/08	0.9816	83.60	3.90	-	8.50
26	PLA/F68/14	0.9789	54.60	3.70	-	5.81
27	PLA/F68/15	0.9820	66.00	4.00	-	7.80
28	PLA/F68/16	0.9758	68.84	3.60	-	8.30
29	PLA/F68/17	0.9781	67.24	3.70	-	9.01
30	PLA/PVA/23	0.9917	39.50	4.70	0.450	5.52
31	PLA/PVA/24	0.9889	50.10	4.14	0.522	8.54
32	PLA/PVA/25	0.9894	49.10	4.30	0.561	9.70
33	PLA/PVA/26	0.9877	58.20	4.10	0.660	12.70

All Polymer and PF 68 variable formulations, follows Baker-Lonsdale model. In PCL polymer NPs, PVA variable formulation follows Higuchi model. In PLGA and PLA polymer NPs, PVA variable formulation follows Korsmeyer-Peppas model. ^{z}Diffusion exponent indicating the drug release mechanism.

Table 5.8 Stability study results of optimized Paclitaxel loaded NPs formulations in three different conditions

S.NO	Stability Conditions	Evaluation Parameters	PCL NPs		PLGA NPs		PLA NPs	
			Observation (months)					
			0	4	0	4	0	4
1	5 ± 2° C	Physical appearances	White	No change	White	No change	White	No change
		Size (nm)	172.03 ± 0.34	175.13 ± 0.81	165.52 ± 2.10	166.23 ± 1.11	152.13 ± 2.10	154.21 ± 0.92
		PDI[a]	0.05 ± 0.53	0.06 ± 0.33	0.20 ± 0.04	0.22 ± 0.05	0.14 ± 0.10	0.24 ± 0.42
		ZP[b]	-25.52 ± 0.44	-26.12 ± 0.14	-25.23 ± 1.45	-24.56 ± 1.23	-30.24 ± 0.16	-30.11 ± 0.89
		DC[c]	4.60 ± 0.12	4.54 ± 0.52	1.65 ± 0.01	1.62 ± 0.22	1.02 ± 0.02	1.01 ± 0.12
2	15 ± 5° C	Physical appearances	White	No change	White	No change	White	No change
		Size (nm)	172.03 ± 0.34	190.13 ± 1.34	165.52 ± 2.10	176.21 ± 1.11	152.13 ± 2.10	165.23 ± 1.11
		PDI	0.05 ± 0.53	0.51 ± 1.42	0.20 ± 0.04	0.52 ± 1.01	0.14 ± 0.10	0.21 ± 0.32
		ZP	-25.52 ± 0.44	-22.52 ± 0.44	-25.23 ± 1.45	-21.89 ± 0.56	-30.24 ± 0.16	-29.11 ± 0.54
		DC	4.60 ± 0.12	4.20 ± 0.42	1.65 ± 0.01	1.59 ± 0.67	1.02 ± 0.02	0.87 ± 0.22
3	37 ± 5 °C	Physical appearances	White	No change	White	No change	White	No change
		Size (nm)	172.03 ± 0.34	193.13 ± 2.34	165.52 ± 2.10	190.23 ± 1.58	152.13 ± 2.10	172.21 ± 1.11
		PDI	0.05 ± 0.53	0.81 ± 0.73	0.20 ± 0.04	0.77 ± 0.24	0.14 ± 0.10	0.67 ± 0.23
		ZP	-25.52 ± 0.44	-21.01 ± 2.44	-25.23 ± 1.45	-20.89 ± 1.01	-30.24 ± 0.16	-23.23 ± 0.32
		DC	4.60 ± 0.12	4.02 ± 0.41	1.65 ± 0.01	1.42 ± 0.40	1.02 ± 0.02	0.67 ± 0.45

[a] Polydispersity index, [b] Zeta potential, [c] Drug loading

5.10 Conclusion

Successfully small with narrow size distribution PNPs, using PCL, PLGA and PLA polymers were prepared by using nanoprecipitation and solvent evaporation method. Based on the results it was observed that quality NPs with 100 % EE, high DC and % recovery were obtained using, 20 mg of PCL (PCL/F68/05), 100 mg of PLGA (PLGA/F68/09) and 50 mg of PLA (PLA/F68/07) with 0.5 % PF 68 as stabilizer. The reason for high EE in case of the prepared PNPs may be due to low aqueous solubility of Paclitaxel, fast rate of precipitation of polymer during preparation and selection of polymer solvent with high vapour pressure and the low viscosity of the internal phase. The prepared PNPs were characterized for their shape and structure using SEM, TEM and AFM. The classy microscopic examination SEM, TEM and AFM analysis revealed the spherical and smooth surface character of the NPs as well as their homogeneous solid matrix without any amorphous arrangements. Though, PVA is the most extensively used stabilizer in the preparation of PNPs, in the present method we studied both PVA and PF 68. Among the two, formulations with PF 68 as stabilizer were selected and used in in-vivo pharmacokinetic and in the in-vivo anti-tumor efficacy study as it provides NPs.

References

9. Saha RN, Vasanthakumar S, Benda G, Snehalatha M. (2010). Nanoparticulate drug delivery systems for chemotheraphy. Mol Membr Biol, 27, 215-223.
10. Sivacharan K, Bende G, Snehalatha M, Saha RN. (2010). Application of rotatable central composite design in the preparation and optimization of poly (lactic-c*o*-glycolic acid) nanoparticles for controlled delivery of paclitaxel. Drug Dev Ind Pharm, 36, 1377-1387.
11. Brittain HG. Profiles of drug substances, excipients, and related methodology. ln: Jauhari S, Singh S, Dash AK, editors. Paclitaxel. UK: Academic Press; 2009. 299-344.
12. Jin C, Li H, He Y, He M, Bai L, Cao Y, Song W, Dou K. (2010). Combination chemotherapy of doxorubicin and paclitaxel for hepatocellular carcinoma in-vitro and in-vivo. J Cancer Res Clin Oncol, 136, 267-274.
13. Gagandeep S, Novikoff PM, Ott M, Gupta S. (1999). Paclitaxel shows cytotoxic activity in human hepatocellular carcinoma cell lines. Cancer Lett, 136, 109-118.
14. Allwood MC, Martin H. (1996). The extraction of diethylhexylphthalate (DEHP) from polyvinyl chloride components of intravenous infusion containers and administration sets by paclitaxel injection. Int J Pharm, 127, 65-71.
15. Snehalatha M, Venugopal K, Saha RN. (2008). Etoposide-loaded PLGA and PCL nanoparticles I: preparation and effect of formulation variables. Drug Deliv, 15, 267-275.

16. Snehalatha M, Venugopal K, Saha RN. Babbar AK, Sharma RK. (2008). Etoposide loaded PLGA and PCL nanoparticles II: biodistribution and pharmacokinetic after radiolabeling with Tc-99m. Drug Deliv, 15, 277- 287.
17. Zhang Y, Huo M, Zhou J, Zou A, Li W, Yao C, Xie S. (2010). An Add-In program for modeling and comparison of drug dissolution profiles. AAPSJ, 12, 263-271.
18. Seremeta KP, Chiappetta DA, Sosnik A. (2013). Poly (caprolactone), Eudragit® RS 100 and Poly (caprolactone)/ Eudragit® RS 100 blend submicron particles for the sustained release of the antiretroviral efavirenz. Colloids Surf B Biointerfaces, 102, 441-449.
19. Derakhshandeh K, Erfan M, Dadashzadeh S. (2007). Encapsulation of 9-nitrocamptothecin, a novel anticancer drug, in biodegradable nanoparticles: factorial design, characterization and release kinetics. Eur J Pharm Biopharm, 66, 34-41.
20. Mitra A, Lin S. (2003). Effect of surfactant on fabrication and characterization of paclitaxel-loaded polybutylcyanoacrylate nanoparticulate delivery systems. J Pharm Pharmacol, 55, 895-902.
21. Zhang Y, Tang L, Sun L, Bao J, Song C, Huang L, Liu K, Tian Y, Tian Ge, Li Z, Sun H, Mei L. (2010). A novel paclitaxel-loaded poly (ε-caprolactone)/Poloxamer 188 blend nanoparticles overcoming multidrug resistance for cancer treatment. Acta Biomater, 6, 2045-2052.
22. Sinjan De, Miller DW, Robinson DH. (2005). Effect of particle size of nanospheres and microspheres on the cellular-association and cytotoxicity of paclitaxel in 4T1 cells. Pharm Res, 22, 766-775.
23. Chakravarthi SS, Sinjan De, Miller DW, Robinson DH. (2010). Comparison of anti-tumor efficacy of paclitaxel delivery in nano- and microparticles. Int J Pharm, 383, 37-44.
24. Mainardes RM, Evangelista RC. (2000). PLGA nanoparticles containing praziquantel: effect of formulation variables on size distribution. Int J Pharm, 5290, 137-144.
25. Lao LL, Venkataraman SS, Peppas NA. (2008). Modeling of drug release from biodegradable polymer blends. Eur J Pharm Biopharm, 70, 796-803.
26. Sahoo SK, Panyam J, Prabha S, Labhasetwar V. (2002). Residual polyvinyl alcohol associated with poly (lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake. J Control Release, 82, 105-114.
27. Dong Y, Feng S. (2007). In-vitro and in-vivo evaluation of methoxy polyethylene glycol-polylactide (MPEG-PLA) nanoparticles for small-molecular drug chemotheraphy. Biomaterials. 28, 4154-4160.

Chapter 6. In-vitro Cytotoxicity and Cellular Uptake Study

6.1 Introduction

The therapeutic efficacy of several types of drugs, particularly anti-cancer drugs, depends on the cellular uptake efficiency. It is expected that in nanoparticulate systems the cellular uptake is enhanced and thus can provide enhanced efficacy. The cellular uptake study in suitable cancer cell is essential to study enhanced uptake and efficacy. Staining the cells/organelles with suitable dye and incubating with the particles labeled with appropriate probe molecule, which can be studied using confocal microscope is the traditional technique for qualitative study of cellular or organ uptake of particles. In case of quantification for the amount of particles taken up by the cancer cells, microplate reader is the universal basic techniques available. Once the NPs reach the cancer cells, it is expected to inhibit the cell proliferation which results in cell death. This cytotoxicity of the drug loaded NPs is studied extensively by in-vitro 3-(4, 5-Dimethylthiazol-2yl)-2, 5, diphenyltetrazolium bromide (MTT) assay (1-5). In this work, the cytotoxicity of the prepared Paclitaxel loaded NPs were studied and the Coumarin 6 (C6) loaded PCL, PLGA and PLA NPs were also subjected for particle uptake study.

6.2 Materials and Methods

Pure drug Paclitaxel and selected nanoparticulate systems, prepared and mentioned in Chapter 5 were used for cytotoxicity study. Breast cancer cell line MCF-7 was obtained from National Centre for Cell Sciences, Pune, India. Chemicals, MTT, fetal bovine serum (FBS), minimal essential medium (MEM), Coumarin 6 (C6), Triton X-100 and propidium iodide (PPI) were obtained from Sigma–Aldrich, Bangalore, India.

6.3 Cytotoxicity study

The cytotoxicity of the prepared Paclitaxel loaded PNPs were measured in MCF-7 cancer cell line using MTT assay at SCTIMST, Thiruvananthapuram, India with ethical approval. The MCF-7 cells were maintained in MEM, supplemented with 10% FBS and antibiotics, 1% penicillin/streptomycin under the conditions of 5 % CO_2 and > 90% humidity at 37°± 1 C. Antibiotics was added to maintain sterility.

6.3.1 Samples of cytotoxicity study

a) Negative control (NC) sample

NC samples are one where theoretically no cytotoxicity effect should be observed. NC sample was included in the study with the intension to prove that the MTT procedure is selective. High density polyethylene is used as negative control. It is prepared by incubating 0.2 gm of high density polyethylene with culture medium containing serum at 37° ± 1 C for 24 ± 2hr.

b) Positive control (PC) sample

PC samples are one which should completely inhibit the cell viability. PC sample was included in the study expecting that it will inhibit the cell growth, to confirm that the MTT procedure is sensitive and selective to cytotoxic material. The positive control used in this study is dilute phenol which is prepared by diluting phenol stock solution (13 mg/ml) to 1.3 mg/ml with culture medium containing serum.

c) Paclitaxel loaded PNPs-Test samples

The cytotoxicity of the prepared Paclitaxel loaded PCL, PLGA and PLA NPs and commercial formulation (CRE-MR) were measured at three different concentrations, 10, 20 and 40 μg/mL.

6.3.2 MTT assay procedure

To determine the cell viability, MCF-7 cells were sub cultured and transferred to 96-well plate to ensure 1 X 10^4 cells per well in 100 μL MEM containing 10 % FBS. Medium was changed every day until 80% confluence was reached. Then the medium was replaced with 100 μL of test samples of 10 μg/mL concentration. The plate was incubated for 24, 48, and 72 hr, then the well content was removed and the wells were washed three times using PBS. MTT reagent, (50 μL from 1mg/mL) was added to the well and incubated for 2 hr. Now, MTT was removed and 100 μL of isopropanol was added to the wells and swayed before the plate was observed for optical density (OD) using spectrophotometer reader (Bio-Tek Instruments, Vermont, USA) at 570 nm. In addition to this, the cells were observed under microscope for there morphological changes. MCF-7 cells without test samples treatment were used as control cells. The cell viability was determined by using the formula

Cell viability (%) = (OD of test cells/OD negative control cells) X 100

6.4 Cellular uptake studies of NPs

6.4.1 Preparation of fluorescence PNPs

The Coumarin 6 (C6) loaded PCL and PLA NPs were prepared by nanoprecipitation and C6 loaded PLGA NPs were prepared by solvent evaporation method. The fluorescence PNPs were prepared as per the Paclitaxel loaded NPs procedure. The fluorescence PNPs prepared was characterized for their size, polydispersity index, zeta potential and drug content to study the cellular uptake efficiency of the prepared PNPs in MCF-7 cells. The shape and size of the C6 loaded NPs were further confirmed through AFM analysis. The cellular uptake efficiency of these prepared C6 loaded PCL, PLGA and PLA NPs were determined by confocal microscopy and micro plate reader technique.

6.4.2 Determination of cellular uptake of NPs by confocal microscopy

The particle cellular uptake study was performed in MCF-7 cells using standard procedure (5, 8). MCF-7 cells were sub cultured and seeded at a density of $1x10^4$ cells/cm^2 in well plate. After 80% confluence, the medium was replaced with C6 loaded PCL, PLGA and PCL NPs dispersion and incubated for 2 hr. After that, dispersion was removed and 1 ml of 70% ethanol solution was added into each well to fix cells. Now the wells with cells in ethanol solution were kept in 37ºC for 20 min, then ethanol solution was removed and PBS was used to wash wells for three times. Subsequently, 10 µL of 5 mg/ml PPI was added to stain MCF-7 cell nucleolus for 30 min, soon after the stain was washed three times using PBS and it is observed under confocal laser scanning microscop (LSM 510 Meta). The C6 loaded particles and PPI staining cell nucleus showed green color and red color, respectively.

6.4.3 Determination of cellular uptake of NPs by micro plate reader

The cells were seeded in the 96-well plate at a density of 1 X 10^4 cells per well. Medium was changed until 80% confluence was reached. The medium was then replaced with 100 µL medium with C6 loaded PCL, PLGA and PCL NPs of three different concentrations 50, 75 and 100 µg/ml. The plate was incubated for 2 hr and the cellular uptake efficiency of C6 loaded NPs was determined by micro plate reader. For each PNPs sample, one control sample without cells was added in the well. After predetermined time interval, NPs suspension was removed and the wells were washed three times using PBS. Now, 100 µl 0.5% triton X-100 in NaOH was added to break the cells and then the plate was measured using a micro plate reader. The excitation wavelength and emission wavelength was 430 and 485 nm, respectively, for C6. The cellular uptake efficiency of PNPs was given by the ratio between the amount of NPs taken up in cells and the amount of NPs in control well.

6.5 Result and Discussion

6.5.1 Cytotoxicity study

As it is important to use sterilized material in cell cytotoxicity study, the prepared PNPs were sterilized by filtering through membrane filteration (6). The cytotoxicity effect of prepared three PNPs was compared to that of commercial formulation, CRE-MR. The concentrations of Paclitaxel 10, 20 and 40 µg/mL was preferred for cytotoxicity study since the plasma level of Paclitaxel is likely to be in this range (7, 8). The MTT test is an assay to identify the proliferation and cell viability by measuring the mitochondrial activity of cells. Metabolically active cells are able to convert the yellow water-soluble tetrazolium salt MTT to water insoluble dark blue formazan by reductive cleavage of the tetrazolium ring (9).

The % cell viability vs. incubation time graph (Fig. 6.1, 6.2 and 6.3) clearly illustrated Paclitaxel loaded PNPs showed strong inhibitory effects on the proliferation of MCF-7 cells when compared to commercial formulation. The negative sample analysis showed almost 80-

90 % cell viability in all the three time points studied. The positive control sample analysis evidently showed that the procedure adopted allows the cytotoxic compound phenol to inhibit MCF-7 cells proliferation completely. The increased cytotoxicity of Paclitaxel loaded PNPs than the CRE-MR is attributed to higher intracellular uptake and drug concentration. The reason for increased cytotoxicity of PNPs could be due to enhanced cell permeation of NPs. The mechanism of enhanced cell permeation is probably due to hydrophilic surface nature of NPs which reduces MDR. Further, the stabilizer used in NPs preparation, PF 68 is also a potent inhibitor of P-gp and CYP3A4 (1), which might have helped in enhanced permeation. The profiles suggest cytotoxic effect is dependent on both, Paclitaxel concentration as well as time of incubation.

Table 6.1 IC_{50} (µg/mL) of MCF-7 cells after 24, 48 and 72 hr of incubation with 10 µg/mL concentration of Paclitaxel loaded PNPs and commercial Paclitaxel formulation

S.NO	**NPs Formulation**	**IC_{50} (µg/mL) at different incubation time (hr)**		
		24	**48**	**72**
1	CRE-MR	19.51	12.27	8.74
2	Paclitaxel PCL NPs	14.30	10.28	7.63
3	Paclitaxel PLGA NPs	13.32	9.88	7.21
4	Paclitaxel PLA NPs	9.79	8.18	6.80

Among the PNPs, Paclitaxel loaded PLA NPs exhibited maximum inhibitory effect. This could be due to increase in cellular uptake of Paclitaxel loaded PLA NPs observed in the particle cellular uptake study. The % cell viability profile illustrated that when the concentration of Paclitaxel loaded PNPs was increased from 10 to 40µg/mL, the cytotoxicity action increases. When the contact time of NPs and MCF-7 cells were increased the proliferation of cells decreased as a result of sustained drug release from PNPs, which is not observed in CRE MR. The cytotoxicity of Paclitaxel, depends on sustain therapeutic concentration rather than maximal plasma concentration, since Paclitaxel need cells to enter M phase (10, 11).

The IC_{50} of the evaluated formulations in MCF-7 cells after incubation for 24, 48 and 72 hr is represented in Table 6.1. IC_{50} is defined as the drug concentration at which 50 % of the cells in culture have been killed in a particular time period. IC_{50} values for commercial formulation (CRE-MR) were found to be 19.51 µg/mL, 12.27 µg/mL and 8.74 µg/mL at 24, 48 and 72 hr incubation respectively. However these IC_{50} values were found to be lower for NPs formulation with lowest value for PLA NPs with 9.79, 8.18 and 6.80 µg/mL at 3 different incubation

periods (Table 6.1). PCL NPs showed maximum IC_{50} value among 3 NPs with 14.50 µg/mL, 10.28 µg/mL and 7.63 µg/mL respectively. There was not much difference in IC_{50} value at 72 hr incubation as in 72 hr all the Paclitaxel are released by 72 hr. During MTT assay, the morphological examination of the cells was observed at 24, 48 and 72 hr through phase-contrast photomicrographs. The morphological examination of cells incubated with PNPs and CRE-MR showed alterations in cell shape compared to negative control and control cells.

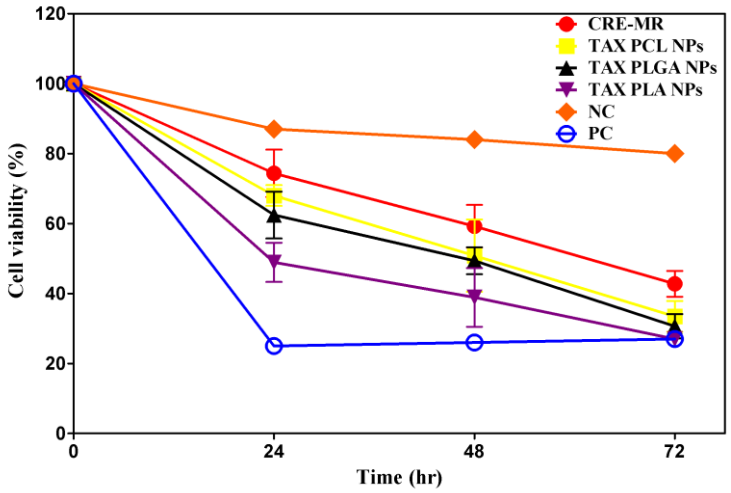


Fig. 6.1 Cell viability of MCF-7 cells treated with commercial product, Paclitaxel (TAX) loaded PNPs at concentration 10 µg/ml. Each data point showed average of five samples

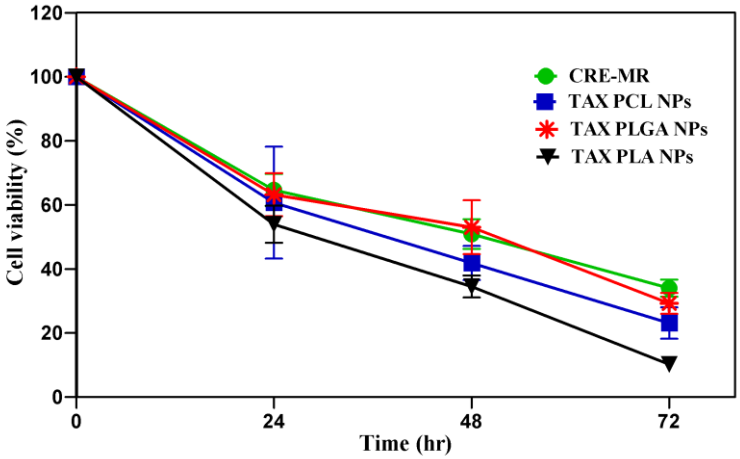


Fig. 6.2 Cell viability of MCF-7 cells treated with commercial product, Paclitaxel (TAX) Loaded PNPs at concentration 20 µg/ml. Each data point showed average of five samples

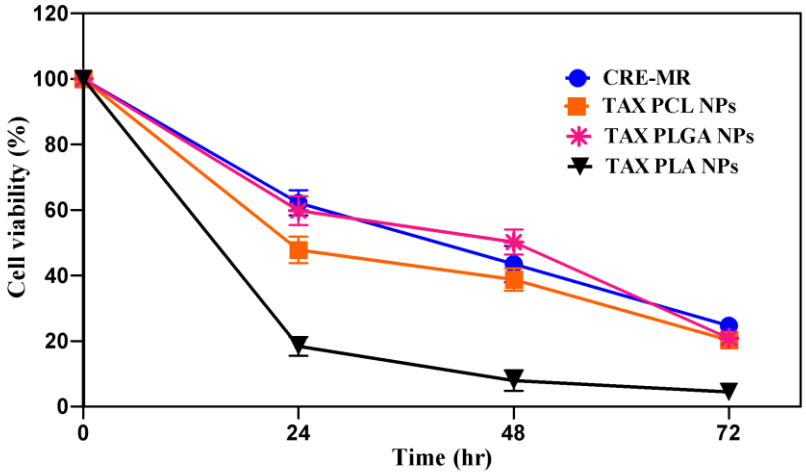


Fig. 6.3 Cell viability of MCF-7 cells treated with commercial product, Paclitaxel (TAX) Loaded PNPs at concentration 40 µg/ml. Each data point showed average of five samples

The negative control cells did not show any change in shape when compared to control cells, this showed that the MTT assay is specific to proliferation inhibiting samples. In positive control group, the shape of the cells was altered drastically or in other hand, phenol inhibited the proliferation of MCF-7 cells.

6.5.2 Cellular uptake studies of NPs

a) Preparation of Fluorescence PNPs

Successfully, C6 loaded PCL, PLGA and PLA NPs were prepared using the optimized procedure of Paclitaxel loaded PNPs as mentioned in Chapter 5. The size and PDI of the prepared C6 loaded PCL, PLGA and PLA NPs were, 171.8 ± 0.80 nm, 0.20 ± 0.03, 165.8 ± 0.14 nm, 0.24 ± 0.12, 154.5 ± 2.20 nm and 0.11 ± 0.01 respectively. The ZP of the prepared C6 loaded PNPs were -25.0 ± 0.9, -21.2 ± 1.20 and -26.0 ± 0.83 respectively. The shape and surface morphology of the prepared C6 loaded PNPs were observed using AFM and they are represented in Fig. 6.4. The C6 loaded PNPs characterization resembles the Paclitaxel loaded PNPs and it is used in the particle uptake study.

b) Confocal microscopic observation of cellular uptake of NPs

The improved drug targeting ability of NPs towards cancer cells depends on internalization and continued retention of particles in target cells. The particle uptake study in cancer cells will be a proof of concept for the better therapeutic effect of drug loaded NPs over the conventional chemotheraphy. The fluorescein isothiocyanate channel was used to observe the NPs and neutral red channel was used to examine the MCF-7 cells. The C6 loaded NPs were represented in green color and the PPI stained MCF-7 cell nucleus was shown in red color. The confocal fluorescence 2D images of MCF-7 cells with C6 labeled NPs after 2 hr incubation is

represented in Fig. 6.5. The 3D view of confocal image of particle uptake evidently confirmed that the C6 loaded PNPs (green color) is located closely around the nucleus (red color), indicating the internalization of NPs (Fig. 6.6).

In addition the cellular uptake is visualized by overlaying images obtained by fluorescein isothiocyanate channel (green), propidium iodide channel (red) and background (black) in Fig. 6.7. The particle uptake image was displayed in three orthogonal projections xy, xz and yz respectively to verify whether the C6 NPs are located outside the top surface of the cells or entrapped intracellularly (12). The orthogonal images (Fig. 6.8) illustrates that the NPs were indeed entrapped within the intracellular spaces.

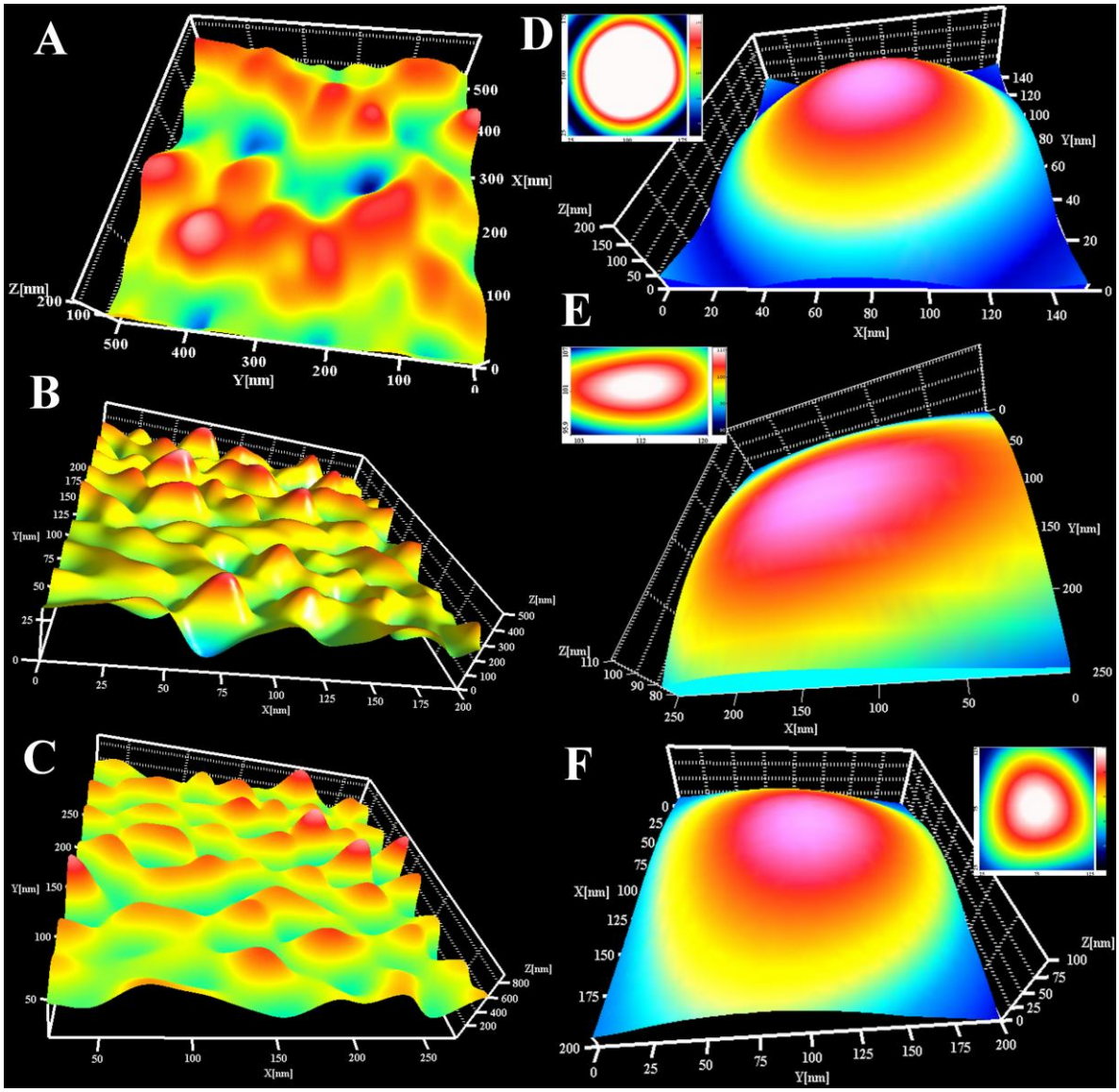


Fig. 6.4 Characterization of Coumarin 6 loaded PNPs by AFM, cluster, single 3D and 2D PCL NPs (A and D) cluster, single 3D and 2D PLGA NPs (B and E) and cluster, single 3D and 2D PLA NPs (C and F)

A)	B)

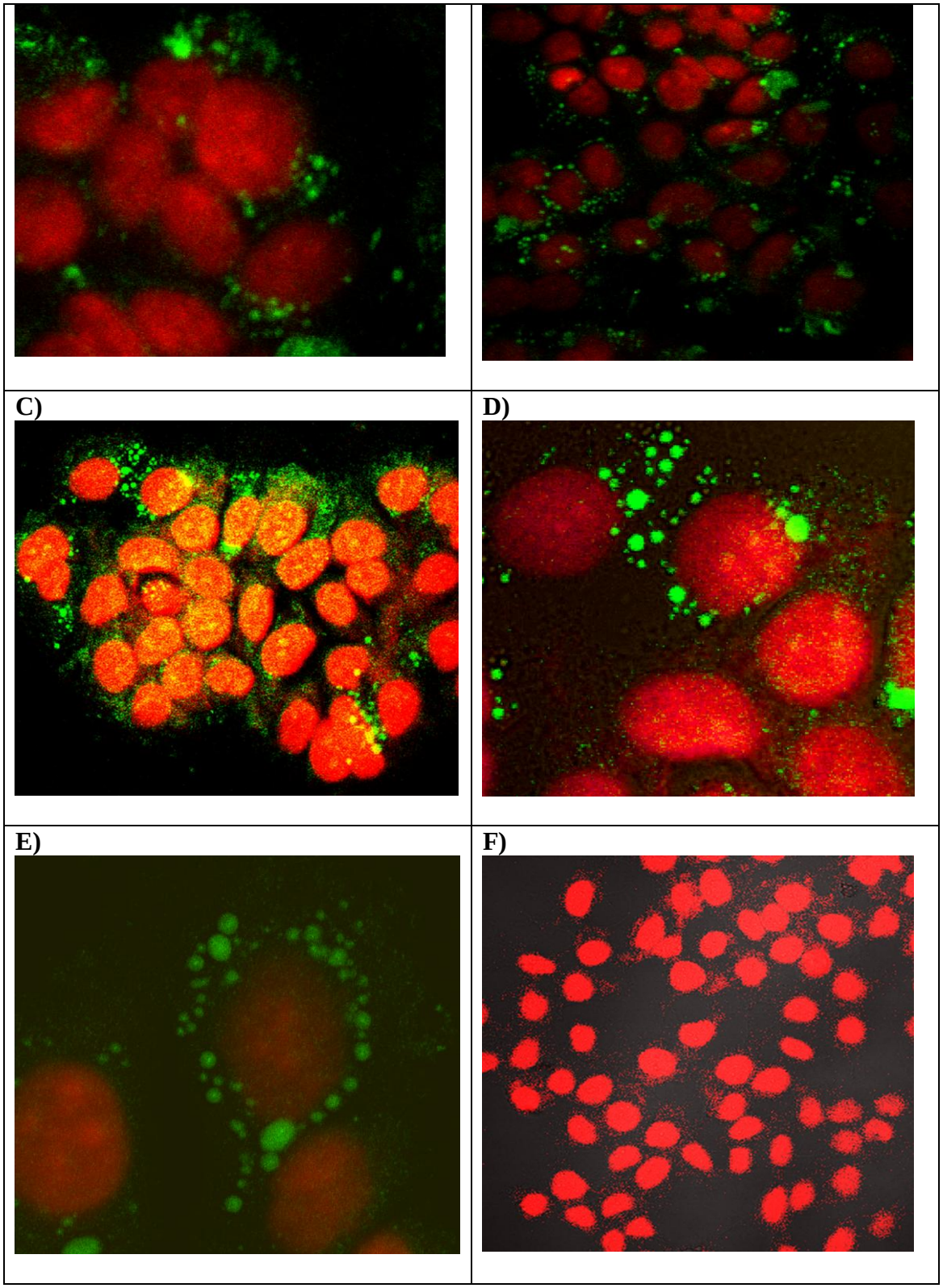
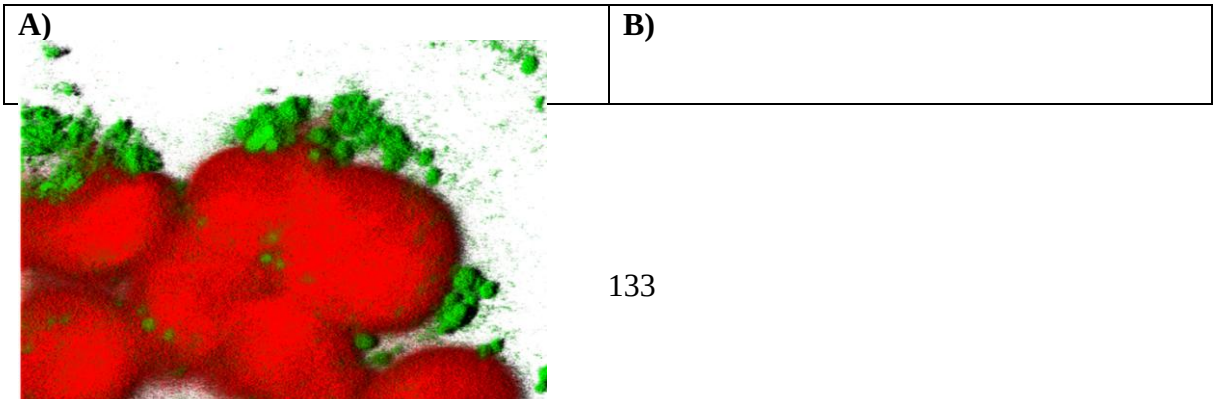


Fig 6.5 Confocal fluorescence 2D images of MCF-7 cells with Coumarin 6 labeled NPs after 2 hr exposure, PCL NPs (A) PLGA NPs (B) PLA NPs (C-E) blank cells (F)



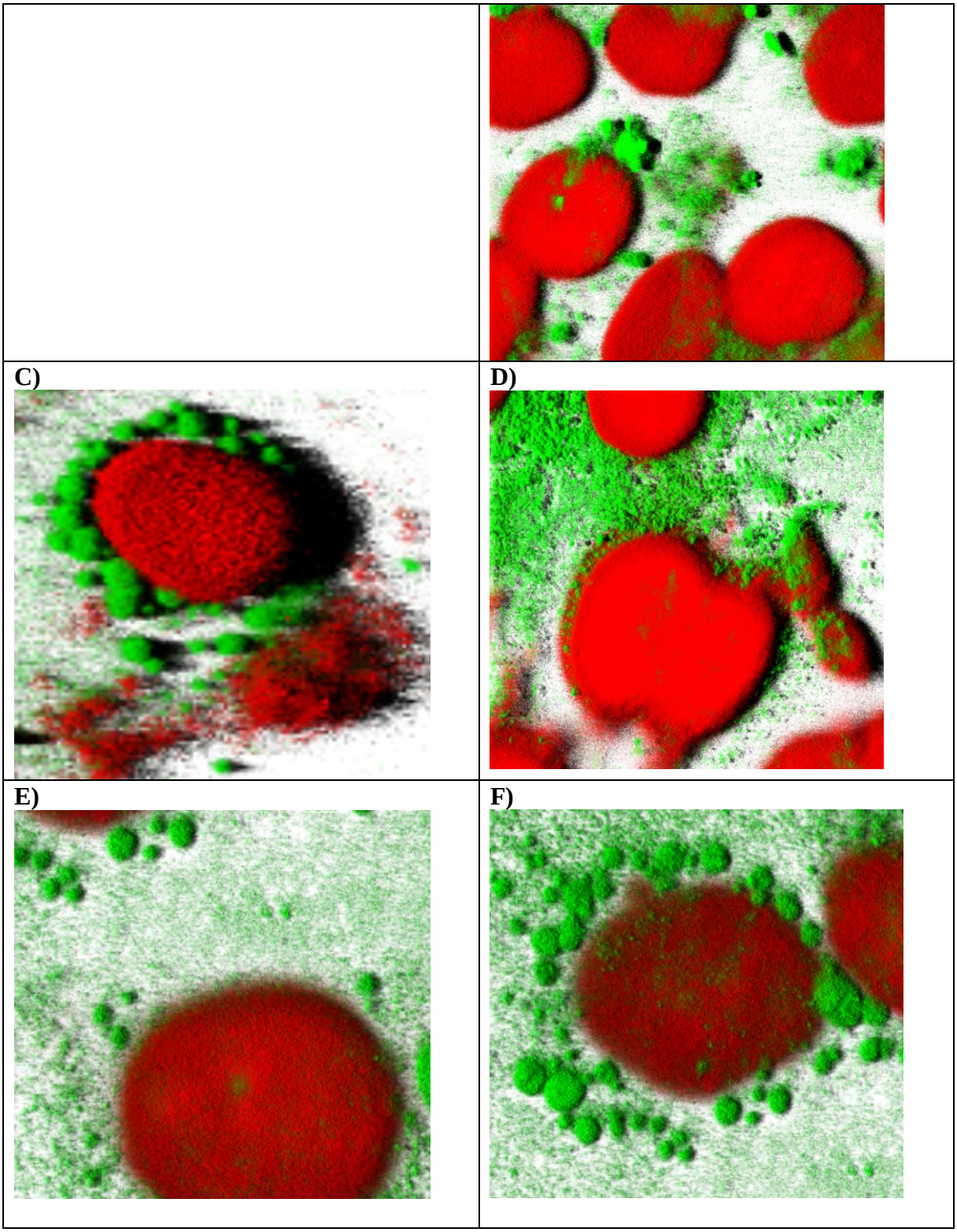
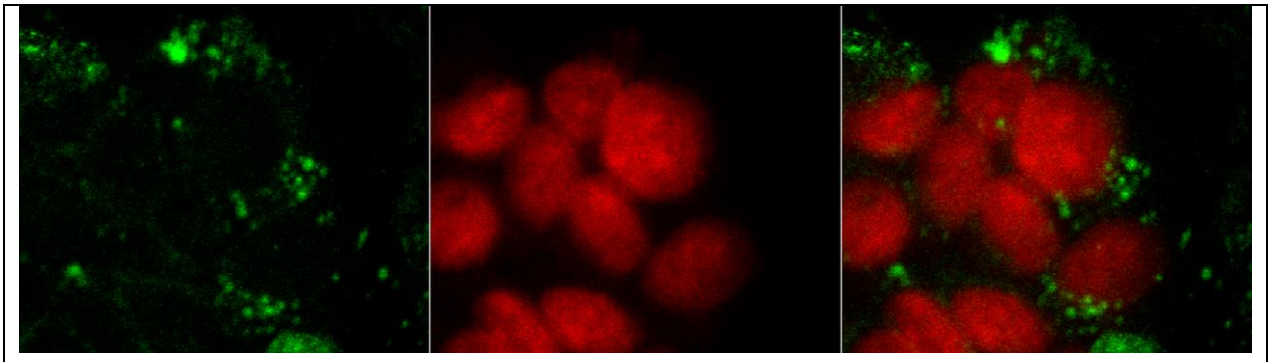
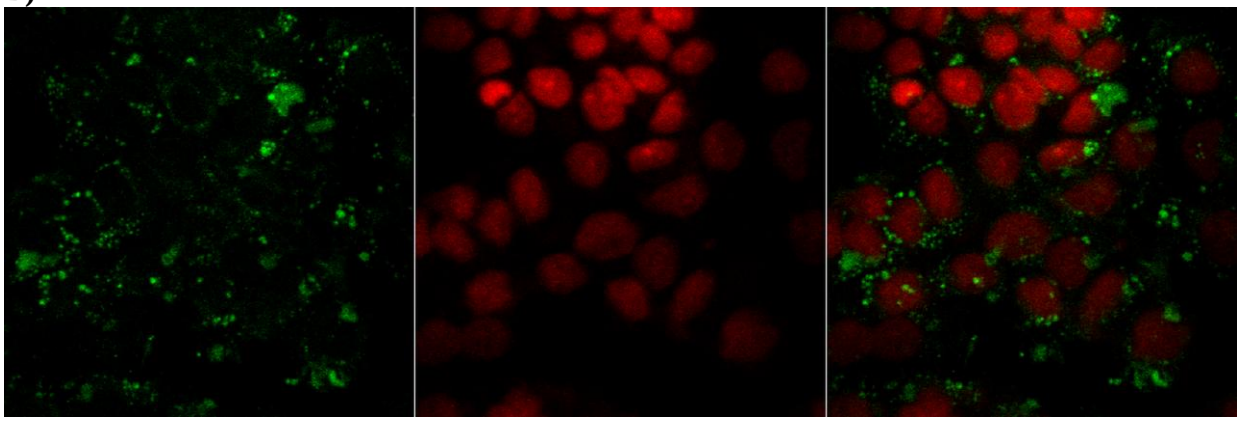


Fig 6.6 Confocal fluorescence 3D images of MCF-7 cells with Coumarin 6 labeled NPs after 2 hr exposure, PCL NPs (A and B) PLGA NPs (C and D) PLA NPs (E and F)

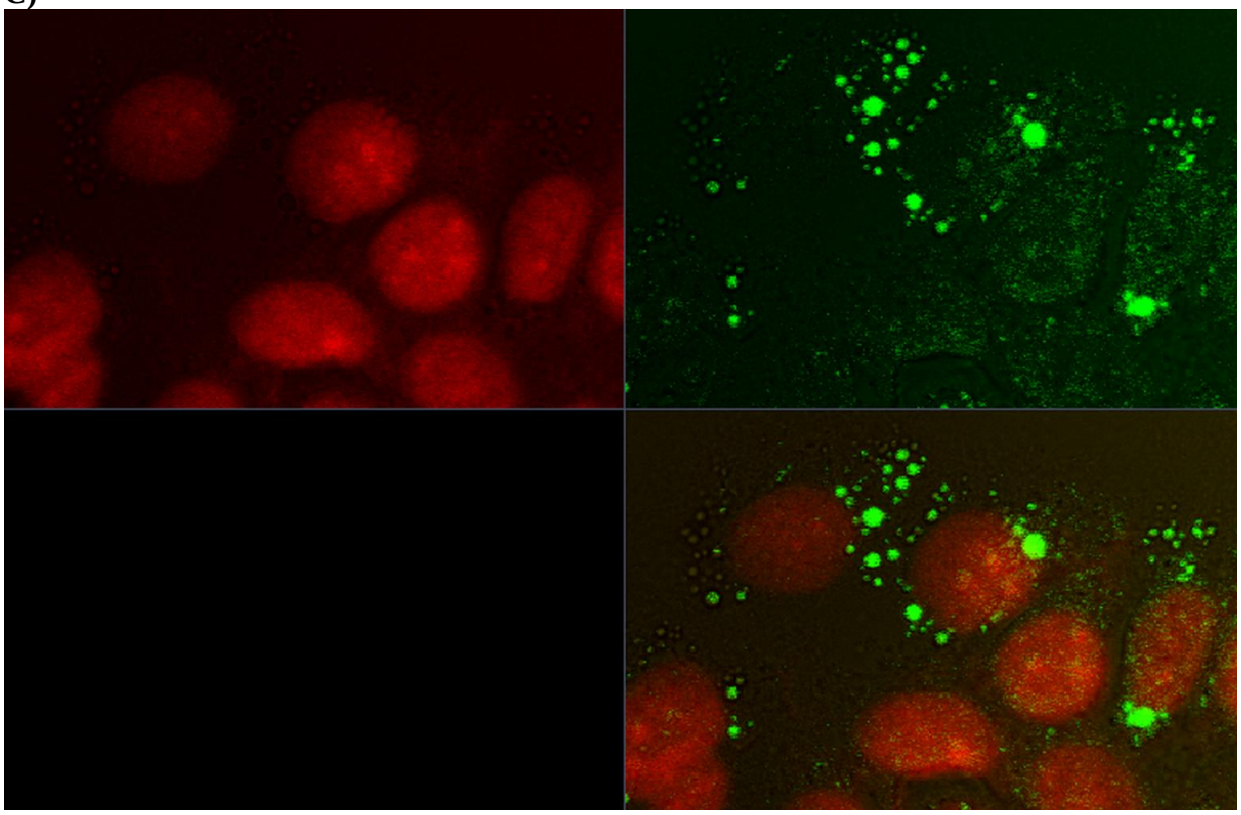
A)



B)



C)



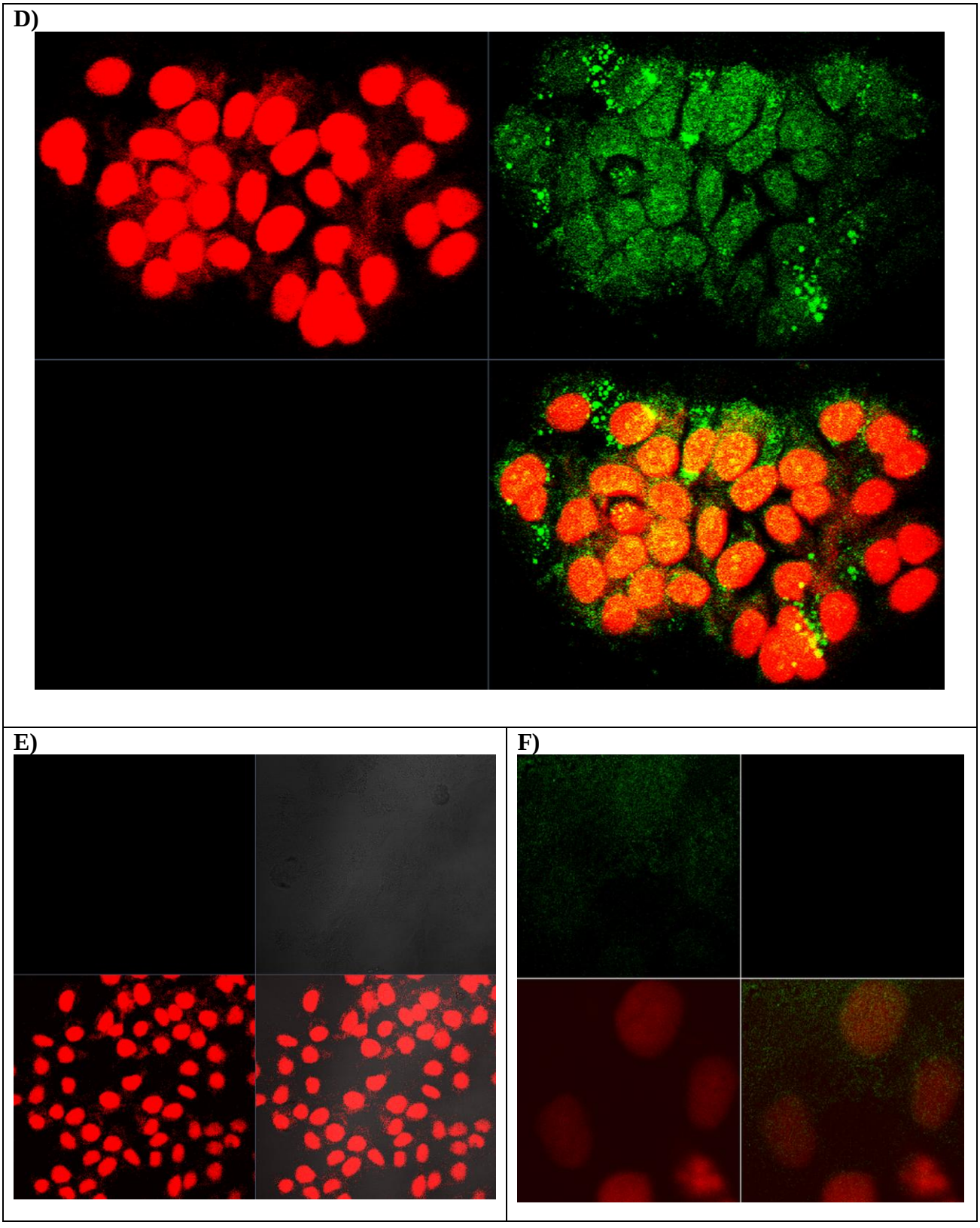


Fig 6.7 Confocal fluorescence microscopy 2D image of MCF-7 cellular uptakes of, PCL NPs (A) PLGA NPs (B) PLA NPs (C and D) blank MCF-7 Cells (E) free Coumarin 6 incubated for 2 hr (F). The cellular uptake is visualized by overlaying images obtained by fluorescein isothiocyanate channel (green), propidium iodide channel (red), background (black) and combination of all three images

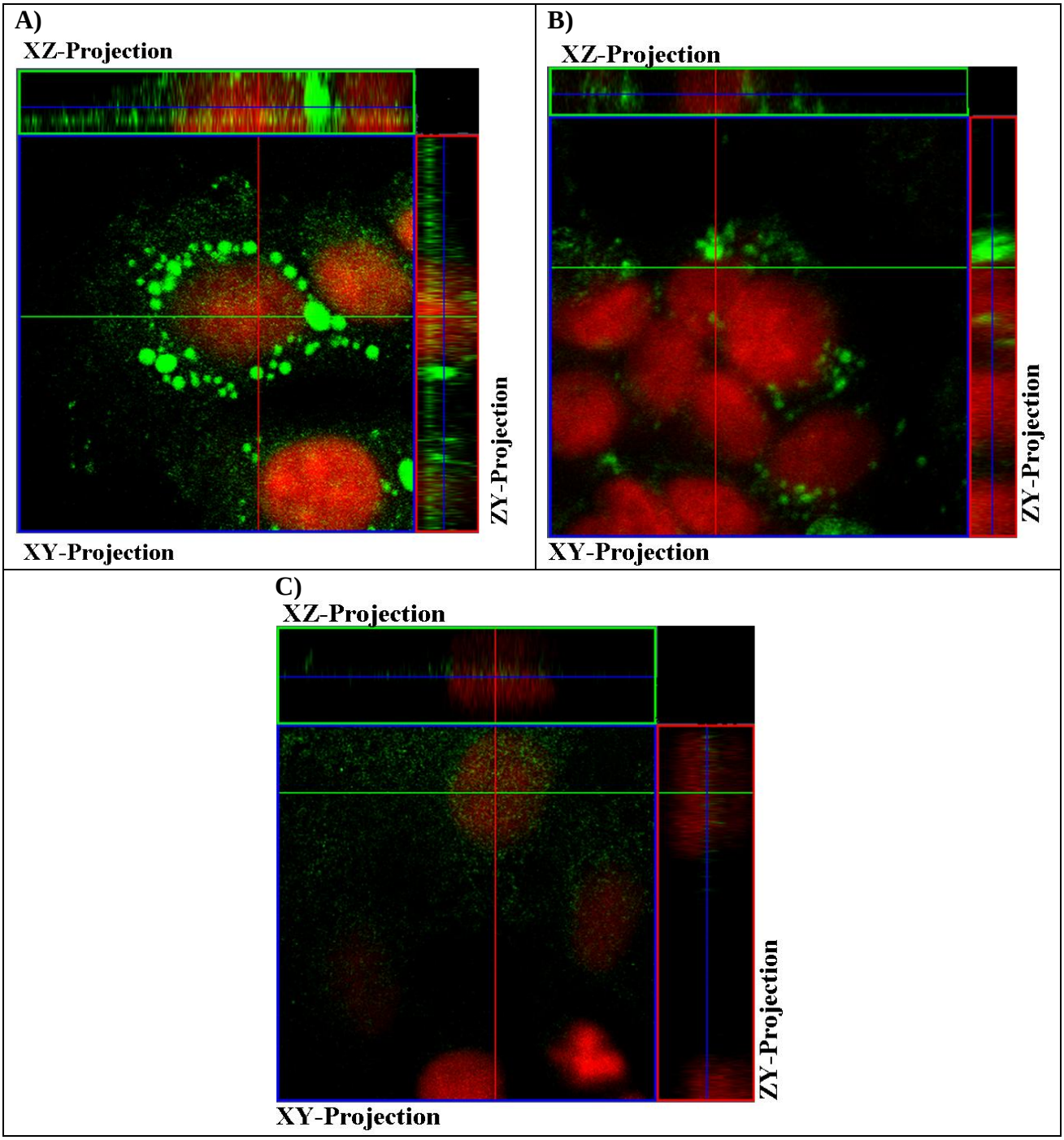


Fig 6.8 Confocal microscopy confirms internalization of NPs by MCF-7 cells. The cell uptake image is displayed in three orthogonal projections XY, XZ and YZ respectively. PLA NPs (A) PCL NPs (B) can be seen within the cytoplasm on the Z-sections (XZ & ZY projection), free Coumarin 6 incubated with MCF-7 cells illustrates less uptake of Coumarin 6 (C)

The orthogonal image of MCF-7 cells incubated with free C6 (Fig. 6.8 C) demonstrated that only C6 loaded PNPs can reach cytoplasm of cancer cells more than the free C6. In general, endocytosis is considered to be the main mechanism for the cells to take up NPs. Particles less than 150 nm reaches cells through pinocytosis and particles larger than 200 nm were taken up by cells through phagocytosis (5, 11). In this study, the nonspecific phagocytosis played major role in the engulfing of C6 PNPs by MCF-7 cells.

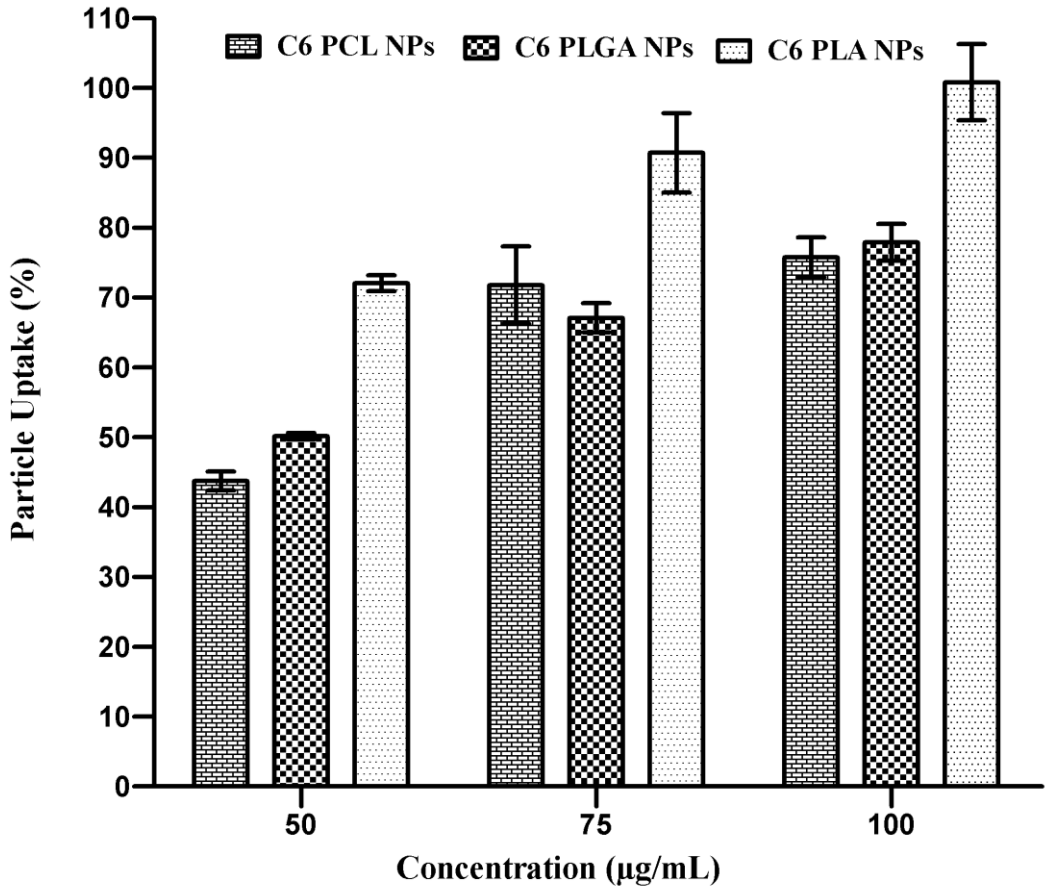


Fig 6.9 Particle uptake of NPs using MCF-7 cells

c) Determination of cellular uptake of NPs by micro plate reader

The quantitative analysis of cellular uptake of C6 loaded PNPs after 2 hr of incubation with MCF-7 cells were represented in Fig. 6.9. It can be observed that when the concentration was increased from 50 to 100 µg/mL the Paclitaxel loaded PCL, PLGA and PLA NPs uptake increased respectively. The increasing order of Paclitaxel loaded NPs uptake observed after 2 hr incubation with MCF-7 cells at 100 µg/mL were, PCL NPs<PLGA NPs<PLA NPs respectively. Among the PNPs studied for cellular uptake, PLA NPs were efficiently taken up by the MCF-7 cells at all the concentrations studied. Researchers demonstrated that the degree of internalization of NPDDS into cancer cells is inversely proportional to the particle size (13, 14). In the present work, PLA NPs cellular uptake is highest, due to small size, 151.13 ± 2.44 nm particles. The particle size of PLGA and PCL NPs was 166.56 ± 1.20 nm and 174.0 ± 9.34 nm respectively. Hence cellular uptake of PCL and PLGA NPs are lower compared to PLA NPs in MCF-7 cells.

6.6 Conclusion

The cytotoxicity study result demonstrated that the prepared Paclitaxel loaded NPs inhibited proliferation of MCF-7 cells more than the commercial formulation, CRE-MR. In addition, the IC_{50} values of PNPs were low for NPs when compared to CRE-MR. This demonstrated the efficiency of Paclitaxel loaded NPs in uptake and cytotoxicity. In overall the cell uptake study established the efficient uptake/target ability of PCL, PLGA and PLA NPs in breast cancer cells, MCF-7. The orthogonal projection of MCF-7 cellular uptake confocal fluorescence image, illustrated that maximum NPs reached the cytoplasm of the cells. The 2D and 3D view confocal fluorescence microscopic image showed that the PNPs were positioned closely around the nucleus. As in this study drug Paclitaxel was not used during identification problem, study is to be done with drug.

References

1. Tahara K, Sakai T, Yamamoto H, Takeuchi H, Hirashima N, Kawashima Y. (2009). Improved cellular uptake of chitosan-modified PLGA nanospheres by A549 cells, Int J Pharm, 382, 198-204.
2. De S, Miller DW, Robinson DH. (2005). Effect of particle size of nanospheres and microspheres on the cellular-association and cytotoxicity of paclitaxel in 4T1 cells. Pharm Res, 22, 766-775.
3. Manju S, Sreenivasan K. (2012). Gold nanoparticles generated and stabilized by water soluble curcumin–polymer conjugate: blood compatibility evaluation and targeted drug delivery onto cancer cells J Colloid Interface Sci, 368, 144-151.
4. Manju S, Sreenivasan K. (2011). Enhanced drug loading on magnetic nanoparticles by layer-by-layer assembly using drug conjugates: blood compatibility evaluation and targeted drug delivery in cancer cells. Langmuir, 27, 14489-14496.
5. Xie J, Wang C. (2005). Self-assembled biodegradable nanoparticles developed by direct dialysis for the delivery of paclitaxel. Pharm Res, 22, 2079-2090.
6. Konan YN, Gurny R, Alle´mann E. (2002). Preparation and characterization of sterile and freeze-dried sub-200 nm nanoparticles. Int J Pharm, 233, 239-252
7. Fonseca C, Simoes S, Gaspar R. (2002). Paclitaxel-loaded PLGA nanoparticles: preparation, physicochemical characterization and in-vitro anti-tumoral activity. J Control Release, 83, 273-286.
8. Raymond E, Hanauske A, Faivre S. (1997). Effects of prolonged versus short-term exposure paclitaxel (Taxol\) on human tumor colony-forming units. Anticancer Drugs, 8, 379-385.
9. Zhang Y, Tang L, Sun L, Bao J, Song C, Huang L, Liu K, Tian Y, Tian Ge, Li Z, Sun H, Mei L. (2010). A novel paclitaxel-loaded poly (ε-caprolactone)/Poloxamer 188 blend

nanoparticles overcoming multidrug resistance for cancer treatment. Acta Biomater, 6, 2045-2052.

10. Yao HJ, Ju R, Wang X, Zhang Y, Li R, Yu Y, Zhang L, Lu W. (2011). The antitumor efficacy of functional paclitaxel nanomicelles in treating resistant breast cancers by oral delivery. Biomaterials, 32, 3285-3302.
11. Jin C, Bai L, Wu H, Song W, Guo G, Dou K. (2009). Cytotoxicity of paclitaxel incorporated in PLGA nanoparticles on hypoxic human tumor cells. Pharm Res, 26, 1776-1784.
12. Ateh DD, Leinster VH, Lambert SR, Shah A, Khan A, Walklin HJ, Johnstone JV, Ibrahima NI, Kadama MM, Malika Z, Gironès M, Veldhuis GJ, Warnes G, Marino S, McNeishe IA, Martin JE. (2011). The intracellular uptake of CD95 modified paclitaxel-loaded poly (lactic-co-glycolic acid) microparticles. Biomaterials, 32, 8538-8547.
13. Lamprecht A, Schafer U, Lehr CM. (2001). Size-dependent bioadhesion of micro- and nanoparticulate carriers to the inflamed colonic mucosa. Pharm Res, 18, 788-793.
14. Desai MP, Labhasetwar V, Amidon GL, Levy RJ. (1996). Gastrointestinal uptake of biodegradable microparticles: effect of particle size. Pharm Res, 13, 1838-1845.

Chapter 7. In-vivo Acute Toxicity and Pharmacokinetic Study

7.1 Introduction

Pharmacokinetic and biodistribution studies of designed formulations are essential for determining therapeutic effectiveness and safety. It is more important for NDDS to asses their benefits. During development of pharmaceutical products, apart from active substances and excipients, several organic solvents are used for various purposes, which is unavoidable. But, if these solvents are not completely removed from the final products, it may lead to serious side effects/adverse effect to the patients. Hence, it is important for the researcher to ensure and check that there is no residual solvent left in the finished products. As per USP and ICH (Q3C) guidelines, the residual solvents in pharmaceutical products should be with in the limits. For any successful formulation it is necessary to find the residual solvent and asses their effect, because it may cause toxicity, which leads to dismissal of such formulation in spite of their potential benefits (1, 2).

The residual solvents are classified as, Class 1, solvents to be avoided, known or suspected human carcinogens and environmental hazards, Class 2 solvents are non-genotoxic animal carcinogens and suspected of other significant but reversible toxicities, Class 3, solvents with low toxic potential and health-based exposure limit is needed (2). In the present study, we have used acetone (Class-3) and dichloromethane (Class-2) during the preparation of NPs. There are different methods to identify and quantify residual solvents in the finished products but widely used ones are the gas chromatography and histopathological studies. Administering the final products to laboratory animals and microscopic examination of tissues for histopathological changes is indirect way of identification of residual solvents exposure. In addition, the presence of residual solvent in the final material can be identified by ^{1}H nuclear magnetic resonance and infrared spectrum, also (1, 2).

In the present work, NMR and indirect method of animal exposure was used to determine presence of residual solvent(s) beyond limit. On the basis of in vitro evaluation, cellular uptake and other studies selective designed products were used for pharmacokinetic and biodistribution studies in animal model. Specifically for anticancer drugs, NPs modify the biodistribution and pharmacokinetic character of drugs and hence can reduce the systemic side-effects accrued due to non-specific delivery and accumulation of drugs to normal cells.The acceptance of NPDDS in oncology field is due its significant favourable pharmacokinetics, biodistribution and toxicity profile when compared to the free drugs. Thus it is important to do pharmacokinetic and biodistribution studies.

7.2 Materials and Methods

Pure Paclitaxel and selected nanoparticulate formulations, prepared and mentioned in Chapter 5 were used for these studies. Surgical instruments scissors, forceps, glass hypodermic syringes,

etc were procured from the Niraj Industries Pvt.Ltd. Mumbai, India. All the animal experiments were done with proper approval from Institute Animal Ethical Committee.

7.3 Residual solvent evaluation

a) ^{1}H NMR

The presence of residual solvent (DCM) in the blank and Paclitaxel loaded PNPs were analyzed by Varian NMR spectrometer, operating at 300 MHz (Varian, Mercury plus, USA). The NPs were dissolved in $CDCl_3$ and the spectrum was recorded for the respective samples and interpreted for presences of solvent peaks.

b) In-vivo acute toxicity

(i) Protocol approval and animal grouping

The in-vivo acute toxicity study for residual solvents was studied in wistar rats (160-220 g) using blank PNPs and Paclitaxel loaded PNPs. The study protocol was approved by the Institute Animal Ethical Committee, BITS, Pilani, India (protocol no: IAEC/RES/5/6/ rev 01). Rats were acclimatised for at least one week before the experiment was initiated. Throughout the experiment, animals were housed in polypropylene cages filled with sterile paddy husk and maintained at 22 ± 2°C and 50-60% RH, under a 12:12 hr light-dark cycle. The animals were separated according to body weight and they were marked with picric acid in head, tail and body. Animals were divided into three groups, saline control, blank PNPs and Paclitaxel loaded PNPs each group having three animals per cage. At predetermined time points tissue samples were taken from respective animals.

(ii) Dosing of animals and sample collection

The acclimatised rats were fasted overnight before sample administration and had access to water ad libitum. Dosing (10mg/kg) through tail vein was started after dilating vein using xylene/hot water to avoid accumulation of drug in muscles, which may cause necrosis. After 24 hr, animals were sacrificed and the respective tissue sections such as heart, lungs, liver, spleen, kidney and brain were isolated and fixed in 10 % formalin saline solution after washing with cold water.

(iii) Histopathological evaluation

The tissue samples were dehydrated by treating with increasing concentration of absolute alcohol and xylene and then embedded in paraffin block. The tissue sections were sliced using a microtome and processed for hydration and for microscopic study. Finally the tissue sections were stained using hematoxylin and eosin then mounted on the microscopic slide and fixed with cover slips using DPX mountant solution. The tissue section slides were observed under light microscope (4X/10X/40X) for any histopathological changes.

7.4 In-vivo pharmacokinetic and biodistribution study

7.4.1 Protocol approval and animal grouping

The pharmacokinetic and biodistribution behaviour of Paclitaxel and Paclitaxel loaded PNPs were performed in wistar rats (160-220 g). The study protocol was approved by the Institute Animal Ethical Committee, BITS, Pilani, India (protocol no: IAEC/RES/5/6/ rev 01). Rats were acclimatised for at least one week before the experiment. Throughout the experiment, animals were housed in polypropylene cages filled with sterile paddy husk and maintained at 22 ± 2°C and 50-60% RH, under a 12:12 hr light-dark cycle. The animals were separated according to body weight and they were marked with picric acid in head, tail and body. Animals were divided into four groups, Paclitaxel solution, Paclitaxel loaded PCL, PLGA and PLA NPs, each group having five cages, each cage containing three animals and for each time point samples were taken from three animals.

7.4.2 Preparation of Paclitaxel solutions and Paclitaxel loaded NPs dispersion

Paclitaxel solution for i.v. injection was prepared by dissolving 90 mg of pure Paclitaxel in mixture of 7.5 mL of ethanol and 7.5 mL of Tween 80 and further diluted with sterile saline (0.9% of sodium chloride) to a final concentration of 3 mg/mL, such that the volume of drug solution injected to rat was below 1 mL (3, 4). The selected PNPs were administered as aqueous dispersion to same strength. The Paclitaxel solution and all the PNPs dispersions were administered at dose equivalent to 10 mg/kg.

7.4.3 Dosing of animals and sample collection

The rats were fasted overnight before Paclitaxel and Paclitaxel loaded PNPs administration and had access to water ad libitum. The dosing (10mg/kg) through tail vein was started after dilating vein using xylene/hot water to avoid accumulation of drug in muscles, which may cause necrosis. For each time point 300 µL of blood samples were collected through cardiac puncher under mild diethyl ether anaesthesia. The samples were immediately transferred into EDTA-Na_2 containing prelabeled 1.5 mL polypropylene micro tubes, immediately after collection and the micro tubes were gently inverted several times to ensure complete mixing with the anticoagulant. The sample collection time points were 0.5, 1, 1.5, 3, 6, 9, 12, 24 and 48 hr after administration. In case of biodistribution study, liver, kidney and spleen samples were collected from rats at 1, 2, 3, 6 and 12 hr after sacrificing the animals. After collecting the tissue samples they were cleaned using phosphate buffer to remove the residual blood and blotted dried with Whatman filter paper. All the samples were stored at -20°C until analysis and thawed every time before analysis.

7.4.4 Estimation of Paclitaxel in plasma and tissue samples

The blood samples were centrifuged at 4000 rpm for 15 min at 4°C to obtain plasma samples.

The plasma samples were processed for extraction of Paclitaxel as mentioned in Chapter 3 for analysis. All the tissue samples were weighed, chopped and transferred to 50 mL tarson tube for homogenizing and 10 mL of acetonitrile was added. The obtained tissue suspension was processed for Paclitaxel estimation as mentioned in Chapter 3. Paclitaxel in the plasma and tissue samples was estimated by HPLC method as mentioned in Chapter 3. The plasma concentration of Paclitaxel vs. time and Paclitaxel amount in per g of tissue vs, time was plotted for pure drug and all the PNPs formulations.

7.4.5 Pharmacokinetic data analysis

All pharmacokinetic parameters were calculated by non-compartment model using WinNonlin software (Version 2.1, Pharsight Corporation and USA) and it is listed in Table 7.1, 7.2, 7.3 and 7.4.

7.5 Result and discussion

7.5.1 Residual solvent evaluation

a) ^{1}H NMR

The ^{1}H NMR spectra of blank PLGA NPs and Paclitaxel loaded PLGA NPs in $CDCL_3$ were obtained and there was no proton peak corresponding to residual solvent dichloromethane used in the preparation of NPs. All the characteristic peaks of Paclitaxel, polymer and stabilizers were observed. This analysis showed that the prepared PNPs were free from the residual solvents or below permissible limit and it can be administered safely to rat by i.v. route.

b) In-vivo acute toxicity of PNPs

Once the NPs are administered, phagocytosis by macrophages of mononuclear phagocytic system results in localization of NPs in the reticulocyte endothelial organs, liver, kidney, lung and spleen (5, 6). If the organic volatile impurities are not removed completely it may results in generation of inflammatory and tissue response at the respective organs (2). Thus the morphology of the respective tissue cells can give clear picture about of the inflammatory condition after systemic administration of PNPs. The histopathology of different tissues (Fig. 7.1) of, blank PNPs and Paclitaxel loaded PNPs treated rats did not illustrate any significant differences in number, arrangement and architecture of respective cells. This confirms that the prepared PNPs or residual solvents (acetone/dichloromethane) did not, induce any histological damage, when administered i.v to rats at 10 mg/kg dose. Hence, the solvents were absent or well within the limits and these formulations can be safely administered by i.v route.

7.5.2 In-vivo pharmacokinetic study

In rat, plasma Paclitaxel concentration above 8450 ng/mL is considered to be maximum tolerable level (MTL) and 43 ng/mL is considered to be minimum effective level (MEL) (7).

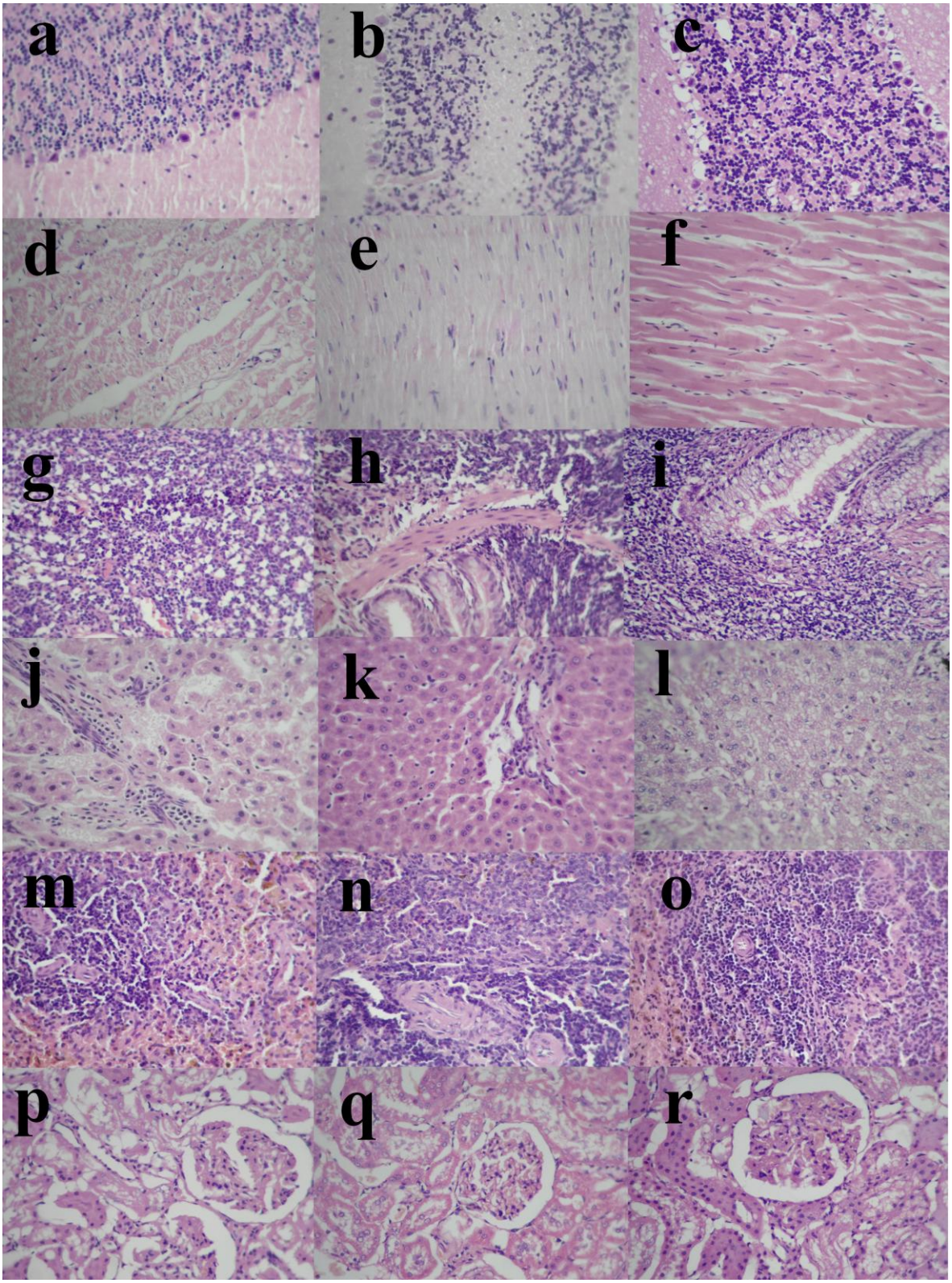


Fig. 7.1 Histopathology staining section of organs, control brain (a), heart (d), lungs (g), liver (j), spleen (m) and kidney (p). Blank PNPs administered brain (b), heart (e), lungs (h), liver (k), spleen (n) and kidney (q). Paclitaxel loaded PNPs administered brain (c), heart (f), lungs (i), liver (l), spleen (o) and kidney (r)

In pharmacokinetic study of all designed PNPs, the plasma concentration at 0.5 hr were in the range of 4000.12 to 3322.49 ng/mL and at 28 hr the concentration was in the range of 195.07 to 161.06 ng/mL respectively. In case of Paclitaxel solution the plasma concentration at 9 hr itself was observed to be 47.78 ± 12.25 ng/mL. It can be observed that in case of all the PNPs the Paclitaxel concentration remained higher, much above 150 ng/mL, within therapeutic window, even after 24 hr (Fig.7.2). These suggested that all the prepared PNPs were safe and are not expected to produce any side effect. Yeh et al. (8) studied the pharmacokinetics of Paclitaxel loaded gelatin NPs in mice at same 10 mg/kg dose, where they reported initial concentration above MTL, and by 6 hr concentration reduced to 14 ng/mL, much below MEL, suggesting possibilities of side effect and very fast elimination.

Straub et al. (9) showed that when Taxol® was administered at same 10 mg/kg dose as i.v. bolus, all the rats died shortly after dosing due to very high initial concentration. In pharmacokinetic study of commercial Taxol® by Dong et al. (7), produced high initial plasma Paclitaxel concentration which reduced to below MEL by 19 hr, suggesting there is possibility of toxicity or side effect. However, designed PNPs in this work produced lower initial Paclitaxel concentration which continued above MEL beyond 24 hr expected to provide long term efficacy.

The MRT (Table 7.1) of all the prepared PNPs were found to be much higher (more than 10 hr) in comparison to Paclitaxel solution which is 3.71 hr. In addition, the MRT of these prepared PNPs were found to be higher than the Paclitaxel loaded micelles and liposomes (in the rage of 3.12 to 10.59 hr) prepared and reported by Dhanikula et al (10). Among the prepared Paclitaxel loaded PNPs, PLGA NPs produced higher MRT and $t_{½}$, 11.91 ± 0.25 hr and 8.56 ± 0.27 hr respectively. Shah et al. (11) studied the pharmacokinetic profile of Paclitaxel loaded PLGA NPs, Pluronic P85-coated Paclitaxel loaded PLGA NPs and transferring conjugated Paclitaxel loaded PLGA NPs in rats at a dose of 20 mg/kg. The MRT and $t_{1/2}$ of these formulations were reported to be 5.46, 6.11 and 7.64 hr and 3.96, 4.33 and 5.43 hr respectively, which are found to be much less when compared to the designed PNPs (Table 7.1). It can be concluded that in case of PLGA NPs, liposome and micelle, Paclitaxel was cleared faster from the body (10, 11).

The antitumor effect of Paclitaxel depends on the sustained therapeutic concentration rather than the maximal or high plasma concentration because Paclitaxel needs the cells to enter into the M phase (3). Thus designed PNPs of this work are expected to be produce enhanced and longer antitumor activity. The V_{ss} (L/kg) of the prepared PCL, PLGA and PLA NPs were 4.1 ± 0.002, 5.0 ± 0.003 and 5.6 ± 0.005 which is much less than Paclitaxel solution (6.2 ± 0.003). This suggested that the distribution of PNPs to unwanted places were controlled and resulted in long circulation of PNPs in blood.

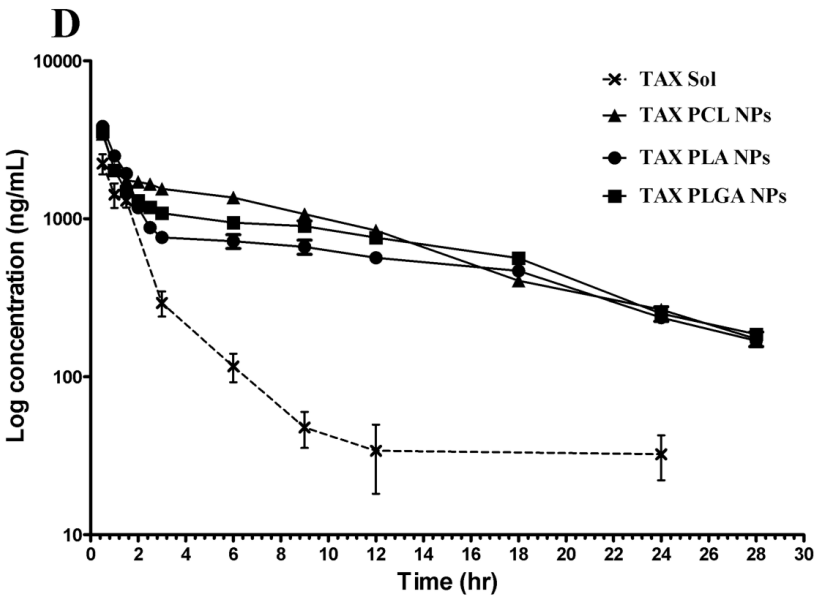


Fig. 7.2 Log plasma concentration-time profile of Paclitaxel and Paclitaxel loaded PNPs

Particles less than 20–30 nm are eliminated by renal excretion and larger particles, greater than 200 nm, will be removed by opsoninization. The ideal size for selective drug delivery is between 70 and 200 nm in diameter which has slow rate of clearance and thus has extended circulation times compared to those with larger diameters (5, 12). In the present work, all the prepared PNPs size were in the rage of 150-175 nm hence they have high AUC, long $t_{1/2}$, low clearances rate and long circulating time (MRT). The main problem in preparing long circulating NPs is opsonization, but in case of smaller particles which have a high radius of curvature, the opsonin cannot bind and clear the particles. Hence the circulation time of the prepared PCL, PLGA and PLA NPs is very high and its clearance rate is low (1). One more reason for the long circulation, low clearances rate or high MRT and $t_{1/2}$ of the prepared PNPs is the adsorption of surfactant (PF 68) on the particle surface. This reduces the opsonization reaction and subsequent clearance by macrophages (10).

7.5.3 In-vivo biodistribution study

The drug amount in rat liver, kidney and spleen vs. time profile and pharmacokinetic parameters of Paclitaxel and Paclitaxel loaded NPs after i.v administrating are presented in Fig. 7.3, 7.4 and 7.5 and Table 7.2, 7.3 and 7.4. The $AUC_{0\text{-inf}}$ for amount of Paclitaxel in liver for drug loaded PCL, PLGA and PLA NPs were increased significantly compared to pure drug (Table 7.2). Highest was found in PLGA NPs, 316 ng.h/mL. The $t_{1/2}$ and MRT of Paclitaxel loaded NPs in liver were in the range of 4.10 to 10.10 hr and 5.10 to 14.04 hr respectively, which is higher than the Paclitaxel solution, 2.50 hr and 3.53 hr respectively.

Table 7.1 Pharmacokinetic parameters of Paclitaxel and Paclitaxel loaded NPs in plasma after i.v administration (n=3)

S.No	Parameters	Paclitaxel solution	PCL NPs	PLGA NPs	PLA NPs
1	$AUC_{0\text{-}inf}$ (ng.hr)/mL	5787 ± 700.99	26220.11 ± 549.21	24214.39 ± 357.33	20813.96 ± 801.72
2	$AUMC_{0\text{-}inf}$ (ng.hr^2)/mL	21484 ± 376.54	273940.08 ± 2546.74	288493.69 ± 10407.66	239565.06 ± 10010.12
3	C_{max} (ng/mL)	3511 ± 59.43	5777.20 ± 556.61	6281.41 ± 536.11	5940.19 ± 604.12
4	$t_{1/2}$ (hr)	3.77 ± 0.13	7.50 ± 0.07	8.56 ± 0.27	8.16 ± 0.15
5	MRT (hr)	3.93 ± 0.32	10.45 ± 0.24	11.91 ± 0.25	11.51 ± 0.34
6	Cl (L/hr/kg)	1.8 ± 0.002	0.4 ± 0.002	0.4 ± 0.004	0.5 ± 0.003
7	V_{ss} (L/kg)	6.2 ± 0.003	4.1 ± 0.002	5.0 ± 0.003	5.6 ± 0.005

Table 7.2 Pharmacokinetic parameters of Paclitaxel and Paclitaxel Loaded PNPs in liver after i.v administration (n=3)

S.No	Parameters	Paclitaxel solution	PCL NPs	PLGA NPs	PLA NPs
1	$AUC_{0\text{-}inf}$ (ng.hr)/mL	1285 ± 243.21	3557.20 ± 452.21	2146.90 ± 312.32	4311.05 ± 522.22
2	C_{max} (ng/mL)	421.93 ± 54.32	308.20 ± 34.67	316.0 ± 30.11	304.90 ± 12.87
3	$t_{1/2}$ (hr)	2.50 ± 0.43	8.12 ± 0.88	4.10 ± 0.44	10.10 ± 0.87
4	MRT (hr)	3.53 ± 0.65	11.91 ± 0.23	6.0 ± 0.11	14.04 ± 0.34
5	Cl (L/hr/kg)	0.008 ± 0.001	0.003 ± 0.001	0.005 ± 0.002	0.002 ± 0.001

When compare to Paclitaxel solution, the Paclitaxel loaded NPs clearances was less in liver tissue (Table 7.2). The biodistribution profile (Fig. 7.3, 7.4 and 7.5) clearly showed that Paclitaxel loaded NPs penetration was high in liver and spleen tissue.

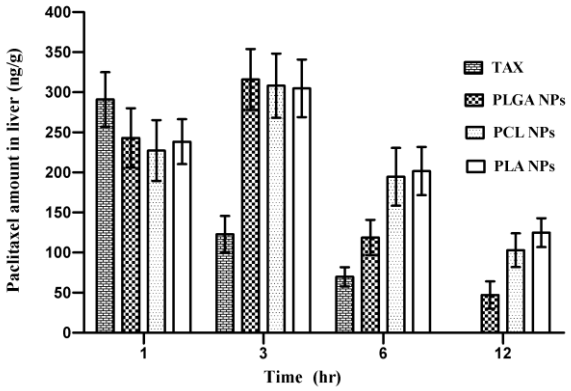


Fig. 7.3 Amount of Paclitaxel and Paclitaxel loaded NPs in rat liver after i.v administration (TAX-Paclitaxel)

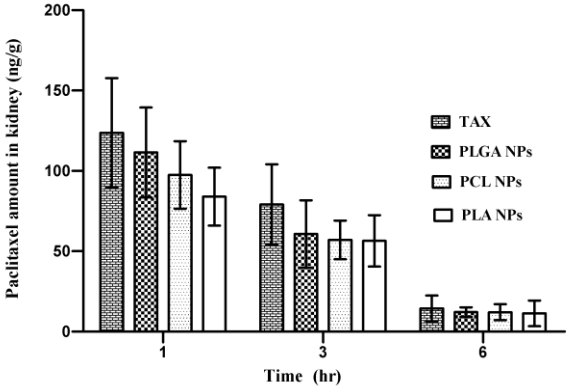


Fig. 7.4 Amount of Paclitaxel and Paclitaxel loaded NPs in rat kidney after i.v administration (TAX-Paclitaxel)

The $AUC_{0\text{-}inf}$ of Paclitaxel loaded NPs in kidney is same as pure drug Paclitaxel and there was no change in C_{max}, $t_{1/2}$ and MRT. Paclitaxel was detected up to 12 hr in liver tissue but in case of spleen it was detected up to 6 hr.

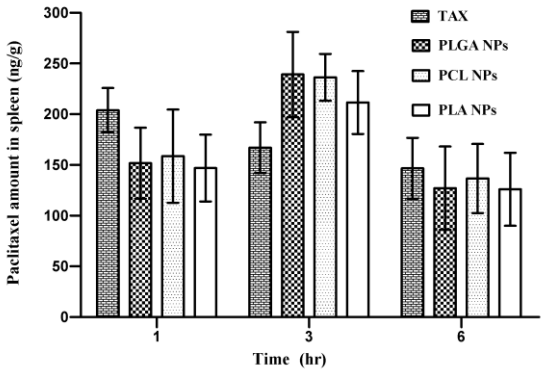


Fig. 7.5 Amount of Paclitaxel and Paclitaxel loaded NPs in rat spleen after i.v administration (TAX-Paclitaxel)

Table 7.3 Pharmacokinetic parameters of Paclitaxel and Paclitaxel Loaded PNPs in kidney after intra-venous administration (n=3)

S.No	Parameters	Paclitaxel solution	PCL NPs	PLGA NPs	PLA NPs
1	$AUC_{0\text{-}inf}$ (ng.hr)/mL	485.20 ± 83.22	377.50 ± 86.21	414.61 ± 50.22	343.11 ± 43.22
2	C_{max} (ng/mL)	154.72 ± 21.32	127.40 ± 17.11	151.30 ± 29.11	102.50 ± 14.87
3	$t_{1/2}$ (hr)	1.60 ± 0.18	1.63 ± 0.67	1.54 ± 0.65	1.70 ± 0.43
4	MRT (hr)	2.50 ± 0.25	2.50 ± 0.22	2.34 ± 0.18	2.60 ± 0.43
5	Cl (L/hr/kg)	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01

Table 7.4 Pharmacokinetic parameters of Paclitaxel and Paclitaxel Loaded PNPs in spleen after intra-venous administration (n=3)

S.No	Parameters	Paclitaxel solution	PCL NPs	PLGA NPs	PLA NPs
1	$AUC_{0\text{-}inf}$ (ng.hr)/mL	3347.01 ± 453.82	4358.60 ± 523.02	3658.0 ± 383.32	3998.80 ± 543.45
2	C_{max} (ng/mL)	225.20 ± 18.04	236.40 ± 21.11	240.0 ± 32.11	211.53 ± 26.34
3	$t_{1/2}$ (hr)	10.83 ± 1.54	16.53 ± 3.54	14.10 ± 1.22	16.50 ± 0.41
4	MRT (hr)	15.70 ± 1.02	23.10 ± 1.42	19.50 ± 2.02	23.11 ± 1.11
5	Cl (L/hr/kg)	0.003 ± 0.001	0.002 ± 0.001	0.003 ± 0.001	0.002 ± 0.001

The amount of Paclitaxel reaching spleen starts decreasing drastically after 1 hr in case of PNPs when compared to pure drug. The $AUC_{0\text{-}inf}$ of Paclitaxel loaded PLA NPs in liver is more in comparison to spleen and kidney tissue. The Paclitaxel amount at 3 hr for PCL, PLGA and PLA NPs was statistically significant in liver ($P<0.001$) and spleen ($P<0.05$) tissues in comparison with Paclitaxel solution. In spleen sample, Paclitaxel eliminated very fast (Fig. 7.5) and there was no drug found after 6 hr, but in case of liver Paclitaxel was estimated up to 12 hr, suggesting PNPs are not cleared fast from the liver tissue. Overall the biodistribution study result showed that Paclitaxel loaded NPs distribute selectively to liver than the other tissue systems studied. This study showed that the prepared NPs can be used to treat liver cancer, because of its selective delivery. Paclitaxel reaches peak concentration in all the tissue samples examined at 3 hr.

7.4 Conclusion

The residual solvent analysis by ^{1}H NMR clearly showed that there was no solvent, DCM in the blank and Paclitaxel loaded PNPs. This study showed that the PNPs formulation is free from the residual solvents and it is safe to administer by i.v to animals. The in-vivo acute toxicity study in rats suggests that the prepared PNPs did not show any toxic effect on the rat tissues and hence there was no change in the architecture of respective organ cells.

After i.v. administration of the PNPs, there were enhanced in plasma concentration, which is a good indication that the Paclitaxel loaded NPs are remaining more in plasma. But drug well distributing into the highly perfused organs. The quick distribution of the NPs to liver may be due to rapid partitioning of the released Paclitaxel in to tissue or direct NPs accumulation/adhering in the interstitial space or macrophage uptake of Paclitaxel loaded NPs. The i.v pharmacokinetic result showed that the Paclitaxel loaded PNPs achieved much larger AUC, much longer $t_{1/2}$ and less clearance of the NPs and less volume of distribution when compared to Paclitaxel solution. The biodistribution study result illustrated that Paclitaxel was found maximum in liver, when Paclitaxel loaded NPs were administered by i.v to rats. The Paclitaxel loaded PCL, PLGA and PLA NPs can be used for liver cancer as targeted drug delivery system for effective therapy.

References

1. United States Pharmacopoeia (USP). Residual Solvents. 31st ed. 2008.
2. Sahana DK, Mittal G, Bhardwaj V, Ravikumar MNV. (2008). PLGA nanoparticles for oral delivery of hydrophobic drugs: influence of organic solvent on nanoparticle formation and release behavior in-vitro and in-vivo using estradiol as a model drug. J Pharm Sci, 97, 1530-1542.

3. Brittain HG. Profiles of drug substances, excipients, and related methodology. ln: Jauhari S, Singh S, Dash AK, editors. Paclitaxel. UK: Academic Press; 2009. 299-344.
4. Saha RN, Vasanthakumar S, Benda G, Snehalatha M. (2010). Nanoparticulate drug delivery systems for chemotheraphy. Mol Membr Biol, 27, 215-223.
5. Owens ED, Peppas NA. (2006). Opsonization, biodistribution and pharmacokinetics of polymer nanoparticles. Int J Pharm, 307, 93-102.
6. Champion JA, Katare YK, Mitragotri S. (2007). Particle shape: new design parameters for micro and nanoscale drug delivery carriers. J Control Release, 121, 3-9.
7. Dong Y, Feng S. (2007). In-vitro and in-vivo evaluation of methoxy polyethylene glycol-polylactide-(MPEG-PLA)-nanoparticles for small-molecular drug chemotheraphy. Biomaterials, 28, 4154-4160.
8. Yeh TK, Lu Ze, Wientjes MG, Au JLS. (2005). Formulating paclitaxel in nanoparticles alters its disposition. Pharm Res, 22, 867-874.
9. Straub JA, Chickering DE, Lovely JC, Zhang H, Shah B, Waud WR Bernstein H. (2005). Intravenous hydrophobic drug delivery: a porous particle formulation of paclitaxel (AI-850). Pharm Res, 22, 347-355.
10. Dhanikula B, Singh DR, Panchagnula R. (2005). In-vivo pharmacokinetic and tissue distribution studies in mice of alternative formulations for local and systemic delivery of paclitaxel: gel, film, prodrug, liposomes and micelles. Curr Drug Deliv, 2, 35-44.
11. Shah N, Chaudhari K, Dantuluri P, Murthy RSR, Das S. (2009). Paclitaxel-loaded PLGA nanoparticles surface modified with transferring and Pluronic P85, an in-vitro cell line and in-vivo biodistribution studies on rat model. J Drug Target, 17, 533-542.
12. Champion JA, Katare YK, Mitragotri S. (2007). Particle shape: new design parameters for micro and nanoscale drug delivery carriers. J Control Release, 121, 3-9.

Chapter 8. Pharmacodynamic Study

8.1 Introduction

Novel drug delivery systems (NDDS) are made for enhanced therapeutic efficacy and better patient compliances. Thus pharmacodynamic study is essential to evaluate designed NDDS. Hepatocellular carcinoma, the first primary malignant tumor of liver and one of the most common malignancies with high prevalence of about 620,000 cases per year worldwide. Hepatocellular carcinoma is the 5th most common neoplasm in the world, 6th most common cancer in male and the 4th most common cause of cancer-related death. Mortality due to hepatocellular carcinoma nearly matches the incidence of this type of tumor (1-3). Targeted drug delivery by systemic administration will be an ideal choice to treat hepatocellular carcinoma patients. Not many researchers have shown the use of NPDDS in hepatocellular carcinoma treatment (1, 2).

Drug targeting to the liver is a very new strategy, which has not been explored extensively (3). Despite increasing interest in sustain/controlled release drug delivery systems in recent years, more attention has been paid to deliver drug to particular target at controlled manner (4). Thus, long term anti-tumor efficacy and extent of selective delivery to liver of Paclitaxel loaded PCL, PLGA and PLA NPs were studied using hepatocellular carcinoma model in rats and reported in this Chapter.

8.2 Materials and Methods

Pure Paclitaxel and selected nanoparticulate systems, prepared and mentioned in Chapter 5 wee used for these studies. Commercial Paclitaxel formulation (INTAXEL 30 MG INJ 5 mL, Fresenius Kabi, Germany) was procured from local Pharmacy shop, Chennai, India. Drug and chemicals were procured as mentioned in Chapter 5. N-Nitrosodiethylamine (DEN, Isopac®) was procured form Sigma-Aldrich chemicals, Bangalore, India. Diethyl ether was obtained from S.D. Fine Chemicals Ltd, Mumbai, India. Surgical instruments scissors, forceps, glass hypodermic syringes, etc were procured from the Niraj Industries Pvt. Ltd, Mumbai, India.

8.3 In-vivo anti-tumor efficacy study

8.3.1 Animals and Hepatocellular carcinoma model in rats

The in-vivo anti-tumor efficacy of Paclitaxel solution, commercial Paclitaxel formulation (CRE-MR) and Paclitaxel loaded PNPs were performed in wistar rats (120-220 g). PCL, PLGA and PLA NPs with high DC and optimal size were selected, based on in-vitro evaluation for the in-vivo anti-tumor efficacy study. The study protocol was approved by the Institute Animal Ethical Committee, VELS University, Chennai, India (protocol no: XIII/VELS/COL/23-I/CPCSEA/IAEC/23.09.11). Rats were acclimatised for at least one week before the experiment was initiated. Throughout the experiment, animals were housed in polypropylene cages filled with sterile paddy husk and maintained at 22 ± 2°C

and 50-60% RH, under a 12:12 hr light-dark cycle. The animals were separated according to body weight, grouped and they were marked with picric acid in head, tail and body. Animals were divided into seven groups, each group with eight animals. Hepatocellular carcinoma model was chemically induced in wistar rats by intraperitoneal administration of N-Nitrosodiethylamine (1, 2) 180 mg/kg for thrice on alternate days (Fig 8.1).

8.3.2 Animal grouping and drug administration

Animals (n=8) were randomly divided into seven treatment groups as follows,

(1) Group 1(HCA) is healthy control animals, treated with normal saline (5 ml/kg i.v)

(2) Group 2 (DCA) is DEN administered control animals, treated with normal saline (5 ml/kg i.v)

(3) Group 3 (D TAX) is DEN administered, treated with Paclitaxel solution (20 mg/kg i.v. daily)

(4) Group 4 (D CRE-MR) is DEN administered, treated with commercial formulation (CRE-MR) (20 mg/kg i.v. daily)

(5) Group 5 (D PCL NPs) is DEN administered, treated with Paclitaxel loaded PCL NPs (20 mg/kg i.v. in alternate days)

(6) Group 6 (D PLGA NPs) is DEN administered, treated with Paclitaxel loaded PLGA NPs (20 mg/kg i.v. in alternate days)

(7) Group 7 (D PLA NPs) is DEN administered, treated with Paclitaxel loaded PLA NPs (20 mg/kg i.v. in alternate days)

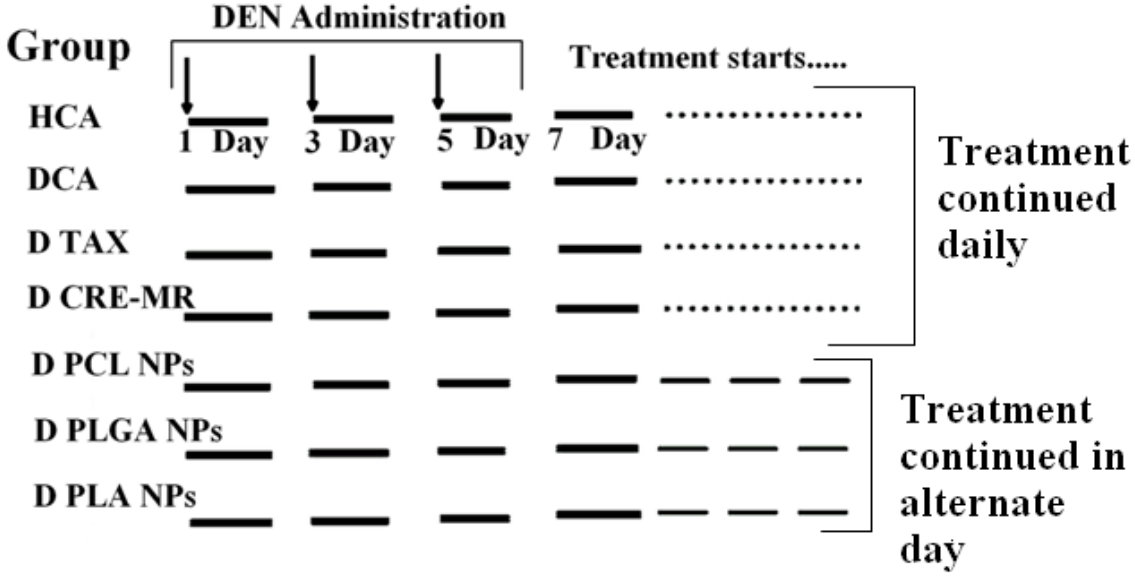


Fig. 8.1 Animal grouping and dosing schedule

8.3.3 Treatment efficiency

The treatment of hepatocellular carcinoma induced rats started on the seventh day after DEN administration on 1st, 3rd and 5th day. The survival of the rats was observed daily and the treatment and study stopped once all the animals died. During the study period, the treatment to all group were continued and the following evaluation were made.

a) Liver weight

Once the animal died during the study period, liver was collected immediately (with in 2 hr) and cleaned using PBS to remove residual blood and blotted with filter paper, then weight was measured. In all the groups last three animals were used for liver weight analysis and average weight of 3 livers were determined.

b) Construction of survival curve

The survival curve was constructed at the end of the study to check the efficacy of the PNPs. The seven treatment groups were compared in terms of, mean survival time (MST), maximal survival time (MXST) and long-term survivor (LTS). The long-term survivor was represented as the number of animals whose survival time is two times higher than the median survival time of group DCA. MST was calculated by taking mean of survival in days, of all the rats. All data were expressed as mean ± SD. The Kaplan-Meier method, using the Log-rank test (Manel-Cox test) was used to analyze the treatment efficiency and survival data. For multiple comparisons, ANOVA was followed as per Bonferroni and Dunnett test. The tests were considered significant when the P values were less than 0.05. All the statistical analysis was done by Graph Pad Prisim version 5, USA.

c) Biochemical estimation

Biochemical study was done with last 3 animals of each group. Rats were anaesthetised under mild diethyl ether to withdraw blood (<2 mL) by cardiac puncture (6). The blood sample collected in the EDTA-Na_2 containing tube was centrifuged at 4000 rpm for 10 min and plasma was separated for biochemical estimation. To evaluate the function of liver, markers like aspartate transaminase (AST), alkaline transaminase (ALT) and alkaline phosphates (ALP) were analyzed (2). The amount of AST, ALT and ALP were expressed in U/L.

d) Histopathological evaluation

The rat livers were isolated, weighed and fixed in 10 % v/v forma saline after washing with cold water. The tissue samples were dehydrated by treating with absolute alcohol followed by xylene and then embedded in paraffin block. The tissue sections were sliced using a microtome and processed for hydration for light microscope. Finally the tissue sections were stained using hematoxylin and eosin

and then mounted on the microscopic slide and fixed with cover slips using DPX mountant solution. The tissues were observed under light microscope (4X/10X/40X) for any histopathological changes.

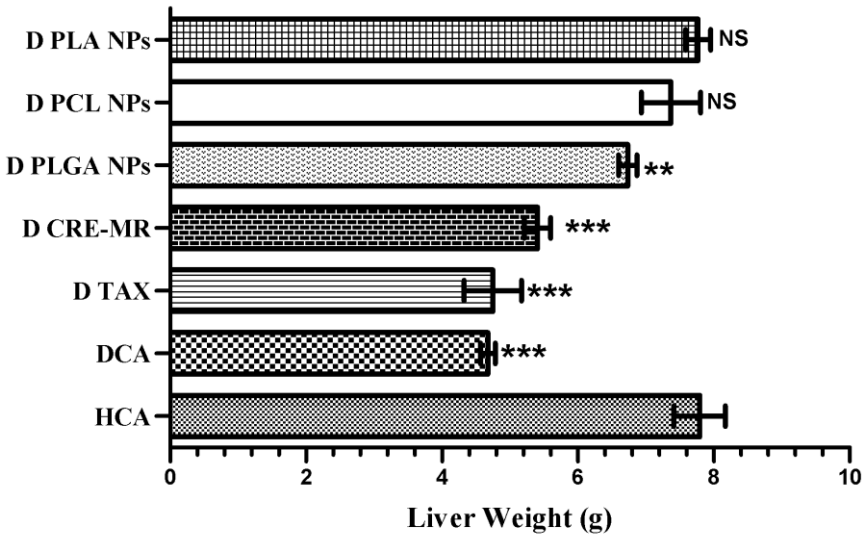
8.4 Result and Discussion

8.4.1 In-vivo anti-tumor efficacy study

a) Liver weight

This DEN model was used widely, since the histological feature of this model is super-imposable to those observed with human hepatocellular carcinoma (5-7). The average liver weight of the rats from seven different groups is represented in Fig. 8.2. There was marked decrease in weight of liver in DEN administered group (DCA), with respect to saline control, due to occurrences of hepatocellular carcinoma caused by DEN. The weight of liver was found to be higher than DCA group in all the Paclitaxel treated groups due to treatment with Paclitaxel. The average liver weight of HCA group was 7.79 ± 0.38 g and in case of DCA group it was decreased significantly (Fig. 8.2) to 4.67 ± 0.11 g. In case of Paclitaxel solution treated D TAX group, the liver weight was marginally increased to 4.75 ± 0.42 g. Liver weight in commercial formulation treated group (D CRY-MR) was recovered significantly to 5.40 ± 0.20 g.

All the three PNPs treated group showed significant increase in liver weight, 6.73 ± 0.14 g, 7.37 ± 0.44 g and 7.77 ± 0.18 g respectively in comparison to DCA and they were close to the liver weight in HCA group. Though, the treatment is made on alternative days, recovery found to much better than treatment with commercial product administered daily. This suggested that the liver damage caused by DEN was recovered better in PNPs treated group (s) animals.



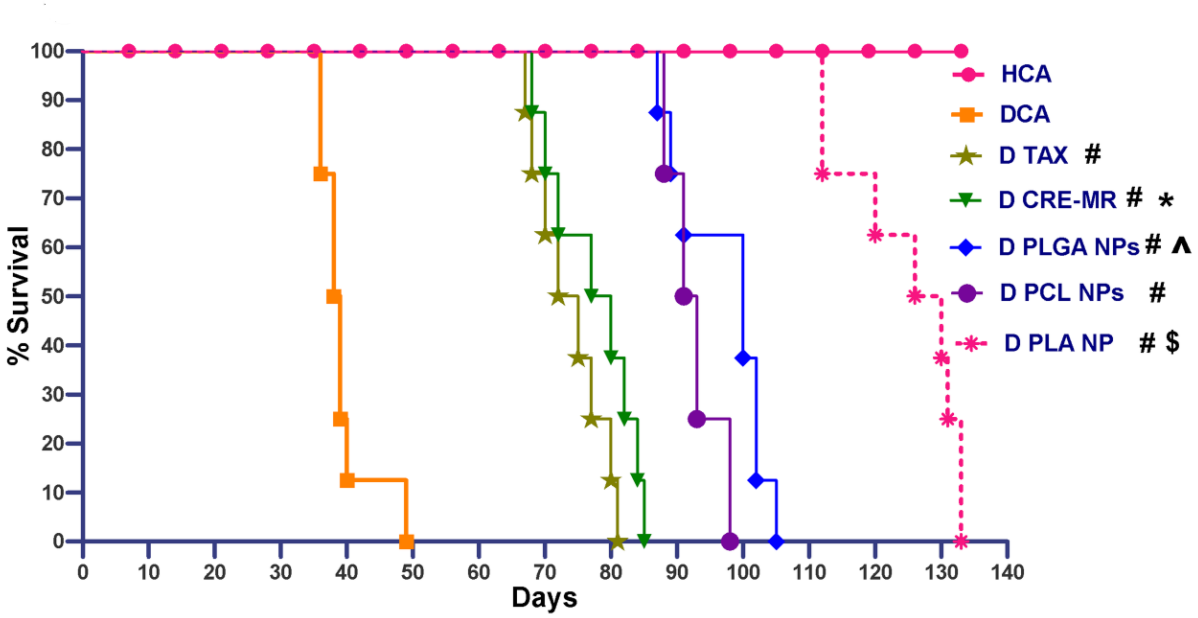
*** Extremely significant (P< 0.001), ** very significant (P between 0.001 to 0.01) and not significant NS (P> 0.05)

Fig. 8.2 Liver weight of rat from seven different groups

b) Survival study

The three PNPs treated animals showed the most significant (P<0.0001) increase in mean survival time (Fig. 8.3), MST (92.5-124.60 days) compared to the Paclitaxel solution (D TAX, 73.5 days) and commercial formulation (D CRY-MR, 78.5 days) treated groups. MST for DEN treated groups was found to be 39.38 days.

Among the PNPs treated groups, the order of increase in MST were, D PLA NPs (128 days) > D PLGA NPs (100 days) > D PCL NPs (92 days). In case of HCA group all the animals were alive even at the end of the study at 133 days. In case of treatment with D TAX and D CRE-MR the mean survival time increased to 73.75 ± 5.34 days and 77.25 ± 6.57 days respectively. No significant (P < 0.05) difference was found in MST between treatment by commercial formulation and Paclitaxel solution. PNPs treated rats showed most significant increases in mean survival time, 97.00 ± 6.89 days in PLGA NPs treated groups and 92.50 ± 3.89 days in PCL NPs and 124.6 ± 8.88 days in PLA NPs treated groups (Table 8.1).



represents that there is no significant (P<0.05) differences in the survival of rats with respect to DEN control group, * represents that there is no significant differences (P<0.05) in the survival of rats with respect to pure Paclitaxel treated group, ^ represents that there is no significant differences (P<0.05) in the survival of rats with respect to PLA NPs treated group, $ represents that there is no significant differences (P<0.05) in the survival of rats with respect to control group

Fig. 8.3 Survival curves of rats in hepatocellular carcinoma model

In all the groups, treatment with PNPs, showed narrow range of 95 % confidence interval indicating statistical significances of the study. The calculated maximum survival time (MXST) found to be 80 days and 84 days for Paclitaxel solution and commercial formulation respectively in comparison to 40 days in case of DEN treated group. But, there was no significant ($P < 0.05$) difference in the MXST value of D CRY-MR group with the D-TAX group.

The MXST was higher in all the three PNPs treated groups in comparison to D TAX and D CRE-MR groups (Table 8.1). Among the three PNPs, the highest MXST calculated to be 131 days in D PLA NPs treated group followed by 102 days for D PCL NPs group, and 98 days for D PLGA NPs. No significant ($P < 0.05$) differenced was observed in calculated MXST value in PLGA NPs treated and PCL NPs treated groups. However in case of PLA NPs treated group calculated MXST value found to be higher ($P < 0.05$) as found in MST and MEST.

Table 8.1 Statistical data of survival study of hepatocellular carcinoma rats [a]

S.NO	Treatment groups [c]	MST [b] (days)	95 % CI for MST	LTS (Nos)	MXST (days)
1	DCA	39.38 ± 4.14	35.92-42.83	-	40
2	D TAX	73.75 ± 5.34	69.29-78.21	2	80
3	D CRE-MR	77.25 ± 6.56	71.76-82.74	2	84
4	D PCL NPs	97.00 ± 6.89	91.24-102.8	3	102
5	D PLGA NPs	92.50 ± 3.89	89.25-95.75	2	98
6	D PLA NPs	124.60 ± 8.88	117.2-132.0	3	131

[a] Survival data were analyzed by the Kaplan-Meier method, using the Log-rank (Mantel-Cox) test, where the survival curves were considered as significant when the P value was <0.05. [b] Average of eight animal ± S.D. [c] All the treatment groups were significantly different with P value <0.0001

Better therapeutic efficacy of the prepared PNPs suggested that the distribution of Paclitaxel to target organ was more in NPs and extended release from NPs produced better survival results even though treatment was made in alternate days (Fig. 8.1). It is well known that efficacy of anti-cancer drugs are more depended on duration of maintaining effective concentration rather that higher concentration (C_{max}). In addition, slight negative charge and particle size of around 150 nm could be another reason for the more distribution and retention of NPs in liver for producing better therapeutic efficacy (7).

It has been showed that the NPs with size range between 70 to 200 nm are localized particularly in the liver, kidney, lungs and spleen (3, 4, 8, 9). Snehalatha et al. (9) showed that when radiolabeled free etoposide, drug loaded PLGA and PCL NPs were administered to rat, highest radioactivity was observed in the liver tissue in PLGA and PCL NPs in comparison to pure drug. However, in their study enhanced delivery of PLGA NPs to liver was found and that may due to smaller size of PLGA

NPs, (105.1 ± 2.38 nm) than the PCL NPs, (257.2 ± 3.96 nm) which helped the NPs to fenestrate the endothelial lining of the liver and the associated parenchymal cells (1, 9, 11). It has been shown that when sterically stabilized particles were administered to rats by i.v it reaches the parenchymal cells of the liver selectively, which suggest the targeting ability of NPs for liver diseases. Gaucher et al (10) showed that when Paclitaxel loaded NPs was administered to animals, higher Paclitaxel concentration was observed in the liver (1.7 fold) and spleen (100-fold) which substantiates the distinct tendency of NPs to accumulate with time inside these organs of the mononuclear phagocyte system. Result in present work also indicated better delivery of PLA NPs to liver due to smaller size (152.17 ± 2.14 nm).

Soma et al (13) showed that when Paclitaxel was administered for peritoneal malignancy in mice model, the median survival time of Paclitaxel dissolved in cremophore EL group was not different from the un-treated control mice receiving saline. However in the present study, D PLGA NPs, D PCL NPs and D PLA NPs groups showed significant increase in the survival of rats which illustrated the targeting ability of the prepared PNPs (Fig. 8.3).

c) Biochemical estimation

The hepatic injury induced by DEN was examined by estimating AST, ALT and ALP levels in blood (Fig. 8.4), which are the indicators of tumor response to therapy. In case of DCA group there was increased levels of AST, ALT and ALP suggesting liver cells damage and which result in the leakage of these enzymes into blood stream (12). Rats treated with Paclitaxel solution (D TAX) and commercial formulation (D CRY-MR) did not show any significant ($P > 0.05$) lowering of ALT and AST levels increased due to DEN. This suggested that the recovery of rats from liver cancer was there but not much in Paclitaxel solution and commercial formulation treated groups. This could be due to non-selective and lesser distribution of Paclitaxel to liver.

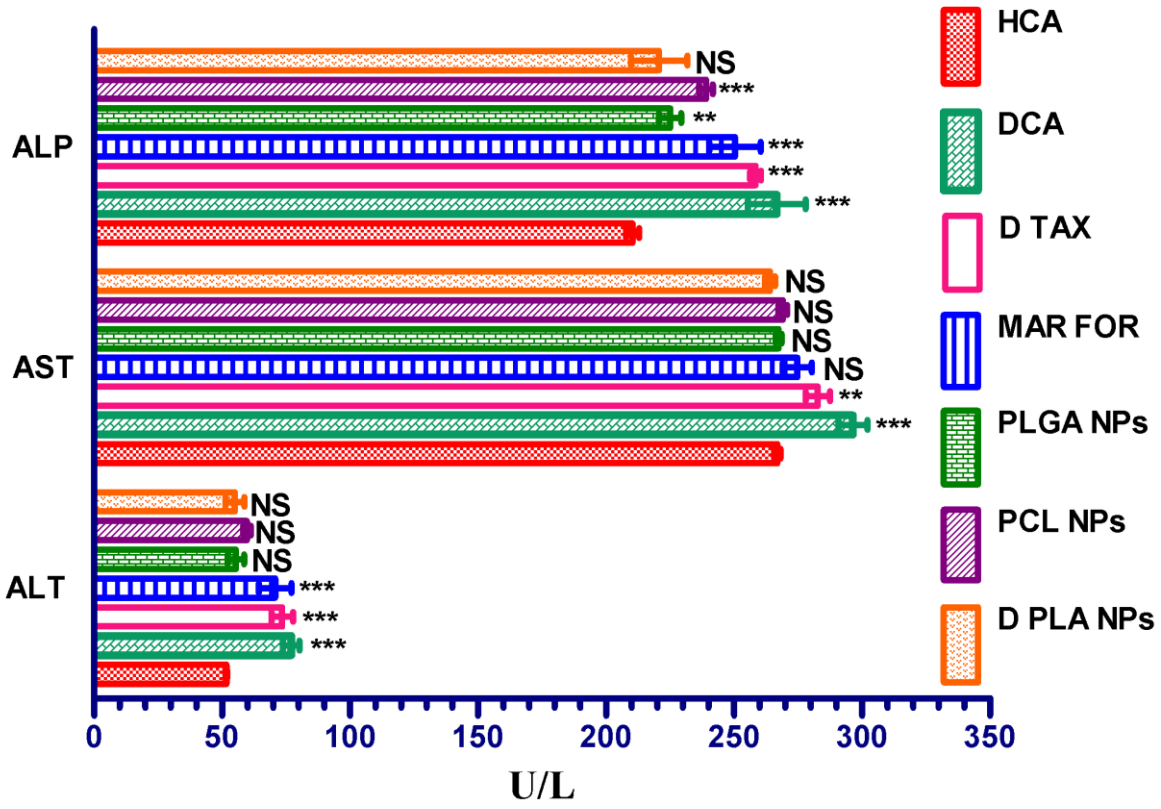
However in case of Paclitaxel loaded NPs treated groups, lowering of ALP, ALT and AST levels was significant ($P > 0.05$) in comparison to enzymes level in DCA group. These results suggested that the Paclitaxel loaded NPs treated groups liver cells are getting recovered from the cancer due to selective deliver of NPs. In D PLA NPs treated group all the three enzymes level significantly lowered (Fig. 8.4) when compared to DCA group. This could be due to healing of hepatic parenchyma cells and the regeneration of hepatocytes because of selective delivery and extended release of Paclitaxel from NPs.

d) Histopathological evaluation

The representative liver photographs and histopathology of all the seven groups was done to identify any histopathological changes in the architecture of liver cells (Fig. 8.5 and 8.6). Histopathological study of liver from HCA group animals showed normal hepatic architecture with liver parenchyma

and portal tracts (Fig. 8.6 A). In DCA group, severe changes was observed due to hepatocytic damage, fatty changes, ballooning degeneration, fibrosis and focal lymphocytic infiltration in liver (Fig. 8.6 A and B).

In animals treated with D-TAX group, there was mild lymphocyte infiltration in the hepatic lobule and the portal tracts triads were normal (Fig. 8.6 C). Animals in CRY-MR group (Fig. 8.6 D) showed almost normal hepatocytes and occasional bi-nucleate cells. Animals treated with D-PLGA NPs showed hepatocytes with focal mild fatty change (Fig. 8.6 E). Animals treated with D-PCL NPs showed liver tissue hepatocytes with regenerative activity (Fig. 8.6 F). The D-PLA NPs treated group animals showed less vacuole formation and absence of necrosis and overall no visible changes observed supplementing the protective effect (Fig. 8.6 G).



Two way ANOVA was performed to the liver function test and the significances (P<0.05) of the data was represented, extremely significant *** (P< 0.001), very significant ** (P between 0.001 to 0.01 or <0.01) and not significant NS (P> 0.05). The results of the test samples were compared to healthy control animals

Fig. 8.4 Estimation of liver markers ALP, AST and ALT in hepatocellular carcinoma study

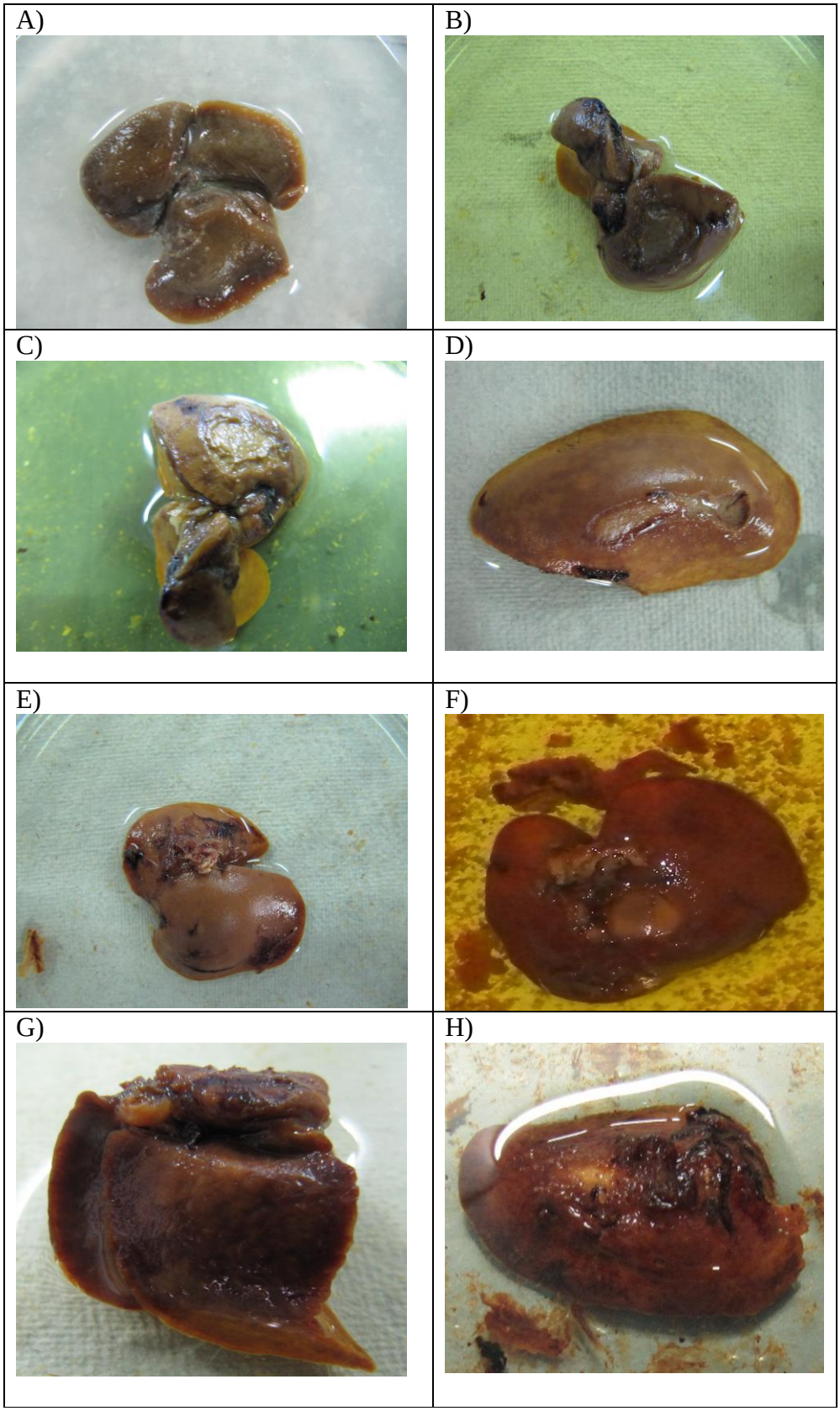


Fig. 8.5 Representative liver photos of, control rats HCA (A), DEN control DCA rats (B and C), Paclitaxel solution treated D TAX rats (D), commercial formulation D CRY-MR treated rats (E), Paclitaxel loaded NPs treated rats D PCL NPs (F), D PLGA NPs (G) and D PLA NPs (H)

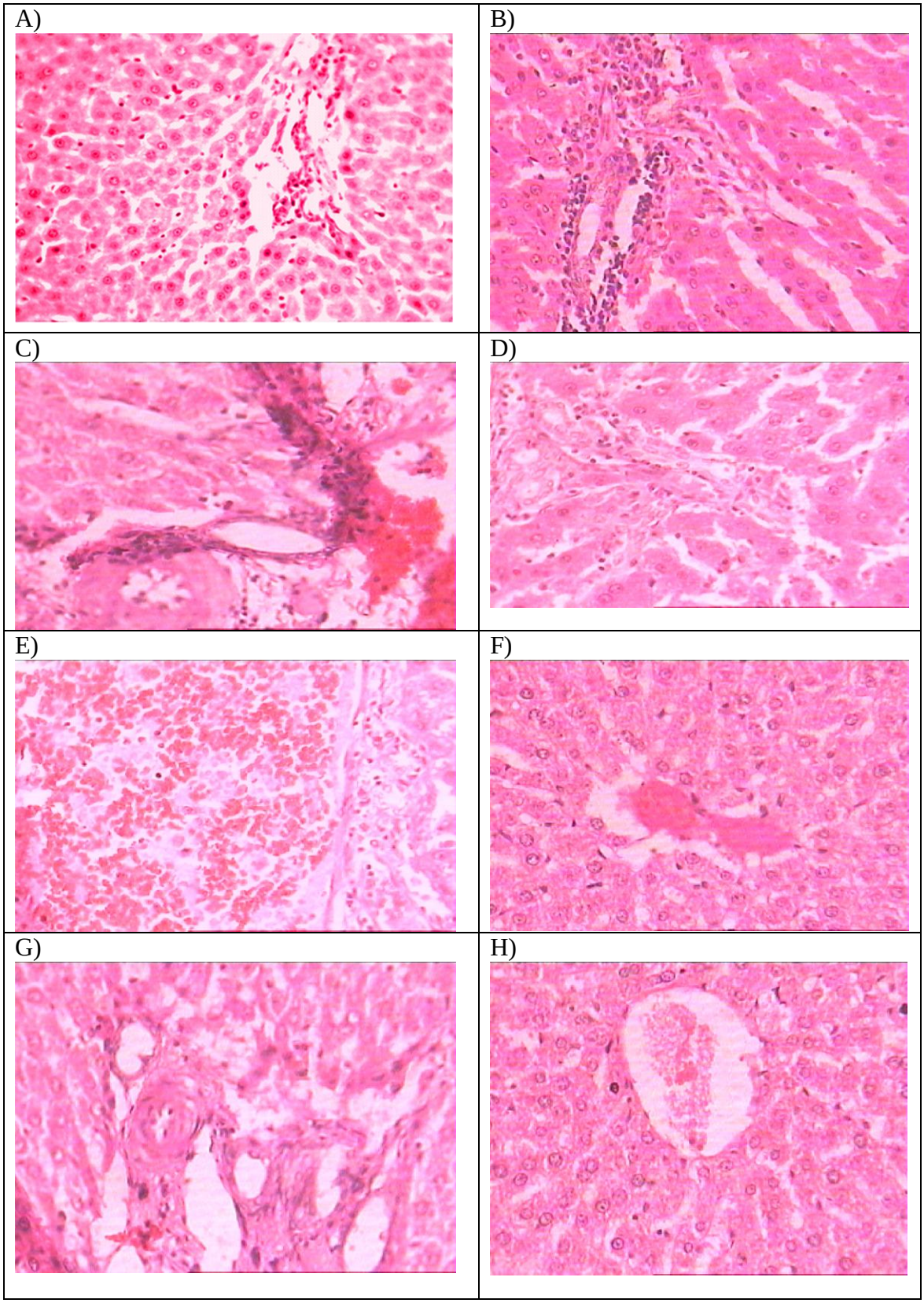


Fig. 8.6 Representative histological staining liver samples of, control rats HCA (A), DEN control DCA rats (B and C), Paclitaxel solution treated D TAX rats (D), commercial formulation D CRY-MR treated rats (E), Paclitaxel loaded NPs treated rats D PCL NPs (F), D PLGA NPs (G) and D PLA NPs (H)

8.5 Conclusion

The therapeutic efficacy of the prepared NPs in case of hepatocellular carcinoma treatment was better than the Paclitaxel solution and commercial formulation. The representative histological staining of liver samples of the PNPs treated hepatocellular carcinoma induced rats showed protective effect and there was improved therapeutic efficiency. The survival curve analysis revels, the survival of the rats was increased significantly in PNPs treated group when compared to the Paclitaxel solution and commercial formulation.

The explanation for the enhanced therapeutic efficiency of the prepared PNPs in hepatocellular carcinoma may be due to selective and effective localization of Paclitaxel at target site liver at therapeutic concentration, restricting its access to non-target areas and extended release from NPs. The therapeutic benefit of PNPs may be due to the fact that when NPs were injected by i.v route it will be taken up by the liver after few minutes due to opsonization process. It has been reported that in solid tumor there is leaky vascular blood vessels and a discontinuous endothelial cell lining which permits the entry of NPs that have limited access to normal tissue. In addition, the impaired lymphatic drainage system makes the NPs to stay in the liver and controls the release of drug from the polymer matrix (14, 15). It has been showed recently (2) that the accumulation of NPs in liver and tumor was 2.7 and 2.4 times higher than the Taxotere® respectively. Particles less than 7 µm are generally taken up by the kupffer cells in liver. Yeh et al. (16) showed that the Paclitaxel loaded gelatin NPs, size ranged from 300 to 900 nm showed longer retention and higher accumulation in the liver tissues. As the target tissue is part of the reticulocyte endothelial system, hence the initial opsonization process is advantage for the liver targeting. It has been found that the uncoated PNPs were more susceptible to hepatic uptake than the PEGylated NPs (17).

References

1. Shirakami Y, Gottesman ME, Blaner WS. (2011). Diethylnitrosamine-induced hepatocarcinogenesis is suppressed in lecithin:retinol acyltransferase-deficient mice primarily through retinoid actions immediately after carcinogen administration. Carcinogenesis, 24, 1-7.
2. Serrano ABG, Martin MA, Bravo L, Goya L, Ramos S. (2009). A diet rich in cocoa attenuates N-nitrosodiethylamine-induced liver injury in rats. Food Chem Toxicol, 47, 2499-2506.
3. Poelstra K, Prakash J, Belijaars L. (2012). Drug targeting to the diseased liver. J Control Release, 161, 188-197.

4. Saha RN, Vasanthakumar S, Benda G, Snehalatha M. (2010). Nanoparticulate drug delivery systems for chemotheraphy. Mol Membr Biol, 27, 215-223.
5. Xu Z, Chen L, Gu W, Gao Y, Lin L, Zhang Z, Xi Y, Li Y. (2009). The performance of docetaxel-loaded solid lipid nanoparticles targeted to hepatocellular carcinoma. Biomaterials, 30, 226-232.
6. Barraud L, Merle P, Soma E, Lefrancois L, Guerret S, Chevallier M, Dubernet C, Couvreur P, Trepo C, Vitvitski L, Hepatol J. (2005). Increase of doxorubicin sensitivity by doxorubicin-loading into nanoparticles for hepatocellular carcinoma cells in-vitro and in-vivo, J Hepatol, 42, 736-743.
7. He C, Hu Y, Yin L, Tang C, Yin C. (2010). Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles. Biomaterials, 31, 3657-3666.
8. Snehalatha M, Venugopal K, Saha RN. (2008). Etoposide-loaded PLGA and PCL nanoparticles I: preparation and effect of formulation variables. Drug Deliv, 15, 267-275.
9. Snehalatha M, Venugopal K, Saha RN. Babbar AK, Sharma RK. (2008). Etoposide loaded PLGA and PCL nanoparticles II: biodistribution and pharmacokinetic after radiolabeling with Tc-99m. Drug Deliv, 15, 277-287.
10. Gaucher G, Marchessault RH, Leroux JC. (2010). Polyester-based micelles and nanoparticles for the parenteral delivery of taxanes. J Control Release, 143, 2-12.
11. Danhier F, Lecouturier N, Vroman B, Jerome C, Brynaert JM, Feron O, Preat V. (2009). Paclitaxel-loaded PEGylated PLGA-based nanoparticles: in-vitro and in-vivo evaluation. J Control Release, 133, 11-17.
12. Prabha SP, Ansil PN, Nitha A, Wills PJ, Latha MS. (2012). Preventive and curative effect of methanolic extract of Gardenia gummifera Linn. f. on thioacetamide induced oxidative stress in rats. Asian Pac J Trop Dis, 2, 1-9.
13. Soma D, Kitayama J, Konno T, Ishihara K, Yamada J, Kamei T, Ishigami H, Kaisaki S, Nagawa H. (2009). Intraperitoneal administration of paclitaxel solubilized with poly(2-methacryloxyethyl phosphorylcholine-co *n*-butyl methacrylate) for peritoneal dissemination of gastric cancer. Cancer Sci, 100, 1979-1985.
14. Kim JH, Kim YS, Kim S, Park JH, Kim K, Choi K, Chung H, Jeong SY, Park RW, Kim I, Kwon IC. (2006). Hydrophobically modified glycol chitosan nanoparticles as carriers for paclitaxel. J Control Release, 111, 228-234.

15. Bardelmeijer HA, Beijnen JH, Brouwer KR, Rosing H, Nooijen WJ, Schellens JHM, van Tellingen O. (2009). Increased oral bioavailability of paclitaxel by GF120918 in mice through selective modulation of P-glycoprotein. Clin Cancer Res, 6, 4416-4421.
16. Yeh TK, Lu Ze, Wientjes MG, Au JLS. (2005). Formulating Paclitaxel in nanoparticles alters its disposition. Pharm Res, 22, 867-874.
17. Owens ED, Peppas NA. (2006). Opsonization, biodistribution and pharmacokinetics of polymer nanoparticles. Int J Pharm, 307, 93-102.

Chapter 9. Conclusion

9.1 Conclusion

Nanoparticulate drug delivery system an alternate drug delivery system with better efficacy for treating various diseases including cancer therapy. It can be beneficial to overcome the difficulties associated with cancer treatment with conventional dosage forms. In this research work, studies were carried out to design and characterize nanoparticulate delivery system to enhance the therapeutic efficacy of Paclitaxel with control release and selective distribution.

As simple, sensitive and accurate analytical methods are essential for design of any delivery systems. UV-spectrophotometric, liquid chromatographic analytical and bioanalytical methods are developed and validated. The developed methods were simple, selective, sensitive, accurate and precise in the estimation of Paclitaxel and these methods have advantages over the reported methods. The analytical methods were used for estimation of Paclitaxel in various preformulation samples, bulk and formulation samples and in in-vitro dissolution samples with accuracy. The developed bioanalytical method was used to estimate Paclitaxel in biological samples, plasma, liver, kidney and spleen for pharmacokinetic and biodistribution studies with high level of accuracy.

In preformulation studies, the Paclitaxel was characterized using XRD analysis. UV-absorption and FTIR spectrum analysis were used to identify the drug successfully. The solubility of Paclitaxel in buffered and un-buffered solution was less than 1µg/mL and not found to be influenced much by buffer system or change of pH. There was no chemical and physical interaction between drug and excipients and hence no incompatibility observed between Paclitaxel, polymer (PCL, PLGA and PLA) and stabilizer (PF 68/PVA). The stability study result showed that stability of Paclitaxel was not affected by pH change and excipients. There was rapid degradation of Paclitaxel in pH 11.00 with high degradation rate constant. The degradation character found to be of first order nature.

Paclitaxel loaded PCL, PLGA and PLA NPs were prepared by nanoprecipitation and solvent evaporation methods. Both process produced NPs with good quality, with narrow size range, high entrapment efficiency and drug content. The microscopic examination revealed the homogeneous solid matrix NPs without any amorphous arrangements. Proposed methods can be used for making quality nanoparticulate delivery system. It was observed that pure Paclitaxel got dissoluted completely with in one hour where as PNPs showed extended and biphasic release pattern, with duration of release varied from 42 to 48 hr for most of the formulations.

The in-vitro cytotoxicity and cellular uptake study of optimized Paclitaxel loaded PNPs was performed with breast cancer cells, MCF-7. Paclitaxel showed strong inhibitory effects on the proliferation of MCF-7, when it is loaded in NPs, than the pure drug and commercial formulation. The cell uptake study established efficient uptake of prepared PNPs by MCF-7 cells. This study clearly

illustrated that cells uptake of prepared NPs are much more than the pure drug and commercial formulation.

The optimized formulations were subjected to in-vivo pharmacokinetic and biodistribution study in rats. Paclitaxel loaded NPs showed multi fold increase in AUC in plasma with longer duration of higher plasma concentration when compared to Paclitaxel solution. The biodistribution study result showed increase in liver uptake of Paclitaxel loaded PNPs in comparison to Paclitaxel solution. The $t_{1/2}$ and MRT of Paclitaxel loaded NPs in liver tissue were found to be many fold higher than the pure drug Paclitaxel indicating selective distribution. Hence the Paclitaxel loaded NPs can be used for liver cancer as targeted drug delivery system for effective therapy.

The selective delivery efficiency and site specific therapeutic efficacy of the optimized Paclitaxel loaded PNPs was studied in hepatocellular carcinoma in rats. The treatment efficacy was better than efficacy of pure Paclitaxel solution and commercial formulation. The survival revels the life f rats increased significantly in treatment with NPs compared to the Paclitaxel solution and commercial formulation. Among the prepared NPs, Paclitaxel loaded PLA NPs produced maximum survival up to 131 days in comparison 80 days for pure Paclitaxel solution.

The explanation for the enhanced therapeutic efficacy of the prepared PNPs in hepatocellular carcinoma may be due to selective and effective localization of Paclitaxel at target site liver. This study showed that the prepared NPs are effective in selective delivery of Paclitaxel to liver and produced better therapeutic efficacy. The histological staining of hepatocellular carcinoma induced rat liver samples of the Paclitaxel loaded NPs treated group illustrated protective effect. It can be concluded that Paclitaxel nanoparticles can be an alternate novel delivery system for selective and better therapy for anti-cancer drugs, as evidences from the results. Selective distribution to different organs/tissue can be achieved by using different polymers or combination of polymers. However study in human patient is required to be done to establish its use for cancer chemotheraphy and commercialization.

9.2 Future scope of work

Further clinical study is required to be done in cancer patients. Also long term toxicological study is also required to be done to determine possible side effects, if any due to presences of polymer and NPs, particular in lungs. Similar study can be done for selective distribution to lungs, brain and others/tissue using different polymers. The study need not be done only for anti-cancer drug but for CNS active drugs also. Study on CNS active drugs, antibacterial, antiviral drugs can also be beneficial if selective distribution can be achieved.

Appendix

International Publication

Vasanthakumar S, Selladurai ST, Saha RN. (2009). A new, simple HPLC method for estimation of paclitaxel in micro-volume rat plasma. AAPSJ, 11, 1953.

Saha RN, **Vasanthakumar S,** Benda G, Snehalatha M. (2010). Nanoparticulate drug delivery systems for chemotheraphy. Mol Membr Biol, 27, 215-223.

Vasanthakumar S, Srinath ST, Saha RN. (2012). A simple and rapid 3D view method for selective and sensitive determination of paclitaxel in micro volume rat plasma by LC-diode array UV and its pharmacokinetic application. J Chro Sci, 50, 259-270.

Vasanthakumar S, Karthikeyan V, Rashid A, Saha RN. (2012). Determination of Paclitaxel by 3D view LC-diode array UV: its application to an *in-situ* closed loop re-circulating intestine absorption study in rats. J Liq Chromatogr R T (Accepted).

Vasanthakumar S, Nazeer Ahamad KFH, Saha RN. (2013). Nanomedicine I: in vitro and in vivo evaluation of paclitaxel loaded poly-(ε-caprolactone), poly (dl-lactide-co-glycolide) and poly (dl-lactic acid) matrix nanoparticles in wistar rats (Communicated).

Vasanthakumar S, Saha RN. (2013). Nanomedicine II: Preparation and characterization of Paclitaxel loaded poly-(ε-caprolactone), poly (dl-lactide-co-glycolide) and poly (dl-lactic acid) matrix nanoparticles: its cytotoxicity and particle uptake efficacy (Communicated).

Book Chapter

Saha RN, **Vasanthakumar S.** Nanotechnology in healthcare. ln: Gupta PD, Udupa N. editors. Nanoparticulate drug delivery system. India: S.P. Publication; 2011. 301-327.

International conferences

Vasanthakumar S, Saha RN. (2009). A new, simple HPLC method for estimation of Paclitaxel in micro-volume rat plasma. Annual Meeting and Exposition, American Association of Pharmaceutical Scientists, Los Angeles, California, USA.

Vasanthakumar S. (2011). Preparation and characterization of PF 68 coated Paclitaxel loaded poly-(ε-caprolactone), poly (dl-lactide-co-glycolide) and poly (dl-lactic acid) matrix nanoparticles. International training event for young professionals in the area of pharmaceutical technology, BASF's Days Pharma program, BASF The Chemical Company, Ludwigshafen, Germany.

National conferences and Symposium

Vasanthakumar S, Saha RN. (2010). Preparation and characterization of nanoparticles (NP) and nanocapsules (NC) for the selected chemotherapeutic agent Paclitaxel. 62nd Indian Pharmaceutical Congress, Manipal, India.

Vasanthakumar S, Saha RN. (2010). Design, characterization and evaluation of nanoparticulate drug delivery system of Paclitaxel. Contemporary Trends in Biological and Pharmaceutical Research, BITS, Pilani, India.

Biography of Vasanthakumar Sekar

Mr. Vasanthakumar Sekar has completed his bachelor degree B. Pharmacy from J.S.S. College of Pharmacy, Ooty, Tamilnadu. He has completed his post graduation, M.Pharmacy (Pharmaceutics) from, The Tamil Nadu Dr. M.G.R. Medical University, Chennai, Tamilnadu. After completing his post graduation he joined as a research scholar at BITS, Pilani, under Chair Prof. R.N. Saha for his Ph D. He has published research and review articles in renowned journals and presented papers in national and international conferences in India and abroad. During the period of Ph.D. work, he was awarded Senior Research Fellow from CSIR, New Delhi, India and he got full funding from DST, India under "Young Scientist International Travel Support" scheme to present his research findings in AAPS, Annual Meeting and Exposition, in Los Angeles, CA, USA in the year 2009. Part of his Ph.D. work has been recognized by leading pharmaceutical excipients company BASF, Germany and got opportunity with full funding to attend their international training event for young professionals in the area of pharmaceutical technology in Ludwigshafen, Germany, in the year 2011.

Biography of Chair Prof. Ranendra N. Saha

Dr. Ranendra N. Saha is Senior Professor of Pharmacy and working as Deputy Director (Research & Educational Development and Administration) in Birla Institute of Technology and Science (BITS), Pilani, India. In 2011 he has been awarded Shri B. K. Birla and Shrimati Sarala Birla Chair Professorship at BITS Pilani. He completed his Bachelor of Pharmacy and Master of Pharmacy (Pharmaceutics) degrees from Jadavpur University, Kolkata and obtained his Doctor of Philosophy degree from BITS, Pilani. Prof. Saha has more than 30 years of teaching, research and administrative experience and guided several doctoral, postgraduates and undergraduate students. He has published research articles in renowned international and national journals and presented papers in conferences India and abroad. Prof. Saha received **'Pharmacy Teacher of India of the Year- 2005'** award given by Association of Pharmaceutical Teachers of India (APTI). Dr. Saha has successfully completed several government and industry sponsored projects. He has obtained patent on novel delivery systems entitled, "Stable Controlled Release Pharmaceutical Composition Comprising Aceclofenac", which is commercialized, and brought to Indian market by Ipca Lab Ltd, Mumbai. He has visited abroad on several occasions on invitation from universities and associations to deliver lectures. He has organized several national and international conferences. He organized one International Symposium at Damascus, Syria on invitation for ASST (Arab Societies of Science and Technology, Syria).

He is also a Senate and Research Board Member of BITS, Pilani. He is on advisory board and selection committee member of a number of Universities in India and abroad. He is also member of many scientific associations and societies like Association of Pharmaceutical Teachers of India (APTI), Indian Pharmaceutical Association (IPA), Indian Society of Technical Education (ISTE), Controlled Release Society Inc., USA, American Association of Pharmaceutical Scientists (AAPS) USA, American Chemical Society USA, Indian Chapter Controlled Release Society.