ABSTRACT

Nanoparticulate drug delivery systems have tremendous application scenarios in the dealing of several diseases, particularly in cancer treatment. The key aim of fabricating nanoparticles in a drug delivery system is to control particle size, surface properties (zeta potential, PDI) and release of drug to attain the targeted action of the active pharmaceutical agent in an optimum range. Polymeric nanoparticles have promising features of easy design, better biocompatibility, broad-structure variability, and distinguished biological characteristics. The main objective of the current research was to develop, optimize and evaluate chitosan and PLGA loaded Bendamustine nanoparticle. The technique employed for the preparation was ionic gelation technique as well as emulsion solvent diffusion technique and the optimization was done with the help of 2^3 factorial designs. In the optimization process a relation between independent (polymer concentration, surfactant concentration and sonication time) and dependent variable (particle size, entrapment efficiency) were developed. The nanoparticles were evaluated by the parameters such as drug excipient compatibility test, mean particle size calculation, morphology, zeta potential, DSC study, % drug entrapment efficiency, % yield, *in-vitro* dug release behavior study.Finally, both the nanoparticles were formulated in dry lyophilized powder dosage form and evaluated.

The present research work, is based on selected drug bendamustine (BM) tested for its recognition by melting point, solubility studies, partition coefficient, UV absorption maxima study and FTIR spectroscopy. Preformulation is the study that emphasis on the physic-chemical characteristics of a newly discovered drug that may influence the drug function, behaviour and the advancement of a dosage form. The study will offer significant material for formulation, strategy and the necessity for molecular variation.

The physical and morphological evaluation of Bendamustine, colour, odour and texture were examined visually. Melting Point determination is the necessary parameter to determine the purity of active drug. The melting point of (BM) is examined with the help of capillary tube technique. Finding out the solubility of bendamustine at various temperatures for three different solutions was taken, both

solutions tested, for the equilibrium solubility 10 ml of each solvent was taken in different test tubes. The maximum absorbance of (BM) was determined by UV-Spectrophotometer (double beam) consuming methanol as solvent and scanning the solution (100µg/ml) between 200-400nm. For the analysis purpose standard solution of Bendamustine prepared in 1mg/ml by dissolving the 100mg of drug in 100ml of methanol. Furthermore the solution was diluted by methanol to obtain standard solutions of 100µg/ml. The active drug spectrum was determined with the help of (FTIR Shimadzu –DRS-8000). Excipient compatibility study is important for maximizing stability of dosage form and to get good quality of product by avoid the incompatibilities during production. The drug and excipient compatibility characterizes a significant stage in the study of preformulation part for the development of all dosage kind forms. The partition coefficient Firstly, 10mg of pure BM was mixed with 25 ml of distilled water and 25 ml of n- octanol too. is described as the equal parts of unionized drug that is dispersed in the organic as well as aqueous phase till the equilibrium achieved. The total quantity of substance present in both phases was determined by UV analysis and related with the amount of the substance initially used. Ionotropic or ionic gelation technique the polymeric nanoparticles can be assessed by the cross-linking process of biodegradable hydrophilic polymer (chitosan) with drug Bendamustine. In this technique electrostatic interaction occurred between positively charged (amine group of chitosan) and negatively charged tripolyphosphate surfactant to produce the coacervates of nanometer range.

In Emulsion solvent diffusion method initially, nano emulsion was formulated. The Polymer (PLGA) was dissolved in organic solvent dichloromethane. After that the desired quantity of bendmustine was dispersed in polymeric solution. The organic solution of drug and polymer was then added gently in aqueous solution of PVA (surfactant) under continuous magnetic stirring followed by sonication process for 5-7 minutes.

After the preparation of both the nanoparticles optimization was require to get a formulation of standard quality for further study. The study was designed to assess persuade of process variables on characteristics parameters that is particle size and entrapment efficiency of nanoparticles. The variables chosen through preliminary studies; single variable always changed at that time while keeping all others constant. Optimization was done in three steps. In step one preliminary studies done

experimentally using the process parameters which was Polymer concentration (0.5%-0.75%), concentration of surfactant (0.5-1%) and sonication time (5-7mim) for Bendamustine chitosan nanoparticle and Concentration of polymer (A) (0.5%-3%) Concentration of surfactant (B) (1.0%-4%) and Sonication time (C) (5-7 min)for PLGA nanoparticle. The chosen independent variables considerably affect the detected responses for the particle size, % EE. In the next step prepared nanoparticle were optimized for selected variables based on the initial study through with Box-Behnken design.

The key effect and interaction factors were obtained by Design-Expert software by Polynomial equations. ANOVA was used for the numerical justification of the equations.$Y = A0 + A1X1 + A2X2 + A3X3 + A4X1X2 + A5X2X3 + A6X1X3 + A7X12 + A8X2\,2 + A9X3\,2 \ldots\ldots equ.1$ Where Y is a kind of measured response also known as dependent variable related individually to factor level combination; A_0 to A_9 are called regression coefficients of particular variables. X_1, X_2 and X_3 are the respective codes of independent variables. The independent variables were denoted by −1, and +1 level related to the maximum and minimum (low and high) separately. The independent variables Y_1 and Y_2 were denoted as PS which is particle size and %EE for drug entrapment efficiency. To get maximum optimized formulation along with least particle size, greatest drug entrapment efficiency, the 8 unlike groups of each Chitosan and PLGA nanoparticles were characterized.

The particle size distribution, PDI and zeta potential of chitosan and Poly lactic co glycolic acid based nanoparticles were calculated by dynamic light scattering using Malvern Instruments. The analysis was performed in a triplicate manner at an angle of 90^0 at the temperature of 25^0C. The nanocarriers of Chitosan and PLGA were estranged from aqueous medium by centrifugation at about 15,000-20,000 rpm and dried up at room temperature then weighed and percentage yield of the formulation. For the calculation of drug loading and entrapment efficiency, initially both chitosan and PLGA nanoparticles were mixed in 10 ml methanol followed by centrifugation with high-Speed cooling Centrifuge for 30 minutes at 14,000 rpm and after that it was filtered with membrane filter. Then the filtrate was diluted with methanol again later scanned with UV Spectrophotometer at 330 nm. Surface methodology and contour of nanoparticles were assessed by TEM. In Transmissionelectron microscopy a beam

of electrons is passed on all the way through a sample to create arepresentation. For the study, initially diluted sample was positioned as drop on a carbon coated copper grid and then marked with a drop of aqueous solution of phosphotungstic acid 2 %. Extra staining was cleaned through filter paper. Samples were dried at room temperature and finally the transmissionelectron microscopy image was captured. For the DSC examination a slight amount of test sample was kept in hermetically sealed aluminum pans and was heated at 50–300 ˚C under the continuous flow of dry nitrogen with heating rate of 10 to 11˚C/min. The lyophilized powder test samples (BM loaded chitosan and PLGA nanoparticle) were kept in the stage and scanned in the range of 2^{θ} to $60^{\theta}$with a specified voltage current for X-ray diffraction study. For the in-vitro drug release study the accurately weighed nanoparticle samples were placed in the donor section of dialysis membrane and was in 10 ml of dissolution medium (phosphate buffer, pH7.4 for BM) at the temperature of $37^{o}C \pm 0.5^{0}C$ by continuous stirring with 100 rpm. Aliquots were withdrawn in a regular interval of time and exactly the similar amount of dissolution medium added during the analysis. All these withdrawn test samples were properly weakened by adding methanol, examined by UV-visible spectroscopy at 329nm. The drug released data is very much useful in concluding the kinetics of drug release and its methodology. To obtain different mathematical models normally one or two circumstances arisen. In the first case, the drug movement method will be Fickian diffusion and case II will be non-Fickian.

Pharmaceutical dosage form is a type of physical form in which a drug has administered. Here the active drug is mixed with excipients considering the parameters like particle size, solubility, and polymorphism. In the current study and research, the best optimized formulation (NPF-4) and (NPF-8) of bendamustine loaded chitosan and PLGA nanoparticle was formulated as dry lyophilized powder and evaluated. Bendamustine causes hydrolysis, so it is formulated as lyophilized powder. Based on the outcomes of in-vitro experiment, entrapment efficiency and cumulative percentage drug release, chitosan and PLGA nanoparticles were formulated into lyophilized powder. These prepared nanoparticles were additionally estimated for in-vitro and experimentation. In-vitro release of BM loaded chitosan as well as PLGA lyophilized formulations and pure drug suspension was assessed through dialysis membrane using modified Franz diffusion cell, was already discussed

in the preparation of nanoparticles. The cytotoxic study of lyophilized powder of both the formulation loading bendamustine was determined using the cell line, Z-138 Lymphocytic Leukemia. After 48 hours of contact with the drug and cell line, cell viability was calculated with the help of MTT assay and IC_{50} (Inhibitory concentration) values. The results were compared with pure drug suspension. Now a days the most normally used technique for the examination of cell growth rate and toxicity. In this method, cells were seeded in 96 well microtiter plates with in minimum vital medium and incubated during the night. After that the cells were treated with MTT then after 4.5 hours the complete medium was drawn out from the wells. The residual form a crystals were mixed in DMSO and the absorbance was measured at 570 nm through 96 well microplate reader. IC_{50} was calculated according to the drug concentration at which the cell viability reduced to 50 %.

Pharmaceutical stability testing and study is very much significantly used in the determination of drug product's shelf life, ideal storage environments, retest time duration, and declaring its general quality for patients.As per the ICH guidelines the stability studies were performed for formulations at room temperature $25^0C \pm 2^0C$ and for accelerated conditions $40^0C \pm 2^0C$ in stability chamber. Both the formulations were stored for 6 months' time period and checked, for any changes in the content of drug at intervals of one, three and six months.

The pure drug bendamustine was appeared as off-white coloured microcrystalline powder with amphoteric properties. The melting point was found to be 150^0C. The bendamustine is freely soluble in methanol and partially soluble in water. A UV absorption maximum of BM in methanol was calculated by scanning the solution (40µg/ml) of BM from 200 nm to 430 nm by UV-Spectrophotometer. The maximum absorbance of BM solution was recorded 329 nm in methanol. The prepared drug solutions of concentration ranging 4-40 µg/ml were scanned at λmax (absorbance maxima) 329 nm and the absorbance was determined. In order to find out the interaction/compatibility between BM, selected polymer (Chitosan), selected surfactant (TPP), FTIR spectra were recorded and the major peaks were determined. The spectra of mixtures of BM with chitosan, TPP showed the occurrence of typical peaks of the drug (BM) at 3414.8 cm^{-1}O-H group stretching, 2953.01 cm^{-1} C-H group stretching, 1502.60 cm^{-1} N-CH_3 stretching and 1634.06 cm^{-1} C=C stretching of aromatic with slight variation or shifting in the peaks. In order to study the

compatibility between BM, selected polymer PLGA and other excipients like PVA, acetone, dichloromethane the spectra was recorded and the main peaks were determined. The spectra of mixtures of BM with PLGA, PVA, acetone and dichloromethane showed the occurrence of typical peaks of the drug peaks (BM) at 3414.8 cm^{-1} due to O-H group stretching, at 2953.01 cm^{-1} C-H group. Though, no additional or new peaks were observed that clarifies the pure drug was completely compatible with all the selected excipients. The partition coefficient of bendamustine was estimated 4.2, confirmed its lipophilic nature. Chitosan and PLGA nanoparticle was successfully prepared through ionic gelation method and emulsion solvent diffusion method. In optimization process total 8 formulations of both the nanoparticles (BM-CH and BM-PLGA) were prepared to find out the relationship between independent variable and dependent variable. The particle size of 8 batches of Chitosan nanoparticle ranged from 110.51±6.2 nm to 169±5.1 nm for three factors, two level combinations and the entrapment efficiency ranged from 50.01%±2.1 to 64.11±3.1 %.3D plot showing the effect between PC-SC, PC–ST and SC-ST in response of particle size and entrapment efficiency. The particle size of 8 formulations of BM-PLGA nanoparticle ranged from 103.5±0.04 nm to150.9±0.51 nm and the entrapment efficiency was ranged between 58% to 82 for 3 factor- 2 levels combinations. The influence of independent variables dependent variable i.e. the quadratic equation was designed to describe the particle size as well as entrapment efficiency. Summary and results of analysis of variance for PS and EE (for BM-PLGA and BM-CH nanoparticle) based on ANOVA model with significant values. The value of determination coefficient (R^2) and adjusting coefficient were greater than 90% which proves that the model is significant. The polydispersity index of BM-CH and BM-PLGA was found to be0.245 and 0.307 respectively. The zeta potential of BM loaded chitosan nanoparticle and BM loaded PLGA nanoparticle was assessed as -21.3 to + 21.3 and -31.9 to +31.9 respectively. TEM scan displays the development of sphere-shaped nanoparticle. TEM graph also discloses that the particles have a relatively uniform size. The particles were segregated with each other.From the *in-vitro* drug release studyit is sure that pure drug suspension of BM releasednearly98.32% of ± 0.40 of drug towards the end of 6^{th}hours and optimized PLGA nanoparticle released 85.2 ± 0.24% of drug at its 48^{th} hour. The formulation exhibited a two phase i.e., biphasic release manner. Both the formulations were followed 1^{st} order i.e. pattern of drug release is non-fickian and also followed the

standard koresmeyer-peppasmodel.finally both the optimized formulations were formulated into dry lyophilized powder. The lyophilized powder (approx. 25 mg) was reconstituted with 10 ml water for injection via shaking. This research showed that no aggregate or clumps were formed during reconstitution with WFI (water for injection) and evaluated for the cellular cytotoxicity and stability study. Therefore, the lyophilized formulation (lyophilized powder) of BM was considered to be more stable at room temperature when compared to the pure drug suspension and not any noteworthy deviations were observed in particle size, Zeta potential and drug content. In the present research work, bendamustine loaded chitosan and PLGA nanoparticle were prepared, optimized, characterized and finally formulated in desired dosage form. Ionic gelation and solvent diffusion methods were used in the preparation of chitosan and PLGA nanoparticles respectively. Both of nanoparticles were selected for parenteral administration due to its nano size range. From this research work it was concluded that, both the techniques (ionic gelation andsolvent diffusion) were useful in the preparation and development of BM nanoparticles but, the emulsion solvent diffusion technique is more suitable and promising method to get nanoparticles of minimum particle size, maximum entrapment efficiency, better percent of drug loading, good stability and desired drug release.

Key words- Nano formulation, Polymer, Anticancer, preformulation, characterization.