7.1 General overview

Many current chemotherapeutic and anticancer drugs have low water-solubility and high lipophilicity. For the solubilization of these drugs require high concentration of surfactant and co-solvents which can cause severe side effects.The nanocarrier drug delivery system can be an alternative to overcome this problem. Numeroustypes of nanocarrier systems are studied broadly for drug delivery of poorlywater-solubleanticancer drugs.

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.

The present research work was classified into three foremostphases.In the first step preparation of both the nanoparticles were done.During the second phase the optimization of chitosan and PLGA nanoparticles was done with the help of preliminary study to select the suitable independent variables and parameters for the preparation of nanoparticle.In preliminary trials suitable range of concentration of polymer, concentration of surfactant andsonication time (independent variable) was determined. These results were again optimized usingdifferent factorial design to find out the greatest optimized formulation of nanoparticles. The optimization was completed through Design Expert Software (Version. 10), here total 8 formulations of chitosan and PLGA nanoparticles were prepared and characterized. These nanoparticles were characterized according totheir respective particle size and encapsulation efficiency. One of the best nanoparticles (chitosan and PLGA) preparation was selected built on lowest particle size and greatestencapsulation efficiency as dependent variable.An experimental batch of optimized formulation was prepared and the % of bias was determined between predicted and observed responses. The best nanoparticle

formulations (BM-CH and BM-PLGA) were then evaluated for zeta potential, PDI (polydispersityindex)Transmission electron microscopy (TEM),DSC(Differential scanning calorimetry), X-ray diffraction, percentage yield of drug, drug loading capacity, cumulativerelease of drug and in- vitrodrug release profile.

In the third and last phase both the best optimized preparations (NPF.4 and NPF.8) were formulated into a dry lyophilized powder. These dry lyophilized powder formulations were evaluated for in vitro study, cytotoxic study and stability study.

7.2. Objective of the research

The objective of the current research work is to develop and formulate and optimized the preparation of bendamustine loaded chitosan and PLGA nanoparticles by using two different methodsi.e.,ionotropic gelation and emulsion solvent diffusion technique, to find out which technique is more suitable and promising for accessing nanoparticle ofnon- toxic, site specific, bettersurface properties, acceptable drug release pattern, narrow size distribution and maximum entrapment efficiency.

7.3. Preformulation study

Thestudies ofactive drug ingredient and excipients beforeformulationmainlyknown as-pre-formulationstudies.The study is very necessary to evaluate the purity of drugby its melting point, drug- excipient interaction or compatibility studies were determined by FTIR and solubility study.

The melting point of bendamustinewas found to be 150ºC. Bendamustine was freely soluble in methanol and dimethyl sulfoxide (DSMO). It was sparingly soluble in water. The aqueous solubility of bendamustine was 0.0618mg/ml. The maximum absorbance of drug in methanol was calculated as329nm. The FTIR spectra of bendamustine displayed characteristic peaks at3315 cm^{-1} (O-H stretching vibration),2715.01 cm^{-1} (C-H stretching,

aliphatic), 1502.60 cm^{-1} (N-CH_3 stretching) and 1634.06 cm^{-1} (C=C stretching, aromatic). All thesespectrawere alike to standard spectra of BM compound. No chemical interaction among drug, excipient and polymer(for ion gelation technique and solvent evaporation technique) were found in compatibility study. The partition coefficient of BM in octanol: water was found to be 4.2 showing that the drug is lipophilic in nature.

7.4. Optimization of chitosan Nanoparticles

Chitosan nanoparticleswere prepared by ionic gelation methodat room temperature maintained 6 minutes of sonication. For the optimizationprocess, preliminary studies were performed to obtainedthevariables influencing the development of chitosannanoparticles. The independent variables examined were, Aconcentration of chitosanpolymer (0.1%-1.0%),B concentration of TPP (Tripolyphosphate) surfactant(0.5%-1.0%) andCsonication time(5-7 min).It was denoted by −1, and +1, analogous to the minimum,andmaximum values and the dependent responses were PS (particle size) (Y1) and %EE (entrapment efficiency) (Y2). On the basis of preliminary studies, opalescent nanoparticleswere obtained at moderate concentration of chitosan (0.1-0.75% w/v) and surfactant (0.5-1.0 % w/v). This range was optimized once againusing factorial Design Expert (Version.10 Stat-Ease Inc., MN) software. Here,response surface methodology was employedusing Box-Behnken design,where 8-runs were performed out with 3-factors and 2-levels to optimize and attain the best formulation.Eight batches of chitosan nanoparticle were prepared and characterized (NPF-4). The outcomes of the test for dependent variables were given significant result ($p < 0.05$). (NSF-4) nanoparticle formulation no.4was having the particle size and entrapment efficiency 130.27±3.4 nm 64.11±3.1%respectively, thatsignified good relation with the predicted values.The composition of formulation attained 0.4% (w/v) polymer concentration, 0.75% (w/v) surfactant concentration and 6minutes ofsonication time. The observed response variables were obtained significant for the dependent variables presented by the

value of $R^2$and results of ANOVA.

7.5. Characterization of nanoparticles

- The optimized nanoparticle formulation was having the zeta potential of-21.3 ± 0.02mV with poly dispersity index 0.245. The drug content and drug loading efficiency were found to be61.12% and 22%±0.14respectively.
- The TEM image of the optimized formulation [NPS-4] assured that the nanoparticles were non-aggregated,almost spherical in shapecontaining a narrow size distribution. TEM image was in aproperarrangement and the graph obtainedfrom particle size analyser.
- The BM and chitosandisplayed an endothermic peak at 155^{o}C and 102^{o}C respectivelyas data obtained from DSC thermogram,showing its melting point. The thermal decomposition of BM occurs at maximum temperature 400ºC because a broad peak was obtained. The chemical interaction between drug and polymer was not found.
- The pure drug BM displayedsharpdiffraction peak in x-ray diffractogram at 20 of 3.3, 11.2, 12.0, 16.6, 7.8, 13.6, 15.4, 23.1 and 32.0. The graph showed the crystalline structure of chitosan.The structure wascompletelydemolishedbecause of thecross linkagewith TPP during the preparation of nanoparticle.The two peaks were disappeared slowly with the pattern which shows the it is amorphous in nature.
- In-vitrodrug releasewas calculated in phosphate buffer pH 7.4 using Franz diffusion cell.It was detected that almost 99.3% of pure drug suspension was released at the end of 6th hour but chitosan nanoparticle was released 80.30% of drug at the end of 48th hours, showed asteady and sustained drug release during completeduration of study. The release pattern ofCH-NPswas in two phasicways, with aprimaryeruptionfollowed byfast release phase and then attained sustainedrelease. In primaryphase, aburstof

nanoparticle and then drug release of 35 % was observed at the initial 30 minutes due to the drug desorption.TheR^2 (coefficient of determination) value of optimized formulation was greater than 0.9 suggesting its first order release. Model confirmed that chitosan nanoparticlepresented a non-fickian (anomalous)transport mechanism for the drug release, here the value of R^2 was calculated as 0.96 and n = 0.78.

7.6. Formulation and evaluation of dosage form

Drug molecules are delivered to sites of action within the body with the help of dosage form.In the present study, the best optimized formulation (NPF-4)chitosan nanoparticle was formulated as dry lyophilized powder and evaluated. As bendamustine suffers hydrolysis, so it is formulated as lyophilized powder. The dry lyophilized powder of BM-CH nanoparticle was successfully prepared with mannitol as cyro-protectant. The lyophilized powder (approx. 25 mg) was reconstituted with 10 ml water for injection withshaking.

The percentage drug content of lyophilized formulation of BM-CHwas assessed by UV-spectroscopic method and was found to be 61.10% as well as the percentage entrapment efficiency of reconstituted lyophilized formulation of BM was found to be 64.09%. After the lyophilization process the particle size ofreconstituted lyophilized formulation was found to be 130.20±3.2 nm with very minute change thePDI 0.24. The zeta potentialof BM loaded chitosan lyophilized formulation was found to be -21.3 ±0.02mV, which specifies that the formulation is in good stability condition

- **In-vitro drug release mechanism**

In-vitro drugrelease of pure drug suspension was almost 99.4% at the end of 6^{th}hrs though 80.16 % drugrelease was detected from BM-CH lyophilizednanoparticles, which presented steady and sustained release throughout thewholeduration of test. The value of R^2 was found to be greater than 0.9 suggestingthat the drug release

pattern was first order. Model fitting confirmed asnon-fickian (anomalous transport)patternfor drug release, here the R^2 was calculated as 0.98 and n = 0.68. The value of n was found to be within limit i.e. (0.5-1.0) for Korsmeyer-Peppas model.

- **Cellular cytotoxicity**

The cellular cytotoxicity/in-vitro anticancer activity of pure drug (BM) and lyophilized formulation of BM was calculatedwitha cell line named Z-138. Next 72 hours contact with drug, cellularviabilityand IC_{50}values of the formulation was calculated by MTT assay.The IC_{50}values of pure drug bendamustine and its lyophilized formulation (NPF-4)was found to be 36.16 ± 0.05 and 18.13 ± 0.12 μM respectively after 72 hours exposure. Results explainedthat the lyophilized formulation havinga noteworthyin-vitroantileukemic activity,when compared with pure drug suspension.

- **Stability study**

In stability testingit was observed that the lyophilized powder of BM degradedabout0.06% of its content in theinitial month and 1% in 6months when stored at 25±2ºC, 60±%RH. In the accelerated stability study (40±2ºC, 75±5%RH)thelyophilized formulation degradedhigher than 1.5% drug during the1stmonth and near about2% in the period ofsix months.Therefore,the lyophilized powder of BM was consideredasmore stable on 25±2ºC, 60±%RH (room temperature)conditions when compared with thedrug suspension and no noteworthy changes were observed in meanparticlesize, zeta potential and drug content.The effectof storage conditions in % residual drug content of BM formulations is 61.06% in 1se month and 60.03% in the end of six months at the room temperature condition.

7.7. Optimization of PLGA nanoparticles

PLGA nanoparticleswere prepared by emulsion solvent diffusiontechnique at room temperature maintained 6 minutes of sonication. For the optimization process, preliminary studies were performed to obtained thevariables influencing the development of PLGAnanoparticles. The independent variables examined were, A concentration of PLGApolymer (1.0%-4.0%), B concentration ofPVA (Polyvinyl alcohol) surfactant (1.0%-5.0%) and C sonication time (5-7 min). It was denoted by −1, and +1, analogous to the minimumand maximum values and the dependent variables were PS (particle size) denoted as Y1 and (%EE) (entrapment efficiency) as Y2. On the basis of preliminary studies, opalescent nanoparticles were obtained at moderate concentration ofPLGA (0.5-3.0% w/v) and surfactant (1.0-4.0 % w/v). This range was optimized once again using factorial Design Expert (Version.10 Stat-Ease Inc., MN) software. Hereresponse surface methodology was employedusing Box-Behnken design,where 8-runs were performed out with 3-factors and 2-levels to optimize and attain the best formulation with lowest particle size and highest entrapment efficiency.Total 8 batches of PLGA nanoparticle were prepared and characterized. The outcomes of the responseare significant ($p < 0.05$). The optimized formulation (NPF-8) was having the particle size and entrapment efficiency of 103.5±0.04nm and 78±4.16% respectively.It signified good relation with the predicted values.The selected formulation (NPF-8) was in the composition of1.55% (w/v) polymer concentration % (w/v) surfactant concentration and 6 minutes of sonication time . The observed response variables were obtained significant for the dependent variables presented by the value of R^2 and results of ANOVA.

7.8. Characterization of PLGA nanoparticles

- The nanoparticleswere having the zeta potential of -31.9 ± 3.07 mV with poly dispersity index 0.307.The % yield and drug loading efficiency were found to be 80.20% and 28%±0.14 respectively.
- The TEM images of the optimized formulation (NPS-8)specifies that the nanoparticles were non-aggregated andalmost in spherical shape withthe narrow size of distribution. TEM images were shown the diameter of each particle in a good arrangement obtained with the help of particle size analyser.
- BM and PLGA (polymer)displayed an endothermic peak at 155^{o}C and 102^{o}C respectively showing its melting point in the DSC thermogram. The thermogram of BM were already discussed. The PLGA polymer established a characteristic peak at 45.43ºC indicatingtowardsglasstransition temperature. No separate peak of its melting point was observed due to the amorphous nature of PLGA. The polymer is thermally stable upto 250˚C. The optimized nanoparticle had not shownany drug material which is crystalline, due toabsence of sharp peak of bendamustine.
- In X-ray diffraction study of PLGA nanoparticlesthe distorted peak of BM was detected, showing that the drug is mixed with PVA and does not exist in free form and comparative reduction in the diffraction intensities was observed in the nanoparticles. Thismight be because of the decrease in the superiority of crystals of BM and this result enhances the change of crystalline drug into amorphous naturehelpful in enhancement of solubility.
- In-vitro release of pure drug suspension was calculated nearly 99.1% of the drug at the end of 6th hrs, though 85.20 % release was observed from PLGAnanoparticles of optimized formulation, which showed good results. Model fitting of PLGA nanoparticle showed non-fickian mechanism for the release of drug where the R^2 was found to be 0.98 and n = 0.67.

7.9. Formulation and evaluation of PLGA nanoparticles

A formulation of desired dosage form is required for targeted drug delivery. In the present study, the best optimized formulation (NPF-8) BM-PLGA nanoparticle was formulated as dry lyophilized powder and evaluated. As bendamustine suffers hydrolysis, so it is formulated as lyophilized powder. The dry lyophilized powder of BM-PLGA nanoparticle was successfully prepared with mannitol as cyroprotectant.The lyophilized powder (approx. 25 mg) was reconstituted with 10 ml water for injection via shaking.

- The percentage drug content of lyophilized formulation of BM (PLGA polymer) was assessed by UV-spectroscopic method and was found to be75.28%as well as the percentageentrapment efficiency of reconstituted lyophilized formulation of BM was found to be 78.13%. The particle size of reconstituted lyophilized formulation of BM was found to be 103.57±3.2 nm with PDI 0.307. Zeta potential was estimated to get information about the surface properties of nanoparticles.The zeta potential of BM loaded chitosan lyophilized formulation was found to be -31.9 ± 3.07, which specifies that the formulation is in good stability condition.
- In the in-vitro study, it was observed that pure drug suspension released almost 99.2% of the drug at the end of 6^{th} hrs while 85.20 % release was observed from PLGAnanoparticles. The R^2 (coefficient of determination) value of BM from optimized formulation in phosphate buffer was > 0.9 signifying first order release pattern. Model fitting confirmed that PLGA nanoparticle showed anomalous transport (non-fickian) mechanism for the release of drug, where the R^2 was found to be 0.98 and n = 0.67. The value of n was found to be within limit i.e. (0.5-1.0) for Korsmeyer-Peppas model.

- The cellular cytotoxicity/*in-vitro* anticancer activity of pure drug (BM) and lyophilized formulation of BM was calculated using Z-138 cell line. The IC_{50} values of pure drug BM and its lyophilized formulation (NPF-8) was found to be 36.16 ± 0.05 and 16.13 ± 0.15µM respectively after 72 hours exposure. Results explained that the lyophilized formulation having significant in-vitro antileukemic activities compared to pure drug suspension.
- In stability testing of lyophilized powder of BM-PLGA, it was observed that the formulation degraded almost 0.03% of drug content in first month and0.09% in 6months when stored at room temperature (25±2ºC, 60±5%RH). In the accelerated condition (40±2ºC, 75±5%RH) the lyophilized formulation lost more than 1.0% drug during first month and near about1.5% in 6 months.Thus, the prepared formulation (lyophilized powder) of BM was found to be more stable at room temperature conditions as compared to pure drug suspension and no noteworthy changes were observed in particle size, zeta potential and drug content. The effect of storage conditions in % residual drug content of BM formulations in the 1st month was 75.25 and 75.14%during the end of 6thmonths at room temperature.

7.10. Conclusion:

From this research work it was concluded that, both the techniques (ionic gelation and solvent diffusion)were useful in the preparation and development of BM nanoparticlesbut, the solvent diffusion technique is more suitable and promising method to get nanoparticles.