4.1 RESULTS AND DISCUSSION

The drug identification studies suggested that drug supplied by Khandelwal Laboratories Mumbai, (India) matches with the standards prescribed in official books for identity and purity.

Identification studies of drug cisplatin indicated that the supplied drug matching the standard as prescribed in Flory, (1973). I.P. procedures were adopted for the identification of drug. U.V. absorption maximum of drug was found to be 301nm (Fig.3.1.1) which conforms for cisplatin. An IR spectrum of the drug was found to be concordant with the reference spectra (Table 3.1.4). Solubility study in different solvents at room temperature revealed that the drug was insoluble in organic solvents, slightly soluble in distilled water and soluble in DMF and freely soluble in DMSO.

Partition coefficient of cisplatin was also revealed its hydrophilic nature as it is found to be 0.179 in n-octanol/water system and 0.234 in n-octanol/PBS pH 7.4. The spectrophotometric method of analysis reported by Cafaggi et al., (1997) was used to estimate the drug in various working samples. The cisplatin was estimated in 0.1 N HCl at 301nm while in water and PBS pH 7.4 it was estimated after developing a chromophore with a mixture of 1 ml each of 4M HCl- 0.4M SnCl₂ and measured absorbance at λ max 398 nm (Fig. no.3.1.1). The data of standard curve was plotted and calculated the value of correlation coefficient i.e. over 0.9 which indicating good linearity between absorbance and drug concentration ranged 2-20µg/ml. The data of standard curve of Cisplatin in PBS (pH 7.4) was linearly regressed and its correlation coefficient was also found to be over 0.9 in the range of 2-20 µg/ml.

The HPLC method reported by Kinoshita et al., (1990) was used to estimate cisplatin in serum. A good linearity was observed between drug concentration (5-500ng/ml) and peak area, as the correlation coefficient was found to be greater than 0.9 (Table 3.1.7 & Fig. 3.1.5)
The compatibility of the drug with ligands (Folic acid, Heparin, Galactose) and Polymer (PLGA, PEG and mPEG-PLGA) was analyzed spectrophotometrically. It was found that drug is compatible with both (ligands and polymers) as no significant change in absorption maxima was observed in any case (Table no.3.1.8).

Different PLGA, PEGylated and, ligand anchored NPs (PEGyNPs, GPNPs, FPNPs and, HPNPs) were prepared by using solvent evaporation method reported by (Patil et al., 2008). This method provides small NPs with spherical shape. Various formulations and process variables i.e. concentration of polymer (PLGA, mPEG-PLGA, Folate-PEG-PLGA, Galactose-PEG-PLGA and, Heparin-PEG-PLGA), drug :PLGA ratio, surfactant concentration, sonication time and, stirring time were optimized by varying one variable at a time and keeping others constant and change in their variable on the characterization of NPs was studied.

The PLGA NPs were prepared by optimizing different concentration of PLGA polymer in respect to particle size of NPs as well as their polydispersity index were determined. It is observed that on increasing the concentration of PLGA (0.5-4% w/v) the size of NPs increased from 152±1.4 to 299±2.6 nm (Table 3.4.2.1, Fig. 3.4.2.1). This could be due to formation of concentrate solution at the higher concentration of polymer which may decrease the shear stress and thus results in increased particle size. The 1 % w/v concentration of PLGA was found to be optimum because it has appropriate sized range (171±1.2 nm) NPs with minimum polydispersity index (0.18).

The PEGyNPs were optimized by varying different concentration of polymer (mPEG-PLGA) in respect to particle size of NPs as well as their polydispersity index were determined. It is observed that on increasing the concentration of mPEG-PLGA (0.5-4% w/v) the size of NPs increased from 162±1.8 to 310±2.82 nm (Table 3.4.2.2, Fig. 3.4.2.2). This could be due to formation of concentrate solution at the higher concentration of polymer which may decrease the shear stress and thus results in increased particle size. The 1 % w/v concentration of mPEG-PLGA was resulted an optimum because it have appropriate sized range (180±1.4 nm) NPs with minimum polydispersity index (0.29). The results were almost similar with PLGA NPs only
PEGyNPs shows higher particle size in all polymer concentration that might be due to presence of more polymer core as compared to PLGA polymer. Excess PEG orientation linked with PLGA increases its polymer concentration and for that particle size was also found increased.

After optimizing the polymer concentration of PLGA and, mPEG-PLGA copolymer, different conjugated copolymers (Folate-PEG-PLGA, Galactose-PEG-PLGA and Heparin-PEG-PLGA) were also optimized in respect of their particle size and polydispersity index. It was found that increase of polymer concentration increases the particle size might be due to decrease of shear stress. Optimized polymer concentration in the case of Galactose-PEG-PLGA copolymer was found to be 1% w/v as it gives optimum particle size of 185±1.83 nm with least polydispersity index (0.21). Whereas for Heparin-PEG-PLGA copolymer and Folate-PEG-PLGA copolymer similar results were obtained. Optimum polymer concentration of Heparin-PEG-PLGA copolymer & Folate-PEG-PLGA copolymer was found to be 1% w/v as it gives optimum particle size of 189±1.39 & 191±1.4 nm with least polydispersity index of 0.22 & 0.20 respectively. Increase in particle size observed in all PEGylated, anchored nanoparticles result were might be same as stated earlier that the availability of more polymer concentration (PLGA, PEG and ligand) increases the particle size.

After optimization of the concentration of polymer, the drugs optimum concentration was optimized by varying its concentration from 50 to 250 (µg/ml). It is noticed that in the case of PLGA NPs maximum amount of drug loaded to the polymer matrix is when drug concentration was 200 (µg/ml) having good entrapment efficiency of 78.8±0.4 & optimum particle size of 169.5±1.6 nm. It was concluded that the size of NPs was increased as an increase of drug concentration on polymer matrix (Table 3.4.3.1, Fig. 3.4.3.1). In case of PEGyNPs the optimum drug concentration was also found to be 200 (µg/ml) as it gives optimum particle size of 184±1.54 with optimum entrapment efficiency of 85.6±1.32. Result conform slight increase in particle size and in drug entrapment. The probable reason will be availability of more polymeric matrix through that more drugs was entrapped (Table 3.4.3.2, Fig 3.4.3.2). Similar results were obtained in the case of GPNPs, HPNPs and FPNPs, in each
Observation, Result & Discussion

case optimum drug concentration was found to be 200 (µg/ml). GPNPs & HPNPs gives optimum entrapment efficiency of 80.1±1.03 nm & 81±1.02 nm respectively whereas FPNPs gives optimum entrapment efficiency of 79.6±0.51 nm. In the case of conjugated copolymer it was clear that increase of drug concentration slightly increases the particle size of nanoparticles too (Table 3.4.3.3 to 3.4.3.5, Fig 3.4.3.3 to 3.4.3.5).

The effect of surfactant concentration was also optimized ranging from 0.5 to 5 % w/v (PVA), it resulted that 1% concentration of surfactant was optimum for giving NPs (PLGA NPs, PEGyNPs, GPNPs, HPNPs and FPNPs) having smaller size and with good entrapment efficiency. It was concluded that 0.5 % surfactant concentration results bigger particle size than 1 % and less Entrapment efficiency as compared to 1 %, the reason behind this is 0.5 % concentration of surfactant was not sufficient to reduce the surface tension of the particle and thus they might aggregated to each other and results in bigger particles. As further increase of surfactant concentration results in a decrease in particle size but the entrapment was also decreased on that. Further increase of PVA concentration results in bigger particle size might be due to increase of viscosity of aqueous phase due to surfactant which results in decrease in shear stress and thus increase in size. Further increase of surfactant concentration from 2.5 to 5 % results in increase in particle size but decrease in entrapment efficiency that might be due to increase of surfactant concentration increases the viscosity and thus decrease in shear stress which results increase in particle size, but the increase of surfactant concentration might causes the increase in partitioning of Hydrophilic drug from polymer matrix to aqueous environment (Table 3.4.4.1 to 3.4.4.5, Fig 3.4.4.1 to 3.4.4.5), thus results in low Entrapment efficiency. So 1 % w/v concentration of surfactant was found optimum to give optimum particle size with optimum entrapment efficiency. The 1 % w/v concentration of surfactant in PLGA NPs gives optimum particle size of 168±1.58 nm with optimum entrapment efficiency of 78±0.05. 1 % w/v optimum surfactant concentration in the case of PEGyNPs gives optimum particle size of 176±1.46 with optimum entrapment efficiency of 84±2. 1 % w/v concentration of surfactant was found to be optimum for GPNPs, HPNPs and FPNPs as it gives optimum particle size of 176±1.53, 179±1.72 and 182±1.67 respectively and optimum entrapment efficiency was found to be 80±2.41, 81±2.01 and 80±1.01 respectively.
Order of particle size and entrapment efficiency was found to be –

\[
\text{PLGA NPs} < \text{PEGyNPs} < \text{HPNPs} < \text{GPNPs} < \text{FPNPs}
\]

The reason for coming particle size and entrapment efficiency in this order will be availability of large polymer matrix to form NPs and to entrap drug.

The sonication time was optimized from 30 sec. to 150 sec. The results conforms that the increase in sonication time results in smaller particle size but decreases the entrapment efficiency too, the probable reason was entrapment efficiency is good in large polymer matrix, due to availability of large matrix on which drug get entrapped on that and thus results in good entrapment efficiency (Table 3.4.5.1 to 3.4.5.5, Fig 3.4.5.1 to 3.4.5.5). The increase in sonication time of nanoparticles (PLGA NPs, PEGyNPs, GPNPs, HPNPs and FPNPs) results in decrease in particle size as well as entrapment efficiency, 120 sec sonication time was optimized for nanoparticles as it gives optimum particle size and entrapment efficiency. 120 second sonication time was found to be optimized for PLGA NPs as it gives optimum particle size of 170±6.5 nm and optimum entrapment efficiency of 77.9±2.3 whereas in the case of PEGyNPs optimum particle size was found to be 178±4.01 nm with optimum entrapment efficiency of 79.2±1.63. Similar decrease in particle size and entrapment efficiency was also found in the case of GPNPs, HPNPs and FPNPs, optimum particle size was found to be 179±1.2 nm, 179±2.7 and 181±4.2 nm respectively with optimum entrapment efficiency 78.4±1.6, 78.9±1.8 and 78.9±3.1 respectively.

After that stirring time was also optimized from 4 to 7 Hrs which conforms that the 6 Hrs stirring time results in smaller particles having good entrapment efficiency. The reduction in stirring time results in increase in particle size probably due to obstruction in complete removal of organic phase solvent the polymer and thus it increases the particle size (Table 3.4.6.1 to 3.4.6.5, Fig 3.4.6.1 to 3.4.6.5) above 6 hrs stirring slight or no reduction in particle size and entrapment efficiency were observed. Due to this observation 6 hrs stirring was found to be optimum for NPs.

Stirring time was optimized according to particle size and entrapment efficiency for PLGA NPs it was found that 6 Hrs stirring results in good particle size (171±1.6 nm)
and good entrapment efficiency (79.8±2.4) (Table 3.4.6.1, Fig 3.4.6.1). Optimum stirring time for PEGyNPs was also found to be 6 hrs as it gives optimum particle size of 179±1.02 nm with good entrapment efficiency of 79±1.96. Stirring time was also optimized for GPNPs, HPNPs and FPNPs results conforms that 6 hrs stirring time gives optimum particle size of 180±1.63 nm, 182±1.24 nm and 181±2.5 nm respectively whereas optimum entrapment efficiency was found to be 77.9±1.66, 78.4±1.31 and 78.6±1.2 respectively.

These prepared nanoparticles (PLGA NPs, PEGyNPs, GPNPs, HPNPs and FPNPs) were characterized for shape and surface morphology, particle size, drug entrapment efficiency and, for Zeta potential. The SEM and TEM photomicrograph exhibit that NPs are spherical in shape but the surface of PEGyNPs, GPNPs, HPNPs and FPNPs is less smooth and PEG chains orientation was clear on that as compared to plain PLGA NPs (Fig 3.4.7.1, 3.4.7.2, 3.4.7.3, 3.4.7.4, 3.4.7.5 for SEM and 3.4.7.6, 3.4.7.7, 3.4.7.8, 3.4.7.9, 3.4.7.10 for TEM). The average size of all NPs was found to be less than 200 nm. The TEM photomicrograph shows the increase in size of PEGyNPs, GPNPs, HPNPs and FPNPs nanoparticles from PLGA nanoparticles due to orientation of PEG chains on the surface (Fig. 3.4.7.7 to 3.4.7.10). The Zeta potential of PLGA NPs was more negative as compared to PEGyNPs, GPNPs, HPNPs and FPNPs that might be due to presence of free –COOH group in PLGA polymer, and the conjugation of free –COOH group from NH₂ group of PEG results in decrease on Zeta potential of all other nanoparticles (Table 3.4.7.1).

Optimized formulations were characterized for in vitro drug release using dialysis bag method. Results shown in Table 3.4.7.2 indicates that drug release from optimized formulations were 24.18±1.4 % after 72 hr in case of PLGA NPs, while PEGylated nanoparticles exhibited 23.48±2.26 %, GPNPs exhibited 22.4±2.21 %, HPNPs exhibited 22.43±2.19 % and FPNPs exhibited 22.41±1.11 % drug release in 72 hr. PEGylated & targeted, PEGylated NPs conformed sustained drug release as compared PLGA nanoparticles, this could be due to presence of an addition barrier layer of PEG over the surface of NPs. Result indicates that the PEGyNPs, GPNPs, FPNPs and HPNPs initially reduces the burst release as compared to PLGA NPs. Data conforms that the GPNPs,
FPNPs and HPNPs gives more sustained release mechanism as compared to PEGyNPs and PLGA NPs.

PLGA nanoparticles, PEGyNPs, GPNPs, FPNPs and HPNPs, were stored at 4 ± 1°C and room temperature (27 ± 1°C) were subjected for stability studies. Optimal storage condition of the formulation assessed by analyzing the particle size and residual drug content after the time intervals of initial, 10, 20, 30, 60 and 90 days.

Average particle sizes of nanoparticles were found to increase on storage, which can be due to aggregation of particles. This effect was least in the case of formulation stored at 4±1°C, which indicate that aggregation can be regulated by regulating temperature and hence ideal storage condition of Nanoparticle formulation is at 4±1°C (Table 3.5.1.1 & 3.5.1.2; Fig. 3.5.1.1 & 3.5.1.2).

Percent residual drug remaining in Nanoparticle by assuming the initial drug content to be 100% revealed that significant percent of drug lost was (9-10%) from the formulation within 90 days, which were stored 27±1°C and only (3-5%) drug was lost from those stored at 4±1°C (Table 3.5.2.1 & 3.5.2.2; Fig. 3.5.2.1 & 3.5.2.2).

The stability testing data indicated that Nanoparticle formulations stored at 4±1°C were more stable than those stored at 27±1°C. Moreover it was also inferred that PEGyNPs, GPNPs, HPNPs and, FPNPs were more stable then PLGA nanoparticles, this may be due to coating of PEG (hydrophilic) in the surface.

Hemolytic toxicity of plain drug solution, polymer (PLGA) or copolymer solution (mPEG-PLGA, Galactose-PEG-PLGA, Heparin-PEG-PLGA and, Folate-PEG-PLGA) & drug loaded NPs were observed. Result conforms that (Table 3.6.1.1) plain drug solution exhibits highest hemolytic toxicity. Whereas PEGylated nanoparticles (PEGyNPs, GPNPs, HPNPs and, FPNPs) exhibited comparatively lesser hemolysis that could be attributed to reduction in hydrophobicity due to presence of hydrophilic layer (PEG coating) around the polymeric core. Ligand conjugated NPs showed least toxicity because of better dispersion and selective affinity to tumor cells. The hemolytic toxicity of various functionalized, targeted, PEGylated, nanoparticles was always less than pure drug because of less interaction of drug with RBCs, which is encapsulated in polymeric core. As hydrophilicity of various nanoparticles goes on increasing, the extent of their...
interaction with RBCs reduces. Thus the order of toxicity of various formulations in decreasing order is-

Plain drug solution > PLGA polymer solution > PLGA NPs > mPEG-PLGA copolymer > Folate-PEG-PLGA copolymer > Galactose-PEG-PLGA copolymer > PEGyNPs > GPNPs > FPNPs > Heparin-PEG-PLGA copolymer > HPNPs.

Photomicrographs in Figure 3.6.1.2 to 3.6.1.13 clearly suggest that conjugated nanoparticles formulation were less toxic and no significant hemolysis were observed, while extent of hemolysis was somewhat higher in PLGA NPs. Least hemolysis was observed in the case of HPNPs the reason behind that might be biocompatibility of heparin and PEG.

In order to study cell uptake and Cytotoxicity of different formulations and different concentrations of ingredients, MDA-MB-231, HeLa cell and A 549 cell monolayers were incubated according to the standard procedures mentioned in concerned section. The cell uptake study of different formulations were carried out by different tumor cells (MDA-MB-231, HeLa cell and A 549), tumor cell monolayer’s were incubated with rhodamine loaded NPs (PLGA NPs, PEGyNPs, GPNPs, HPNPs and, FPNPs) and control, for different time intervals i.e. 24, 48 and 72 hours at 37ºC. To distinguish surface bound NPs, tumor cells were washed with either cold PBS to remove unassociated NPs or acidic saline to strip bound but not internalized NPs. The cells were then lysed and assayed for residual cell fluorescence using the protocol described in concerned section.

MDA-MB-231 cells-

In Fig; 3.6.2.1.1 it shows that drug (Cisplatin) concentration in the range of 12.5 µg/ml was highly cytotoxic than other drug concentrations. Fig; 3.6.2.1.2 explains that PLGA concentration in the range of 750µg/ml was more cytotoxic to the cells than other polymer concentrations. According to Fig 3.6.2.1.3 it was concluded that PLGA nanoparticles & PEGylated Nanoparticles loaded with drug (optimized drug and polymer conc.) having less cytotoxic effect as compared to FPNPs, GPNPs, HPNPs loaded with drug. Fig; 3.6.2.1.3 conforms that FPNPs are more cytotoxic then compare to other
targeted PEGylated Nanoparticles (GPNPs & HPNPs). Whereas in the case of Fig; 3.6.2.1.3 FPNPs loaded with drug (optimized drug and polymer conc.) exhibited highest cytotoxic effects compare to other PEGylated Nanoparticles (GPNPs & HPNPs), which might be due to the presence of highly expressed receptors present in cells, so that it was internalized through Receptor Mediated endocytosis (RME) method.

According to Fig. 3.6.2.1.12 highest uptake of rhodamine loaded FPNPs was found to be increased up to 84.45 % in 72 hrs (Fig. 3.6.2.1.12), whereas PLGA nanoparticles and PEGyNPs uptake was found to be 65.26 % & 75.23 % respectively in 72 hrs (Fig. 3.6.2.1.12) which confirms that FPNPs was highly taken up by the cells via receptor mediated endocytosis method and thus uptake was gradually increased as time increases. Whereas PLGA nanoparticles and PEGyNPs were taken up by the cells via non receptor mediated endocytosis. Enhanced endocytic activity and leaky vasculature of malignant cells might favors accumulation of PLGA NPs and PEGyNPs thus increases (65.25 % & 75.23 % in 72 hrs) in up take was seen but the efflux mechanism of glycoprotein receptors (GPR) efflux PLGA NPs out, thus after 48 hrs cell uptake was found to be low (65%) as compared to FPNPs (84%) & PEGyNPs (75.23 %). Other GPNPs and HPNPs also confirmed the receptor mediated endocytosis thus their uptake was increased as compared to PEGyNPs and PLGA NPs, but expression of their receptors are limited as compared to folate receptors thus uptake was found to be low. Result conforms that folate saturated cells (Fig. 3.6.2.1.12) also explained non receptor mediated simple endocytosis mechanism (due to complete saturation of receptor from excess available ligands (folate)) and the results were almost same with slight difference to PLGA NPs & PEGyNPs.

**HeLa cells**-

In Fig; 3.6.2.1.1it shows that drug (Cisplatin) concentration in the range of 10.0 µg/ml was highly cytotoxic than other concentrations. Fig; 3.6.2.2.2 explains that PLGA concentration in the range of 750µg/ml was more cytotoxic to the cells than other polymer concentrations. According to Fig 3.6.2.2.3 it was concluded that PLGA
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nanoparticles & PEGylated Nanoparticles loaded with drug (optimized drug and polymer conc.) having less cytotoxic effect as compared to FPNPs, GPNPs, HPNPs loaded with drug. Fig; 3.6.2.2.3 also confirms the highest cytotoxicity effect of FPNPs as compared to other targeted nanoparticles (GPNPs and, HPNPs).

Almost same cell uptake results were obtained in the case of HeLa cells. Highest uptake was found from Folate targeted PEGylated Nanoparticles might be due to highly expression of their receptors on HeLa cells (Fig. 3.6.2.2.11). The order of cell uptake was-

FPNPs > GPNPs > HPNPs > PEGyNPs > PLGA NPs

A 549 cells-

In Fig; 3.6.2.3.1 it shows that drug (Cisplatin) concentration in the range of 12.0 µg/ml was highly cytotoxic than other concentrations. Fig; 3.6.2.3.2 explains that PLGA concentration in the range of 750µg/ml was more cytotoxic to the cells than other polymer concentrations. According to Fig 3.6.2.3.3 it was concluded that PLGA nanoparticles & PEGylated Nanoparticles loaded with drug (optimized drug and polymer conc.) having less cytotoxic effect as compared to FPNPs, GPNPs, HPNPs loaded with drug. Fig; 3.6.2.3.3 conforms that GPNPs are more cytotoxic then compare to other targeted PEGylated Nanoparticles (FPNPs & HPNPs). Whereas in the case of Fig; 3.6.2.3.3 GPNPs loaded with drug (optimized drug and polymer conc.) exhibited highest cytotoxic effects compare to other Targeted, PEGylated Nanoparticles (GPNPs & HPNPs), which might be due to highly expression of asialoglycoprotein receptors present in cells, so that it was internalized through Receptor Mediated endocytosis (RME) method. The order of cell uptake was-

GPNPs > HPNPs > FPNPs > PEGyNPs > PLGA NPs

In the case of A 549 cells highest cell uptake were obtained from GPNPs as previously reported that galactose targeted carriers are highly accumulated in the lung due to increased expression of Asialoglycoprotein receptors. Other results were almost same as found in other melanoma cells (MDA-MB-231 & HeLa cells) (Fig. 3.6.2.3.11).
Acridine Orange Study-

MDA-MB-231 cells

Acridine orange study results that FPNPs and GPNPs shows nuclear morphology changes similar to that of apoptotic cell morphology in cancerous cells. Highest apoptosis occurred in FPNPs might be due to excess expression of its receptors in the cells, after that GPNPs conforms for apoptosis in the cell (Fig; 3.6.2.1.17 and 3.6.2.1.18). Apart from that other NPs (PEGyNPs, HPNPs, PLGANPs) and plain drug solution resulted less morphological changes due to either non receptor mediated endocytosis or quick efflux from the melanoma cells. Whereas for other cancerous cell culture of HeLa cells again FPNPs conforms the nuclear morphological changes (Fig; 3.6.2.2.1). In the case of A-549 cells GPNPs and FPNPs (Fig; 3.6.2.3.17 and 3.6.2.3.16) shows nuclear morphological changes. In A-549 cells GPNPs shows maximum apoptosis (Fig; 3.6.2.3.17) as compared to other targeted NPs and plain drug solution might be due to excess expression of Asialoglycoprotein receptors in the lung carcinoma cells.

In this study the FPNPs and GPNPs are found to be efficient to produce apoptosis whereas other NPs and plain drug solution are less effective to produce apoptosis.

From Acridine orange study it is conformed that targeted nanoparticles with excess expression of receptors are localized in the inner portion of cell and thus they produces maximum apoptosis as compared to other Ligand anchored nanoparticles without sufficient expression of their receptors as well plain drug solution.