SYNOPSIS
1. INTRODUCTION
Cancer is a condition of cells characterized by the loss of normal cellular growth, maturation and multiplication. Liver cancer is one of the most widespread forms of cancer and the major reason for tumor related mortality in Asia. It ranks fifth with respect to incidences of cancer in the male population and seventh in females. Globally, more than 6,00,000 cases of death per year are associated with this cancer type. Although, liver cancer is observed more commonly in the developing countries, its instances are increasing at an alarming rate throughout the world.
Hepatic cancers are a diverse group of benign and malignant cancers comprising of primary cancers which arise from hepatobiliary system and secondary cancers which metastasize to the liver from other parts of body. Liver is the second most common site for metastasis after regional lymph node. Metastasis occurs most frequently in patients with primary cancers originating in organs drained by the portal vein, including cancers of stomach, small intestine, colon, pancreas, gall bladder and extra-hepatic biliary tract.
Primary benign liver cancers include hemangiomas (cluster of abnormal blood vessels forming a swelling) and adenomas (clumps or knots of liver tissues) and hepatocellular carcinoma (liver cells). Malignant liver cancers include cholangiocarcinoma (bile ducts in the liver), angiosarcoma (blood vessels in the liver), lymphomas (immune cells in the liver) and carcinoids (hormone making cells in the liver).

2. LIMITATIONS OF CURRENT TREATMENT OPTIONS
A variety of treatment options are available at present for liver cancer which include surgery, liver transplantation, chemotherapy, transarterial chemoembolization, ethanol injection, cryosurgery, radiofrequency ablation, microwave coagulation therapy and laser thermotherapy. Liver resection is the treatment of choice for cirrhotic subjects, but holds limitations like suitability for small tumors and effectiveness in selected patients. Liver transplantation proves to be a boon for patients with tumor size of less than 5 cm, but suffers from restricted amount of donors. Ideally, cancerous cells should be exposed to suitable concentrations of drug over an extended time period. Current chemotherapy treatments are given over a short period of time with time intervals of 2-3 weeks. This causes speedy growth of tumors and thus results in weakening of the therapeutic effects of drug with untoward side effects. Also, it is seen that prolonged exposure to tumors produced better results rather than short term exposure to elevated
drug concentrations. Resistance to anticancer drugs is another aspect to be considered for chemotherapy treatment. Drug delivery to the tumors occurs through endothelial junctions, fenestrations and vesicular organelles. Factors like P-glycoprotein mediated drug efflux and changes in enzyme activities add to failure via chemotherapy. Chemoembolization, although a well established technique for localized tumors, suffers from drawbacks like patient discomfort and lack of suitable embolizing agents. Radiation therapy involves destruction of cancerous cells via x-rays or gamma rays and is associated with various problems like vomiting, diarrhea and gastrointestinal toxicity. Recurrence of disease is seen in patients on most of these treatments along with marginal improvement in survival. Hence, despite wide variety of treatments available for cancer, there is a need for a better and specific treatment option. In this context, selective delivery of drugs to tumor has become the most widely explored area in cancer research.

3. RATIONALE OF THE RESEARCH PROJECT

The prime focus of any cancer treatment is to direct the drug selectively to the desired tumor location along with reduction in side effects. In this context, nanotechnology is gaining a lot of popularity due to properties of nanocarriers like their ease of preparation, site specific delivery, reduced toxicity and greater efficacy as compared to conventional modes of therapy. Nanoparticles are solid spheres in the range of 100-1000 nm where the drug is either entrapped or encapsulated in polymer matrix. Nanoparticles loaded with anticancer agents can successfully act at cellular levels, enhancing antitumor efficacy. They can be endocytosed/ phagocytosed by cells, with resulting cell internalization of the encapsulated drug. Also small size of these colloidal carriers makes them suitable for parenteral delivery of drugs. Inherent selectivity of some drugs is low resulting in toxic effects. Nanoparticle drug delivery using polymers/lipids can solve this problem by providing a less harmful, targeted delivery of the drugs to the desired site of action. Other advantages of nanoparticles for cancer therapy include minimum drug degradation, prolonged circulation in the body, controlled release properties and high drug loading.

It is observed that various nanocarriers have the tendency to preferentially accumulate in pathological tumor areas by a process called as enhanced permeability and retention (EPR) effect. Tumor vasculature is found to be ‘leaky’ i.e. it is permeable to large molecules as well as
nanosized particles unlike vasculature of normal cells. Also, the tumor tissues are devoid of lymphatic drainage system which is responsible for flushing out macromolecules. The rapidly expanding tumor vasculature often has a discontinuous endothelium that may be several hundred nanometers large, which allows for passive targeting of nanoparticles to these tissues. Passive targeting depends on EPR effect for the nanoparticle accumulation in tumors. However, high concentrations of administered nanoparticles can accumulate at specific locations in malignant cells via active targeting. Active targeting is a term which involves coupling of ligands or other specific molecules/components with nanoparticles resulting in selective attachment to the desired receptor at the site of action. This type of targeting reduces side effects of drugs and maximizes the therapeutic outcome. An optimum targeted nanoparticulate methodology would reduce the toxicity related to antineoplastic agent, thus allowing the utilization of lower doses for treatment. The final beneficial effects could be achieved by selective recognition techniques like antigen-antibody identification and ligand-receptor attachment. There are mainly three components of an active targeted system viz. anticancer drug, carrier/polymer and the targeting ligand. Various types of molecules which are capable of selective delivery are targeting ligands, antibodies and enzymes.

Nanoparticles of biodegradable polymers exhibit long term stability and can deliver drugs to the liver via phagocytic uptake. Also, these nanoparticles can incorporate various active moieties, proteins, vaccines and biological macromolecules. Various parameters to be taken into account for final selection of the polymeric carrier include preparation conditions of nanospheres, drug-polymer compatibility, drug release pattern and desired route of administration. Hence, in order to overcome the drawbacks associated with the parenteral drug delivery of anticancer drugs and other therapies, an attempt will be made to design an alternative drug delivery system for anticancer drug in the form of nanoparticles.

4. AIMS AND OBJECTIVES

The present research work aims at developing stable, biodegradable intravenous nanoparticle based drug delivery system for an anticancer agent using suitable polymers. Optimization and characterization of the finalized formulations would be carried out to determine the physicochemical parameters and suitability of the formulations for intravenous administration. Further, the project aims at establishing the in-vitro efficacy, pharmacokinetics and in-vivo...
Synopsis

biodistribution pattern, benefits and safety to determine the potential of developed nanocarriers to treat liver cancer.

5. PLAN OF WORK
1. Literature survey on anticancer drugs, various biodegradable polymers and polymeric nanocarriers
2. Selection, procurement and standardization of drug candidate terms of solubility, pH and pKa profiles
3. Selection and evaluation of suitable polymers and excipients
5. Forced degradation studies using HPLC
6. Drug – Excipient Compatibility Studies
7. Fabrication and optimization of the polymeric nanoparticles for intravenous administration
8. Characterization of the anti-cancer agent loaded developed nanoparticles
9. Determination of in-vitro release of the drug from the developed formulations using dissolution apparatus to determine the release pattern of drug from nanoparticles
10. Sterilization of the developed intravenous formulations using gamma sterilization technique
11. Stability studies of the optimized formulations
12. In-vitro studies on HepG2 liver cancer cell line of the developed nanoparticulate intravenous formulations
13. In-vivo pharmacokinetic and biodistribution studies of the developed intravenous formulations
14. Toxicity assessment of the fabricated intravenous formulations

6. EXPERIMENTAL WORK

6.1. PART-I
a. Literature survey for identification and selection of drug and excipients: Extensive literature search was carried out from peer reviewed scientific journals, academic books,
electronic databases and patents to select a suitable drug candidate and biodegradable polymers for intravenous administration.

b. Selection of drug candidate and polymers:
   
i. **Cisplatin**: Cisplatin was selected for the present research work based upon its physicochemical properties and its utility in treatment of liver cancer.  
   **Drug profile**: Cisplatin (cis-diamminedichloroplatinum) is an inorganic compound which is widely used for the treatment of hepatoma. It has a molecular formula of $\text{Cl}_2\text{H}_2\text{N}_2\text{Pt}$ with a molecular weight of 300.1. Cisplatin has a solubility of 1 mg/ml in aqueous media and administered to the patients by intravenous route.
   
   ii. **Gelatin**: It is a biodegradable material obtained from hydrolysis of collagen which is extracted from skin and bones of animals. For the present research work, Type A gelatin (acidic) with a bloom strength of 200 was utilized for the preparation of nanoparticles.
   
   iii. **Bovine serum albumin**: Bovine serum albumin (BSA), Fraction V; is a serum albumin protein and is often used as a protein standard. It is a low cost protein with similar properties to human serum albumin and hence, it was decided to work with this polymer before proceeding with human serum albumin.
   
   iv. **Human serum albumin**: Human serum albumin (HSA) is a plasma protein with a molecular weight of 65 kDa. It a biodegradable, biocompatible, FDA approved polymer for human application and hence was selected for the present work.
   
   c. **Standardization of Cisplatin and polymers**: Cisplatin was standardized as per the monograph given in Indian Pharmacopoeia 1996. Gelatin, BSA and HSA were standardized as per respective Certificate of analysis and Handbook of pharmaceutical excipients.
   
   d. **Spectral characterization of Cisplatin by I.R and U.V spectrometry**: This was performed to confirm the identity and purity of selected drugs and to identify the $\lambda_{\text{max}}$.
   
   e. **Forced degradation studies**: Forced degradation studies were carried out to determine the stability of Cisplatin under various conditions. They were performed using HPLC analysis on Shimadzu (LC-10 AT) VP system with 20 μl loop capacity and sensitivity of 0.010 AUFS on L8 column which is aminopropyl silane chemically bonded to porous silica gel, particle size
10 μm (Spherisorb NH₂, 10μm) with 25 cm/0.4 cm column dimension. Mobile phase used was Ethyl acetate: methanol: dimethyl formamide: water:: 25:16:5:5 and the flow rate was 1 ml/min. The wavelength of detection was 310 nm and data analysis was performed using Spinchrome software. The degradation pattern of the drug under stressed conditions of 0.1 N HCl, 0.1 N NaOH, 0.1 N H₂O₂ and heat was studied using the developed HPLC method.

f. Analytical method development for estimation of drug in formulations: A colorimetric method was developed for estimation of Cisplatin in formulations for routine analysis. Briefly, Cisplatin at a concentration of 10 μg/ml was dissolved in PBS 7.4. OPDA was dissolved in dimethyl formamide at a concentration of 1.2 mg/ml solution. The drug and reagent solutions were mixed in 1:1 volume ratio (2 ml of each) and heated at 100°C for 10 min. The resulting blue coloured solution was scanned in the visible range (400-800 nm). The concentration of OPDA and time for complexation was optimized for the above method. The method was developed and validated for linearity, precision, accuracy, range, LOQ, LOD, robustness etc as per ICH guidelines.

g. Analytical Method Development for estimation of Cisplatin in plasma and organ tissues during biodistribution studies: Inductively plasma coupled atomic emission spectrometer (ICP-AES) was selected for analytical method development of Cisplatin for estimation in plasma and organ samples. The method was validated for various parameters using ICH guidelines. Briefly, for estimation of the drug in plasma and organ samples, 0.5 ml of Cisplatin solutions in the above developed range were added to 0.5 ml of plasma or 0.5 gm of organ homogenates, and the samples were digested with aqua regia (combination of HCl: HNO₃:: 3:1) under steam bath at 100°C for 2 hrs for acid digestion of CDDP to platinum. The solutions were filtered through Whatman filter paper no. 42 and analyzed for platinum content by ICP-AES.

h. Drug excipient compatibility studies: Compatibility of Cisplatin and selected excipients was investigated using Differential Scanning Calorimetry (D.S.C) by preparation of physical mixtures of the drug and excipients. Thermal behavior of drug, excipient, blank nanoparticles, physical mixture of the drug and polymer and drug loaded nanoparticles were studied using S11 nanotechnology (Seiko) DSC6220. The sample was heated under nitrogen
purging at rate of 10°C/min, in the temperature range of (-170 to 725°C). Empty pan was used as reference standard and DSC thermograms were obtained.

6.2. PART-II

a. Formulation development: The following nanoparticulate formulations were developed for intravenous administration to liver tumors:
   I. Cisplatin Gelatin nanoparticles using Type A injectable grade Gelatin for passive targeting to liver tumor
   II. Cisplatin Bovine serum albumin nanoparticles for passive targeting to liver tumor
   III. Cisplatin Human serum albumin nanoparticles for passive targeting to liver tumor
   IV. Galactosamine coupled Cisplatin human serum albumin nanoparticles for active targeting at asialoglycoprotein receptors on liver parenchymal cells
   V. TG cross-linked Cisplatin human serum albumin nanoparticles for passive targeting

b. Selection of suitable methods for preparation of protein nanoparticles: A two step desolvation technique was employed for the preparation of gelatin nanoparticles. Single step desolvation technique was used for fabrication of BSA and HSA nanoparticles.

c. Synthesis of Galactosamine coupled human serum albumin nanoparticles: The concentrations of ligand and EDC were optimized for this process. For ligand attachment, initially glycine was added to the prepared nanoparticle solutions to cap the free aldehyde groups on the surface of nanoparticles. 10 mg EDC was added separately to 10 mg of ice cold Galactosamine-PBS 7.4 solution. 9 ml of the nanoparticle solution was added to the above EDC-Gal solution and stirred at room temperature for 24 hours. Ligand attached nanoparticles were further purified by ultracentrifugation and stored at 4°C.

d. Optimization and finalization of the developed formulations: Developed formulations were optimized for parameters like polymer and drug concentration, pH value, type and amount of desolvating agent, rate of addition of desolvating agent, concentration, volume and type of cross linker. Various process parameters like stirring speed and stirring time were also optimized. Particle size and Polydispersity index (P.I) were monitored at each stage as dependent variables for the optimization process.
e. Characterization of nanoparticulate formulations:

i. Particle size, polydispersity index and zeta potential: Developed nanoparticles were characterized for mean particle size and polydispersity index using photon correlation spectroscopy using Malvern Zetasizer 3000 HAS. Zeta potential was measured by micro electrophoresis using a dip cell at a temperature of 25°C (Malvern instruments, U.K).

ii. Determination of entrapment efficiency of Cisplatin in nanoparticles: Entrapment efficiency of the developed nanoparticles was determined after ultracentrifugation at 30,000 rpm at 4°C for 1 hour. Amount of Cisplatin entrapped in the nanoparticles was calculated by subtracting the amount of free drug in the supernatant from the initial concentration of Cisplatin used in the formulation. Amount of free Cisplatin present in the supernatant was estimated using U.V spectrophotometer by a colorimetric method after reaction with OPDA at 703 nm.

iii. Determination of drug content: 1 ml of nanoparticle solution was taken and diluted to 100 ml with PBS 7.4. 10 mg of trypsin was added in this solution so that the concentration of trypsin was 1 mg/ml. This solution was heated at 37°C for 8 hours under dark for degradation of albumin nanoparticles. The solution was filtered and analyzed spectrophotometrically at 704 nm.

iv. Determination of morphology of nanoparticles by scanning electron microscopy: A drop of the nanoparticle sample was placed on a specimen holder i.e. specimen stub and kept for drying. The dried sample was then coated with platinum by high vacuum evaporation. It was then placed inside the specimen chamber.

v. Determination of morphology of nanoparticles by transmission electron microscopy: Morphology of nanoparticles was investigated using Transmission Electron Microscopy (CM200 machine PHILIPS Model) operated at voltages of 20-200 kV with a resolution of 2.4 Å. A drop of nanoparticulate solution was placed on the copper slide of Transmission electron microscopy instrument provided with carbon grids. The sample was allowed to dry on the slide, the slide was introduced into instrument and scanned under microscope for viewing of particles.

vi. X-Ray Diffraction (X.R.D) analysis: X-ray diffractometer (Philips Expertpor MPD, PANalytical Inc. Germany) with a resolution of 0.001Å was used in the present study. Vacuum grease was applied over a glass slide to stick the sample. About 10 mg of sample was sprinkled
Synopsis

over it to make a layer having a thickness of ~0.5 mm. The sample slide was placed vertically at an angle of zero degree in the sample chamber. An X-ray beam (Philips Cu target x-ray tube) was allowed to fall over the sample and was exposed to all lines ($\lambda=1.54056$). The scanning angles ranged from 5° to 40° of 2θ. The current used was 30mA and voltage 40kV using a step size of 0.2° at a scan speed of 1 s/step.

vii. Confirmation of ligand attachment on nanoparticle surface by I.R and N.M.R: Infrared (I.R) and Nuclear Magnetic Resonance (N.M.R) studies were performed to confirm the covalent attachment of galactosamine on the nanoparticles. I.R spectrums were obtained by placing a drug pellet prepared in KBr in the path of IR light and scanning them from 3500 cm$^{-1}$ to 400 cm$^{-1}$ (JASCO-FTIR- 5300). For N.M.R analysis, samples were solubilized in DMSO and analyzed on Mercury plus 300 N.M.R spectrophotometer (Varian, USA).

viii. Lyophilization of formulations for long term stability: Lyophilization of the polymeric dispersions was carried out using Martin Christ freeze dryer operated at a temperature of 4°C. The nanodispersion was mixed with various concentrations of cryoprotectants and subjected to freeze drying for 63 hours. Increase in particle size post lyophilization was determined by critically optimizing the lyophilization cycle, type and cryoprotectant concentration. Particle size and P.I of the particle pre-lyophilization and post- lyophilization were determined.

ix. Gas Chromatography analysis: Shimadzu-GC-2014 system was used for the study with helium as the carrier gas at a temperature range of 50-300°C and pressure of 20kPa. RTX-Biodiesel TG was used as the column and flame ionization detector was used. The freeze dried sample was dispersed in suitable solvent and filtered before injecting into the GC column. The retention time was recorded by the instrument as chromatograms to check the presence of residual amounts of acetone and ethanol.

x. Viscosity measurement: Viscosity of the developed formulations was determined by a Brookfield viscometer at a temperature of 36.2°C to determine their syringeability and suitability for intravenous administration.

xi. In-vitro drug release studies: For drug release studies, 3 ml of nanoparticle dispersion was placed inside a dialysis bag with molecular weight cut off of 12-14 kDa (Himedia Laboratories Pvt Ltd, Mumbai). Dialysis bag was tied and placed in a beaker containing 100 ml of phosphate
buffer saline 7.4, maintained at 37°C and stirring under 100 rpm. Dissolution medium (2 ml) was withdrawn at suitable time intervals and replaced with same volume of fresh dissolution medium. Amount of Cisplatin in the release medium was determined by a colorimetric method with o-phenylene diammine at 703 nm. All the dissolution measurements were done in triplicate.

xii. In-vitro hemolytic assay: Haemolysis was studied by incubating erythrocytes and nanoparticles in combination and the resultant dispersions were centrifuged at 3000 rpm for 10 min. The supernatant were carefully separated and the absorbances of the supernatant were recorded at 540 nm by U.V-spectrophotometric analysis (UV-JASCO Spectrophotometer). PBS treated erythrocytes and Triton X-100 treated cells were used as controls for 0% and 100% haemolysis, respectively. Interaction was determined for synthesized plain and ligand attached nanoparticles.

xiii. Sterilization of developed formulations: Formulations were sterilized by γ-radiation from Co⁶⁰ at a dose of 10 K Gy and were evaluated for physicochemical parameters and subjected to sterility testing.

6.3. PART III

In-vitro studies on HepG2 cancer cell line: HepG2 cell line is derived from the liver tissue of fifteen year old male with differentiated hepatocellular carcinoma. It is the most widely available and used cell line in liver cancer studies and hence was chosen for the present work.

a. In-vitro cytotoxicity by SRB assay: Cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. Cells were inoculated into 96 well microtiter plates in 90 μL at plating densities, depending on the doubling time of individual cell lines and the microtiter plates were incubated at 37°C, 5 % CO₂, 95 % air and 100 % relative humidity for 24 hrs prior to addition of experimental drugs. Experimental drug were solubilized with appropriate solvents and tested at concentrations ranging from 10⁻³ M to 10⁻⁷ M. After compound addition, plates were incubated at standard conditions for 48 hours and assay was terminated by the addition of cold TCA. The supernatant was discarded; the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (50 μl) at 0.4 % (w/v) in 1 % acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by
washing five times with 1% acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on an Elisa plate reader at a wavelength of 540 nm with 690 nm reference wavelength.

b. Cellular proliferation using MTT cytotoxicity assay: MTT assay was carried out using HepG2 cells/well seeded at density of 2500 and 1500 in 96-well culture plates. The cells were exposed to pure drug Cisplatin, HSA, Galactosamine HSA and HSA enzyme linked blank and Cisplatin loaded nanocarriers. Pure drug and formulations were diluted with the prepared DMEM medium at concentrations of 0.1-100 μg/ml. These plates were further incubated at 37°C for 24, 48, 72 and 96 hrs in incubator. The MTT solution at a concentration of 1 mg/ml was added to all the wells and incubated overnight. The formazan crystals formed were dissolved in DMSO and the readings were taken using ELISA plate reader at dual wavelengths of 540/690 nm respectively. IC_{50} for pure drug and formulations were calculated i.e. the concentration required to kill 50% of the cells.

c. Colony formation assay: HepG2 cells at a density of 600 cells/plate were seeded and allowed to adhere. Cells were then treated with formulations, placebo formulations and pure drug. The formulations were removed after an incubation period of 24 hr, and further incubated in the presence of complete media for a period of 8-10 days. The cells were fixed using 70% chilled methanol and were stained with 1% crystal violet. The colonies were counted and colonies containing 50 or more cells were plotted.

d. Cellular morphology in presence of Cisplatin loaded in nanoparticulate formulation: Haematoxylin-Eosin (HE) staining was performed to observe the morphological changes in presence of Cisplatin loaded in nanoparticulate formulations. The sub-confluent cells were allowed to grow on coverslips and the cells were treated with pure drug, drug loaded nanoparticulate formulations and placebo formulations. Cells were fixed using 70% chilled methanol. The coverslips stained using haematoxylin and eosin were mounted on glass slides using DPX mountant and were observed under Zeiss upright microscope.

e. Cell cycle analysis using flow cytometry: Sub-confluent cells were treated with sub-toxic concentrations of the drug and formulations. Cells were harvested, washed twice with PBS and then fixed in chilled 70% ethanol. The cell pellet was then treated with RNase (MBI Fermentas
USA) at a concentration of 0.5 mg/ml and finally stained with propidium iodide (50 μg/ml). Ten thousand events were acquired on Becton-Dickinson FACS scan and analyzed using Modfit software.

f. Cellular uptake—qualitative assessment using Confocal microscopy: The qualitative assessment of uptake of nanoparticles was performed using particles loaded with FITC (0.2 mg/mL). The sub-confluent cultures of HepG2 grown on coverslips were treated with FITC and FITC loaded nanoparticles for 2 hrs respectively. The cells were treated with DAPI and thereafter cover slips were washed thrice using PBS. The cover slips were mounted using 2.5% (DABCO) on glass slides. The acquisition was done using LSM 510 confocal microscope from Zeiss at 63X.

g. Cellular uptake—quantitative assessment using FACS: The quantitative assessment of uptake of nanoparticles was performed using particles loaded with FITC (0.2 mg/mL). The cells were grown in 35 mm culture plates till a confluency of 60-70% and were treated with FITC and FITC loaded nanoparticles for 2 hr as carried out earlier. Cells were harvested and fixing was carried out using 1% PFA. The cells were washed twice with PBS and finally suspended in the same. The cells were acquired using FACS Calibur and the analysis was done using CellQuest software.

6.4. PART IV

a. In-vivo pharmacokinetic study: For this study, male wistar rats (180-200 gm) were selected. Pure drug, marketed formulation (Cytoplatin) and nanoparticle formulations were administered via intravenous tail vein injection. Blood at time points of 0.5, 1, 2, 4, 6, 8, 24, 48 and 72 hours and was withdrawn from each animal through retro-orbital technique. Removed blood was centrifuged at 4,000 rpm for 10 mins and plasma was collected. All the samples were stored at -20°C for further analysis by ICP-AES.

b. Biodistribution study: For biodistribution study, three animals treated with different formulations under investigation were sacrificed at time point of 24, 48, 72 and 96 hrs. Major organs viz. brain, heart, lung, liver, spleen and kidney were carefully removed. These organs were thoroughly washed with PBS 7.4 and were immediately subjected to homogenization process. Approximately, 0.5 gm of the organs were homogenized and the prepared homogenate
was centrifuged at 5,000 rpm for 20 mins. The supernatant was separated and all the samples were stored at -20°C samples till further analysis by ICP-AES.

c. Toxicity studies: Acute and Repeated dose toxicity studies were performed to assess the toxicity of the administered developed nano-formulations. Histopathological studies to identify any morphological changes or gross necroscopy occurring in the cells/tissues due to the drug/formulations were assessed. Other parameters like physical parameters, hematological and bioclinical parameters were studied to assess the toxicity by the developed nanoparticles.

6.5. PART V

Stability studies: Selected nano-formulations were subjected to stability studies as per ICH guidelines. The samples were withdrawn periodically and assessed for relevant physico-chemical characteristics like particle size, polydispersity index, zeta potential and drug content.

7. RESULTS AND DISCUSSION

7.1. Preformulation studies and analytical method development

Cisplatin and selected polymers were found to comply as per the specifications given in monographs and COA’s. The identity and purity of the drug was confirmed by I.R spectroscopy. Cisplatin was found to be stable in acidic conditions and under heating and degraded in the presence of base and oxidation. Also, it was stable in presence of normal laboratory light indicating that it will not be degraded under formulation preparation conditions. Cisplatin was found to be sensitive to U.V at concentrations at or above 1 mg/ml and hence a U.V colorimetric method was developed for the same. The concentration of OPDA and time of complexation for the reaction were finalized at 1.2 mg/ml and 10 mins respectively. The method was found to be linear in the range of 1-10 µg/ml. Cisplatin could be analyzed by various methods like HPLC, atomic absorption spectroscopy and ICP-AES for estimation of drug in plasma samples. However, HPLC method mentioned in the literature involved post column derivation procedure which increased the complexity during analysis. Also, the developed HPLC method was linear in the range of 100-1000 µg/ml which was not suitable for estimation of Cisplatin in bioanalytical samples. Interference with results of plasma samples is seen in case of U.V spectroscopy. Hence, it was decided to employ ICP-AES for estimation of CDDP in plasma samples and organ tissues. The amount of aqua regia for ICP analysis was optimized at an amount of 1 ml/sample.
developed method was found to be linear in the range of 0.375-15 µg/ml. Cisplatin was found to be compatible with the excipients selected for the research work.

7.2. Formulation I: Cisplatin Type A Gelatin nanoparticles for passive targeting

Nanoparticle formulations were synthesized with injectable grade type A gelatin as the polymer. This polymer has an isoelectric pH ranging from 7-9 and these nanoparticles were synthesized at a pH value far from isoelectric pH value. Also, since Cisplatin is unstable at alkaline pH and stable at acidic pH, the selected polymer was found to be justified for the preparation of nanoparticles. A two step desolvation technique was selected for the above process due to the inherent heterogeneity (presence of both low and high molecular weight fractions) in the gelatin molecules. The prepared nanoparticles were optimized for time and speed of stirring, polymer and drug concentration, type and amount of desolvating agent and other parameters. Optimized formulation exhibited a mean particle size in the range of 150-180 nm with low P.I values ranging from 0.1-0.3. Positive zeta potential values were obtained for gelatin nanocarriers which is very typical of cationic polymers. Developed nanoparticles were found to be discrete and spherical with high entrapment efficiency and devoid of residual solvents. Trehalose (1:1) was optimized for the lyophilization process and nanoparticles showed minor increase in size and polydispersity after freeze drying process. Sustained release of Cisplatin was observed from the formulation which was further reinforced by slow killing of the liver cells with these nanoparticles as seen from SRB assay in comparison to pure drug. Pharmacokinetic study demonstrated the enhancement in half life of the pure drug with the increase in AUC seen for the sustain release nanoparticles with biodistribution to liver, lung and kidneys.

7.3. Formulation II: Cisplatin BSA nanoparticles for passive targeting to liver tumor

Bovine serum albumin possesses similar properties to human serum albumin and is relatively cheap as compared to HSA. Hence, it was decided to work with this polymer before shifting to synthesis of HSA nanoparticles. BSA nanoparticles were prepared by a desolvation process using ethanol as the desolvating agent, followed by chemical cross linking with glutaraldehyde. The prepared particles were optimized for various process and formulation parameters and finalized formulations were characterized for various parameters to determine their particle size, polydispersity, shape, entrapment efficiency and release pattern. Developed nanocarriers displayed particle size in the range of 90-300 nm with polydispersity values from 0.2-0.4. Zeta
potential indicated presence of negative charge on nanoparticle surface. Negatively charged particles are a good substrate for uptake by liver cells and other RES components. TEM analysis revealed discrete, irregular shaped nanoparticles. In-vitro studies demonstrated slow release of the drug from nanoformulation indicating its suitability for administration as a controlled release product. Increase in particle size is observed with albumin nanoparticles after storage for a long period of time. Hence, cryoprotectants in varying concentrations were tried for freeze drying process so as to obtain an optimum particle size and P.I after reconstitution. Lyophilization process was optimized using sucrose (1:5) as the cryoprotectant. Negligible change in particle size and polydispersity was seen after reconstitution of the lyophilized powder in saline as well as dextrose solution. Gas chromatographic analysis revealed absence of ethanol in freeze dried product. SRB assay revealed sufficient cytotoxicity of the formulation on HepG2 cells. Pharmacokinetic and biodistribution studies revealed low peak platinum concentrations in the plasma and overall increase in liver concentrations, the desired site of action as compared to platinol injection. Hence, due to the promising results with BSA, it was further decided to develop human serum albumin nanoparticles.

7.4. Formulation III: Cisplatin Human serum albumin nanoparticles for passive targeting to liver tumor

The major portion of the research work was focused on formulations prepared using human serum albumin. Due to its proteinaceous nature, HSA nanoparticles are naturally biodegradable and non-toxic, since they can be metabolized by natural mechanisms into harmless end products. HSA nanoparticles can be easily prepared under mild conditions by simple coacervation, or desolvation process and their size distribution can be engineered by controlling the process parameters. Cisplatin was insoluble in most of the organic solvents and hence methods like homogenization, emulsion cross linking were excluded. Lactic acid cross linking method was tried for albumin nanoparticles but suffered from reproducibility in results. Heat cross linking was successful at an albumin concentration below 1% and worked well for only for blank formulations. In the present research work, nanoparticles were produced by a desolvation technique followed by chemical cross linking. Preparation of nanoparticles was optimized for various formulation and process parameters. Polymer concentration, rate of addition of desolvating agent and glutaraldehyde concentration were found to influence the particle size,
stability and drug release to maximum extent. Mean particle size of developed nanoparticle formulation was in the range of 150-300 nm with low polydispersity values. Zeta potential of the optimized nanoparticle batch was negative indicating that the particles possess a chance for preferential liver uptake. Cisplatin entrapment efficiency in the nanoparticles was found to be in the range of 75-80%. Although Cisplatin is a hydrophilic moiety, it is known to exhibit high plasma protein binding in the blood. Hence, Cisplatin could be entrapped to a greater extent in albumin nanoparticles. Developed nanoparticles were irregular in shape with smooth surface. Cisplatin showed a sustained release pattern from albumin nanoparticles thus indicating their advantage to conventional injections in terms of reduced dosage, frequency and patient compliance. In lyophilization study, best results were obtained with 1:3 sucrose as the cryoprotectant. Sucrose is known to protect the nanoparticle against freezing and drying stresses. Other excipients also gave minor increase in particle size and P.I however, sucrose was chosen due to its additional benefit of low cost in comparison to other cryoprotectants. Absence of residual ethanol along with low viscosity of the formulation suggested its appropriateness for intravenous administration. No disturbance in the integrity of erythrocyte membrane by the nanoparticles was seen in in-vitro hemolytic assay.

Additional studies were performed on HepG2 cell line to understand the anti-proliferative effect of drug on cancer cells. The functional assessment was performed using in-vitro studies such as MTT, colony formation and cellular morphology. The cellular uptake of formulated nanoparticles was investigated using flow cytometry and confocal microscopy. Results indicate that action of Cisplatin nanoparticles on the inhibition of cell growth was time and concentration dependent which was well correlated with the in-vitro release pattern. γ-sterilization was a suitable method of sterilization for the prepared nanocarriers.

Animal studies were performed for the developed nanoparticles as per the protocol approved by Institutional Ethical Animal Committee (IAEC). Pharmacokinetic study demonstrated the enhancement in half life of the pure drug with the increase in MRT seen for the sustained release designed nanoparticles. Developed nanoparticle formulations exhibited no toxicity as seen from the histopathological analysis however, pure drug and marketed formulations displayed slight toxicity in the kidneys.
7.5. Formulation IV: Galactosamine coupled Cisplatin human serum albumin nanoparticles for active targeting at asialoglycoprotein receptors on liver parenchymal cells

It has been reported that asialoglycoprotein receptors on liver cells have receptors on their surface which can recognize sugars and sugar like compounds. Hence, for the present research work, galactosamine was selected as the targeting ligand. This ligand was coupled on surface of nanoparticles using carbodiimide chemistry. The formation of amide bond between amine of galactosamine and free carboxylic group on nanoparticles was confirmed by I.R and N.M.R. There was a slight change in the particle size and P.I after ligand loading as compared to bare nanoparticles, probably because of the low concentrations of ligand used in the reaction. Concentrations of EDC and ligand were optimized for the above reaction. *In-vitro* hemolysis assay was performed for the developed ligand coupled nanoparticles to assess their safety. Lyophilization process was optimized using sucrose as the cryoprotectant but was required in slightly higher concentration. SEM and TEM revealed smooth and spherical particles. The formulations were devoid of toxicity as seen from the histopathological analysis and had better distribution in the liver tissues as compared to other organs.

7.6. Formulation V: TG cross-linked Cisplatin human serum albumin nanoparticles for passive targeting

After applying the desolvation method for manufacturing albumin nanoparticles, particles of reproducible size, size distribution and yield could be gained. As glutaraldehyde is consumed during the cross-linking reaction and residuals are removed by particle purification, consequently no adverse effects could be observed. Nevertheless, aiming at potential future applications of albumin nanoparticles, substances like glutaraldehyde with a residual risk should be replaced with safer alternatives. It is known that a particular TG is already used as a biocatalyst to covalently cross-link proteins in food chemistry and has been utilized in the present work. Nanoparticles were optimized using formulation and process parameters and finalized formulation obtained was stable with a desired particle size and low polydispersity values. The enzyme alone did not exhibit any inherent cytotoxicity as confirmed from SRB assay. The cross linking of TG with albumin nanoparticles was confirmed by NMR. Lyophilization process was optimized using 1:3 sucrose as cryoprotectant with low viscosity values as determined from Brookfield viscometer. TEM analysis revealed perfectly spherical and smooth particles. *In-vitro*
cell line studies revealed superior action of the nanoparticles over plain drug solution. Pharmacokietic studies revealed increase in half life as compared to marketed platolin injection which is typical characteristic of controlled release preparations. No toxicity was observed for the formulation as seen from histopathological studies.

8. CONCLUSION
Nanoparticulate based drug delivery systems for treatment of liver tumors have been developed and assessed for their in-vitro and in-vivo performance using various techniques. Integrating the results and findings of the various sections of the work it could be concluded that nanoparticles possess a great potential in the area of hepatic tumor therapy.

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10. REFERENCES

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