CHAPTER 8

SUMMARY AND CONCLUSION
The research work presented in this thesis deals with the development, characterization, in-vitro and in-vivo evaluation of polymeric nanoparticles for therapy of Hepatic cancer.

Introduction

Liver cancer is one of the most common malignancies worldwide with an annual occurrence of at least one million new cases. Hepatocellular carcinoma (HCC) is one of the most severe forms of liver cancer greater than 95% incidence worldwide. Despite many available treatment options and advances in technologies, the prognosis of HCC remains poor. Hence, there is a constant need for efficient and selective mode of therapy for HCC treatment. Nanoparticulate drug delivery systems have revolutionized the therapy of cancer due to their small size, large surface area, smooth passage though the tumor interstitium, tunable properties and ability to target cancer cells by selective recognition properties. Fabrication of nanoparticles with biodegradable polymers is preferred in drug delivery due to their ease of degradation within the body, biocompatibility and safety.

The rationale of utilizing nanoparticles in HCC therapy is to maximize tumor deposition of the drug with minimum systemic toxicity. Hence, the focus of present research work was to develop nanoparticulate drug delivery systems of Cisplatin based on biodegradable polymers for the treatment of hepatoma. Attempts were made to develop patent non-infringing Cisplatin nanoformulations for targeted drug delivery to hepatocellular carcinoma. This research work primarily focused on the liver targeting ability of the nanoparticles via passive and active targeting techniques.

Chapter 1 of the thesis comprised brief information of HCC, its types, existing treatments, advantages of various nanoparticulate delivery systems viz. liposomes, polymeric nanosystems, niosomes, solid lipid nanoparticles etc, nanoparticle targeting strategies, their methods of preparation and examples of commercial nanocarrier based products. The plan and rationale of research work has been presented in Section 1.5.

Preformulation and drug-excipient compatibility studies

The preformulation studies on drug and excipients are described in Chapter 2. FDA approved, injectable grade albumin and gelatin were selected for the present work. Cisplatin and selected polymers bovine serum albumin, gelatin and human serum albumin with >98% purity and other
excipients for the study were procured, characterized as per pharmacopoeial standards and used without further purification. The identity of the polymers, drug and excipients was confirmed by D.S.C analysis and I.R spectroscopy. Drug excipient compatibility studies perfomed by I.R spectroscopy exhibited overlapping drug and polymer spectras, indicating the compatibility of Cisplatin with BSA, HSA and Gelatin.

**Analytical method development**

Analytical methods for the estimation of Cisplatin in developed formulations, dissolution medium and in biological samples are developed as mentioned in Chapter 3. An HPLC based method as mentioned in USP with minor modifications in the column was tried for estimation of Cisplatin in formulations. However, the sensitivity of this method was found to be low for estimation of the drug (Range 100-1000 µg/ml) in formulations and biological samples. Sensitivity of Cisplatin to U.V spectroscopy was also less and it was improved by complexometry with ortho-phenylenediamine. The developed method was validated for linearity, precision, accuracy, specificity, LOD and LOQ and was found to be linear and sensitive in the range of 1-10 µg/ml. The concentration of OPDA and time of complexation for the reaction were optimized at 1.2 mg/ml and 10 mins respectively.

Forced degradation studies were performed on Cisplatin by developed and validated HPLC method under conditions of heat, light, acid, base and oxidation. Cisplatin was found to be stable under laboratory light, heat & acid and degraded in basic conditions and under oxidation. However, none of the above methods could be employed for estimation of Cisplatin in plasma and organ samples.

Other analytical methods reported for Cisplatin involve liquid chromatography with post column derivatization, LC-MS, GC-MS which are either too complex or time consuming and costly for routine purpose. Hence, ICP-AES method was utilized for bioanalytical purposes based on its simplicity, high sensitivity, accuracy and ease of recovery. Cisplatin was analyzed based on its single step conversion to platinum metal with combination of strong acids like aqua regia. The developed method was validated and was linear in the range of 0.375-15 µg/ml. Hence, a simple, low cost, one step, sensitive technique was developed for bioanalytical purposes in the present research based on ICP-AES.
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Formulation development

Formulation development of Cisplatin polymeric nanoparticles has been presented in Chapter 4. BSA and HSA nanoparticles were fabricated by desolvation technique. Gelatin nanoparticles were fabricated by two step desolvation process. The main advantages offered by both these techniques include absence of surfactant for the process and reproducibility of the method.

Various formulation variables which could affect the formation and nature of nanoparticles were evaluated. Polymeric nanoparticles were prepared by varying pH values, polymer concentration, type of aqueous phase, type of desolvating agent and its rate of addition, ratio of aqueous phase: desolvating agent, type of cross linkers, concentration of cross linker and their volume, type and concentration of neutralizing agents and drug concentration. Additionally, the method of preparation was optimized using different process variables like stirring speed, stirring time and ultracentrifugation time. The effect of the above variables was explored and the formulations were optimized based on their particle size, polydispersity index, zeta potential and stability.

The pH value in desolvation was found to be a critical factor in preparation of bovine albumin nanoparticles. pH was adjusted away from the isoelectric point of all the protein based formulations for optimum size and stability. The size of nanoparticles was found to increase with increase in concentration of the polymer. BSA nanoparticles were most stable when 10 mM NaCl was utilized as the aqueous phase. Desolvation was successfully achieved with ethanol as the desolvating agent at a ratio of 1:3 and rate of addition of 1 ml/min. Optimum particle size was obtained using glutaraldehyde as the cross linker, stirred at a speed of 400 rpm as discussed in Chapter 4, Section 4.3.1.

Gelatin nanoparticle preparation was primarily influenced by the temperature during desolvation process and pH value. The time period for first desolvation was optimized at 120 seconds. Optimum nanoparticle characteristics were obtained at a temperature of 40°C, pH of 5.5; employing acetone as the desolvating agent, added at a rate of 2 ml/min at a ratio of 1:3 as described in Chapter 4, Section 4.3.2.

The particle size of human serum albumin nanoparticles were found to be comparatively higher than the BSA nanocarriers. Optimized HSA nanoparticles were obtained using ethanol as the desolvating agent at a ratio of 1:2 of aqueous: ethanolic phase at 1 ml/min using glutaraldehyde as the cross linker (Chapter 4, Section 4.3.3).
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Active targeting to hepatocellular carcinoma at asialoglycoprotein receptors was attempted in the present work using covalent linking of galactosamine ligand on surface of HSA nanoparticles using a carbodiimide methodology viz. use of water soluble EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. This process was optimized based on the amounts of EDC, galactosamine and stirring time (Chapter 4, Section 4.3.4).

Novel TG-linked albumin nanoparticles were proposed in the present work as a replacement for the toxic cross linker glutaraldehyde. The main parameters influencing formulation of TG nanocarriers were TG concentration, temperature and type of medium (Chapter 4, Section 4.3.5).

Characterization of nanoparticles

Physicochemical characterization of the optimized polymeric nanoparticles was performed as mentioned in Chapter 5. The optimized BSA nanoparticles were found to exhibit a mean particle size of 238.3 nm with a P.I value of 0.281, zeta potential of -24 mV and 70.22% entrapment efficiency. High entrapment efficiency was achieved in the present study owing to the potential of plasma protein binding of Cisplatin. D.S.C and X.R.D analysis confirmed the entrapment of drug in nanoparticles. S.E.M and T.E.M studies revealed presence of smooth, irregular and discrete particles with potential release of drug for 48 hours. BSA nanoparticles exhibited non-Fickian type of release for Cisplatin. The freeze drying process was optimized with 1:3 ratio of formulation: sucrose and there was slight increase in particle size and polydispersity after reconstitution with dextrose and saline. Viscosity of the developed formulation was found to be 1.88 cps, indicating suitability for i.v administration.

The particle size and polydispersity index of Gelatin nanoparticles was around 161 nm with polydispersity of 0.084 and positive zeta potential of 11.2 mV. Higher entrapment efficiency of 80.22% was observed with this polymer due to the ligand exchange reaction between the chloride groups of Cisplatin and carboxylic moieties of Cisplatin. Smooth, spherical particles with a 72 hour release profile were seen for CDDP gelatin nanocarriers. Lyophilization process was optimized with 1:1 ratio of nanoparticle dispersion: trehalose which gave a free flowing powder, demonstrating slight increase in size and P.I post lyophilization.

Human serum albumin nanoparticles exhibited particle size in the range of 150-300 nm with low polydispersity values of 0.115, indicating homogeneity in the formed nanocarriers. Negative zeta potential of -17 mV was seen for this formulation. Entrapment of Cisplatin in albumin matrix
was verified by D.S.C and X.R.D analysis. Around 60% of Cisplatin was released from the nanoparticles within 15 hours, followed by slow release thereafter for 96 hours due to the covalent bonding of nanoparticles with the crosslinker, wherein the release profile exhibited Higuchi kinetics. Minor increase in size and P.I of the optimized formulations of human serum albumin was seen employing 1:3 ratio of formulation: cryoprotectant. Human serum albumin was found to be superior to the developed BSA and gelatin nanoparticles because of the higher entrapment efficiency of Cisplatin.

Upon attachment of galactosamine on the surface of HSA nanoparticles, the particle size was found to be 277.2 nm with P.I of 0.129 and zeta value of -29.4 mV. The presence of amide bond formed between the ligand and the polymer was confirmed by the characteristic C=O stretching at 1600 cm⁻¹ and N-H bending at 1520 cm⁻¹. In the NMR spectra, N-H stretching at 3400 cm⁻¹ confirmed the formation of amide bond. The presence of amide bond from the NMR spectrum was verified by the presence of single amide peak in the form of a doublet at 5.8 ppm (Section 5.3.13).

TG was employed as a substitute for the toxic glutaraldehyde in the present research work. The absence of inherent cytotoxicity of TG was confirmed by SRB assay. The secondary amide formation between the carboxyamide group of TG and amino group of lysine of albumin was estimated by NMR spectroscopy at 5 ppm. Particle size of TG anchored nanoparticles was higher than all the synthesized protein nanoparticles. Optimum particle size was 310 nm with P.I of 0.17 and zeta potential ranging from -20 to -30 mV. Scanning and transmission electron microscopy revealed the presence of smooth, discrete, perfectly spherical nanoparticles with entrapment in the range of 80-90%. Cisplatin was found to release from these nanoparticles for 72 hours, following Higuchi kinetics. Trehalose (1:1) was found to be the optimized cryoprotectant for the lyophilization process.

Nanoparticles were sterilized by gamma radiation (25 kGy from Co⁶⁰) for a period of 4 hours. The effect of sterilization process on the physicochemical properties of nanocarriers was assessed and the sterility testing was performed as per Indian pharmacopoeia. The optimized radiation dose was found to be 10 kGy with insignificant changes in nanocarriers after sterilization.
Optimized formulations were subjected to stability testing as per ICH Q1A R2 guidelines under refrigeration for 6 months and under accelerated conditions for 3 months. Samples were withdrawn at regular intervals and evaluated for physical appearance, pH, particle size, polydispersity and drug content. No significant changes were observed in the physical properties of nanoparticles under refrigeration, however changes were prominently observed at room temperature. The results of stability studies indicated that refrigeration at 5°C ± 3°C was the ideal conditions for storage of nanoparticulate formulations.

**In-vitro liver cell line and biocompatibility studies**

Chapter 6 explored the in-vitro efficacy of the optimized formulations against HepG2 human liver cancer cell line using different in-vitro cell line assays. Cisplatin BSA nanoparticles exhibited slow cytotoxicity on HepG2 cell lines (49.5 μM) due to the sustained release of the Cisplatin from developed nanoparticles in SRB assay as compared to Cisplatin alone (3.6 μM). Blank nanoparticles did not exhibit any cytotoxicity (>100 μM), indicating the absence of toxicity due to the formulation excipients per se. Cisplatin gelatin nanoparticles also exhibited a high value of GI50 than the pristine drug in HepG2 cells during SRB assay (18.6 μM), attributed to the sustained release of Cisplatin from gelatin matrix.

Human serum albumin nanoparticles developed in the present work were tested against free Cisplatin in order to determine their efficacy in human hepatocellular carcinoma cell line HepG2. All the tested formulations were found to exhibit time and concentration dependent cytotoxicity on HepG2 cells with significantly less (p<0.05) IC50 values for HepG2 cell line as compared to B16F10 murine melanoma cell line. This was substantial in proving the specificity of albumin nanoparticles towards hepatocellular carcinoma, rather than melanoma, due to which the cytotoxic action of formulations was more prominent on HepG2 cell line as compared to the B16F10 murine melanoma cell line.

In colony formation assay, Cisplatin prevented the anchorage independent growth of HepG2 cells which was evident by the dose dependent decrease in number of colonies in drug administered and formulation groups. Also, it was seen that both the drug and drug loaded nanoformulations caused changes in the cell structure, swelling of cells, spindle formation and consequent cell damage with equivalent efficacy in Leighton tube assay. Developed human
albumin nanoparticles were found to be superior to pure drug by acting on different phases of cell cycle viz. G0-G1, S phase and G2/M phase.

In both qualitative and quantitative cell uptake studies, it was observed that the uptake of ligand attached nanoparticles (Formulation D) was more in HepG2 cells as compared to plain albumin nanoparticles. The uptake of nanocarrier formulations in HepG2 and B16F10 cancer cell lines were found to be in the following order:

B16F10 melanoma < HepG2 cells
Plain albumin nanoparticles < Ligand attached nanocarriers

In-vivo studies on developed formulations

Chapter 7 demonstrated the in-vivo performance of formulated polymeric nanoparticles. Pharmacokinetic profiles and organ distribution studies after intravenous administration were carried out using Albino rats, Wistar strain. These studies were performed as per the protocol number IAEC/CUSCP/33/2012-13, S.N.D.T Women's University, Mumbai. Cisplatin nanoparticles equivalent to the therapeutic dose were administered to rats. Plasma and organ samples were collected at different time intervals and platinum concentration was analyzed by ICP-AES method. The safety studies on developed formulations were carried out and the formulations were subjected to single and repeated dose toxicity in wistar rats. Rats were observed for mortality, behavioral and weight changes. Complete blood analysis, serum biochemistry and histopathological examination of major organs were performed. The obtained results were subjected to suitable statistical analysis using the software Graphpad prism 5 (version 5.04).

It was observed that the pharmacokinetic parameters were significantly different for Cisplatin when formulated into nanoparticles ($p<0.05$), tested at 95% confidence interval (CI) as compared to commercial injection (Section 7.4.2). It was observed that the peak Cisplatin concentration after intravenous administration was lower ($6.1 \pm 2.78$ µg/ml) from BSA nanoparticles than that of the marketed injection ($9.612 \pm 3.34$ µg/ml). In case of BSA nanoparticles, the drug levels were seen to reduce after the $C_{max}$ was attained and found to be maintained upto 48 hours. The difference in the pharmacokinetic parameters was attributed to the slow diffusion of the drug from the developed bovine albumin formulations over a period of 48
hrs. Also, there was 4 times rise in the AUC and AUMC with reduced clearance and MRT of 11 hours, indicating higher bioavailability of the formulation and reduced toxicity of Cisplatin.

Similarly, the half life of Cisplatin from gelatin and plain human albumin nanoparticle and TG linked formulations were found to increase due to the slow release of drug from nanoparticles. This was accompanied by increase in AUC, MRT and lowering of clearance of drug from these formulations.

The values of area under the concentration-versus-time curve (AUC_{0\rightarrow\infty}), mean residence time (MRT) and half life of galactosamine human albumin nanoparticles were similar to that of human albumin nanocarriers without the ligand \((p>0.05)\), suggesting that the formulations behave identical despite the presence of ligand. This could be attributed to the fact that targeted nanoparticles generally do not lead to alteration in ADME parameters but rather assist in the internalization of nanoparticles in the tumor cells upon extravasation.

In biodistribution studies, it was seen that Cisplatin was distributed throughout the body after intravenous administration; however its concentration was higher in liver as compared to all the organs from both the formulations. Cisplatin was also distributed to kidneys and lungs after intravenous administration from commercial injection. The concentration of Cisplatin in heart, brain and spleen were found to be lower for both the formulations, although some amount of commercial formulation reached the brain. Hence, these results signify that administration of pure solution would result in unwanted toxicity to other organs apart from liver and thus concurrent side effects. Commercial platinol injection was found to be superior to pure solution in terms of distribution to hepatic tissue.

BSA and gelatin nanoparticles were found to be non-specifically distributed to other organs apart from liver viz. lungs and kidneys. However, the amount of these formulations was reduced in the kidneys as compared to pure drug solution, suggesting reduced nephrotoxic potential of Cisplatin when formulated into nanocarriers.

High liver concentration of Cisplatin was achieved by the targeted galactosamine nanoparticles and was significantly elevated as compared to all other organs \((p<0.05)\). There was no significant difference between the nanoparticle concentrations in the liver for non-targeted as well as targeted formulations at 48 hours. However, this difference was significant for Cisplatin concentrations at 72 and 96 hours from both the formulations. This could be related to the better
uptake, internalization and retention of the ligand attached nanoparticles in the ASGPR sugar receptor and hence would lead to selective action of the anticancer agent on hepatocellular carcinoma with reduced non-specificity to other organs in the body (Section 7.4.2).

Pharmacokinetic studies also indicated that HSA-TG nanoparticles could serve as non-toxic alternative to glutaraldehyde linked nanoparticles by presenting high liver concentrations for treatment of hepatoma.

Acute and repeated dose toxicity studies were performed on developed human albumin nanoparticles. Pristine Cisplatin and commercial platinol injection exhibited toxicity by causing changes in the hematological, biochemical and histopathological parameters in both the studies. However, these abnormalities were not recorded for animals treated with fabricated CDDP loaded human serum albumin nanoformulations on acute and repeated administration. Hence, the results of in-vivo studies indicated potential of HSA nanocarriers in liver cancer treatment.

Various polymeric nanoparticulate based drug delivery systems for hepatocellular carcinoma have been developed in the present research project and have been proven to be effective in hepatoma treatment by various in-vitro and in-vivo manifestations. Hence, from the results and findings of this work, it can be concluded that developed nanoparticulate systems could act as potential treatment options for HCC.

**Highlights of the research work**

Surfactant free, polymeric nanoparticles were developed in the present research work with enhanced efficacy in hepatocellular carcinoma cell line HepG2. Novel Transglutaminase cross linked nanocarriers were successfully developed and tested against HepG2 cell line as an alternative to toxic glutaraldehyde linked nanoparticles.

**Future perspectives**

- Scale up studies at intermediate and industrial level to ensure easy transfer of technology from laboratory scale to industrial setup.
- Therapeutic efficacy of the developed polymeric nanoparticles in liver tumors and extrapolation of the same at clinical level in patients.
- Estimation of cellular targeting of the developed nanoparticulate formulations.