5 MATERIAL AND METHODS

5.1 Material

The drug sample was received from the pharma industry as a gift sample on request and all the chemicals were analytical grade.

5.2 Method:

5.2.1 Introduction

Preformulation is the study that emphasis on the physic-chemical characteristics of a newly discovered drug that may influence the drug function, behavior and the advancement of a dosage form. The study will offer significant material for formulation, strategy and the necessity for molecular variation. These studies are intended basically to discover the conditions in which the molecule will be more stable [94, 95]. It is required to progress a well-designed, stable, effective and safe dosage form by founding the compatibility with added excipients and by determining the physical and chemical parameter of newly discovered drug materials.

Before starting a preformulation study following points are required:

- The study of existing physical and chemical data with chemical structure, potency of the new products and the dosage form are required [96].
- Probable dosage quantity and the planned route of drug administration is necessary.
- It provides condition and progress schedule of newly discovered dosage form.

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.

5.2.2 Physical and morphological evaluation of drug

In physical and morphological evaluation of Bendamustine, color, odor and texture were examined visually.

Procedure: 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. A minor amount of powdered sample was located into a capillary tube. The drug filled tube was then positioned in the melting point apparatus. The temperature at which powder started to melt as well as the temperature when the powder melted completely, was noted [97].

5.2.3 Solubility study

Procedure: 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. Then exactly weighed quantity of each drug (BM) (approx.10 mg) was added in small increment and shaken for 5 minutes every time till precipitation occurs [98].

5.2.4 Determination of absorption maxima (λ max):

Procedure: 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 [99].

5.2.5 Preparation of standard solution:

Procedure: 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 [100].

5.2.6 Determination of calibration curve of Bendamustine:

Procedure: From the prepared standard solution 20ml of solution withdrawn in 100 ml of volumetric flask to obtain 40µg/ml solution. From this stock solution of 40 µg/ml aliquots of 0.5,1,2,3,4,5------10 ml was withdrawn and further diluted with 10ml of methanol to achieve dilutions of 4, 8, 12, 16, 20, 24, 28, 32, 36 and 40 µg/ml. The absorbance of resultant dilutions was determined at 329 nm [101].

5.2.7 FTIR Spectroscopy of pure drug

Procedure: The active drug spectrum was determined with the help of (FTIR Shimadzu –DRS-8000). In which no need to make KBr pellets, just directly 1-2mg of drug was located in the sample holder after that the spectrum was recorded [102].

5.2.8 Drug –Excipient Compatibility by FTIR

Drug-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. Drug particles having several sensitive and reactive functional groups [103].

Procedure:

For the compatibility study drug and excipients were prepared in 1:1 ratio i.e., 10 mg of pure drug, polymer and drug plus polymer samples taken and directly assessed by FTIR (Shimadzu – DRS-8000).

5.2.9 Determination of Partition coefficient:

This is described as the equal parts of unionized drug that is dispersed in the organic as well as aqueous phase till the equilibrium achieved.

$$Po/w = (Coil/Cwater)\ at\ equilibrium$$

Procedure: For the Assessment of degree of lipophilicity i.e. partition coefficient for BM was completed separately by shaking flask process. Firstly, 10mg of pure BM was mixed with 25 ml of distilled water and 25 ml of n- octanol too. Then the blend was shaken separately up to half an hour duration. After that both the phases (oil and water) were mixed together in a separating funnel and shaken for 4 hrs on a mechanical shaker. After shaking the mixtures, allow them to stand till the phase separation process ended. The total quantity of substance present in both

phases was determined by UV analysis and related with the amount of the substance initially used [104].

5.3 Preparation and characterization of nanoparticle [105]

5.3.1 Ionic gelation technique: In ionotropic or ionic gelation technique the polymeric nanoparticles can be assessed by the cross-linking process of biodegradable hydrophilic polymer (chitosan) with drug Bendamustine.

Chitosan nanoparticles have grown more reflection as carriers' system due to its enhanced stability, very less toxicity, easy preparation method and providing versatile routes of administration[105].

5.3.2 Emulsion solvent Diffusion technique:

In this method initially, nano emulsion was formulated. The Polymer (PLGA) was dissolved in organic solvent dichloromethane. After that the desired quantity of bandmustine was dispersed in polymeric solution. Then the sample was added gently in aqueous solution of PVA (surfactant) under continuous magnetic stirring followed by sonication process (Prob sonicator, meco Ultrasonics, India) for 5-7 minutes. For the smooth conduction of emulsification process, magnetic stirring (Remi, India) was continued for 5-6 hours. After that Oil in water emulsion was formed and in this step dichloromethane diffused in the aqueous phase and therefore, nanoparticles formed by polymer precipitation. For the whole diffusion of DCM the extra amount of water was added. Finally, the nanoparticles were obtained by centrifugation method [106].

5.3.3 Freeze drying

This process involves the total exclusion of water and other solvents of the given sample by sublimation process. This method is also known as lyophilization. It keeps the temperature of the sample very low as much as essential throughout the procedure to skip the variations of the dried sample appearance and characteristics[107].

- **Preparation of Bendamustine loaded Chitosan Nanoparticles by ionic gelation technique:**

In this technique, different concentration of chitosan polymer ranging from 1-5mg/ml was dissolved in 1% v/v solution of acetic acid. The chitosan solution pH was adjusted to 4.5-4.6 with 0.1 N NaOH. Then the calculated amount of 2 mg/mL Bendamustine in methanol mixed with chitosan solution (5ml). In the next step cross linking agent or surfactant tripolyphosphate was also mixed in deionized water. Finally, magnetic stirring was done in that the surfactant solution was added in the chitosan solution by carefully adding drop by drop at room temperature for some period of time followed by sonication for 5-7 minutes, until an opalescent suspension was obtained. Then centrifugation process for 30 minutes at 15,000 rpm also water was used for washing of nanoparticles. After that they were kept for freeze drying and treated by 3% of mannitol used as protecting agent in lyophilization process.

- **Preparation of nanoparticle through solvent diffusion technique:**

The second method was emulsion solvent diffusion technique adopted for the for Bendamustine nanoparticles. The precisely weighed quantity of PLGA and drug was mixed in (10 ml) of dichloromethane. After that the obtained polymeric drug solution added to aqueous solution of PVA (4%w/v) with continuous stirring for 4 hours, until the oil in water emulsion was formed. 30 ml of deionized water was also added for complete diffusion of organic phase into water.

The finally Nanoparticles separated by centrifugation technique at 10,000rpm for 30min afterward separation of the supernatant from precipitants was completed. This precipitant containing nanoparticles was freeze dried and evaluation studies were performed.

5.4 Optimization of Chitosan based nanoparticles prepared by ionic gelation technique

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 nanoparticles.

These factors are very much important in to control size, size distribution and other physiochemical parameters of nanoparticles. In the next step process parameter based on pre study was selected once again and optimized. Total 8 batches of formulations were selected and characterized. In last step the suitable nanoparticle formulation obtained through drug entrapment efficiency with minimum particle size. With the help of this method the best

experimental formulation with same concentration ingredient was prepared and percentage bias was determined [108].

5.4.1 Process and formulation parameter of Chitosan Nanoparticles

Procedure

The prepared nanoparticles were optimized for selected variables based on the preliminary study applying the Box-Behnken design in which 8 batches were selected using 3 factors and 2 levels and data was generated for each batch. Amount of polymer A, Amount of Surfactant B and sonication time C called the independent variables (three factors) which would affect the dependent variable. The optimization was done by Design expert (version 10) software. Through the optimization process a formulation with greatest desirability factor was carefully chosen and data was studied [109].

Table1.2: Process and formulation parameter of Chitosan Nanoparticles prepared by Ionic gelation technique[8]: `

1) Preliminary studies for the formation of CS nanoparticles		
Variables	**Range**	**Selection Parameter**
Concentration of chitosan	0.1-1.0%w/v	Formation of nanoparticle
Concentration of Surfactant	0.5-1.0%	
2) Selection and optimization of key variables		
Key variable	Range	Selected parameter
Polymer Concentration	0.5-0.75%w/v	Particle size and Drug entrapment efficiency
Surfactant Concentration	0.5-1.0%	

Sonication time	5-7 min	

Table1.3: Process and formulation parameters for PLGA Nanoparticle prepared by emulsion solvent Diffusion technique:

1) Preliminary studies for formation of nanoparticles		
Variable(s)	**Range**	**Selection parameter**
Conc. of PLGA (Polymer)	1.0–4.0 %w/v	Formation of Nanoparticle
Conc. of PVA (surfactant)	1.0-5.0 % w/v	
2) Selection and optimization of key variables		
Key variable	Range	Selection parameter
Conc. of PLGA (Polymer)	0.5-3.0% w/w	Particle size and drug entrapment efficiency
Conc. Of PVA (Surfactant)	1.0-4.0 %	
Sonication time	5-7 min	

In the next step prepared nanoparticle were optimized for selected variables based on the initial study through with Box-Behnken design. The quadratic model produced by the design was:

$$Y = A0 + A1X1 + A2X2 + A3X3 + A4X1X2 + A5X2X3 + A6X1X3 + A7X12 + A8X2\,2 + A9X3\,2 \ldots\ldots equ.1$$

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 represent the measured responses; 3D graph was plotted to describe the connection among independent variables as well as dependent variables. The value of % bias expressed the reliability of the model. The value of bias was determined by using equation

$$\%Bias = \frac{Predicted value - Experimental value}{Predicted value} \times 100 \quad \textbf{.....equation.2}$$

The independent parameters also selected from preliminary studies were also used for optimization through design expert software. The range of parameters selected for Chitosan nanoparticle were based on various parameter i.e.

a) Amount of polymer (0.5% - 0.75%) and

b) Amount of surfactant (0.5% - 1.0%)

c) Sonication time (5-7min)

The coded values for variables (independent, dependent) in experimental plan in table 1.4.

Table.1.4: Highest and lowest range of selected independent variable for ionic gelation technique.

Independent variables	**Levels**		**Dependent Variables**
	Low (-)	**High (+)**	
Polymer Concentration (A)	0.5	0.75	(Y1) Particle size and (Y2) drug Entrapment Efficiency
Concentration of Surfactant (B)	0.5	1.0	

Sonication time (C)	5	7	

Table 1.5: The independent variables with their coded and actual values of 8 batches of Chitosan Nanoparticle

Nanoparticles Formulation	Independent variables Coded values			Independent variables Actual values		
	A	**B**	**C**	**A**	**B**	**C**
NPF1	1	1	1	0.75	1	7
NPF2	-1	1	1	0.5	1	7
NPF3	1	1	-1	0.75	1	5
NPF4	-1	1	-1	0.5	1	5
NPF5	1	-1	1	0.75	0.5	7
NPF6	-1	-1	1	0.5	0.5	7
NPF7	1	-1	-1	0.75	0.5	5
NPF8	-1	-1	-1	0.5	0.5	5
Dependent variables						**Limits**
Y1 - Particle size (nm)						Minimize
Y2 – Drug Entrapment efficiency (%)						Maximize

where, A = polymer concentration , B = surfactant concentration , C= sonication time and (-1), and (+1) low and high level individually for independent variables

The formulation parameters of PLGA prepared nanoparticles through solvent diffusion technique were optimized meant for minimum particle size and maximum entrapment efficiency using response surface quadratic model. Created on the preliminary study, PLGA (polymer) and PVA (surfactant) were optimized by design expert software respectively. For the outcome of dissimilar variables on formulation parameters such as particle size (PS), (Y1) and Entrapment % EE (Y2) of the already prepared nanoparticles factorial design response was used. The independent variables were as follows:

a) Concentration of polymer (A) [0.5%-3%]

b) Concentration of surfactant (B) [1.0%-4%] and

c) Sonication time (C) [5-7 MIN]

The coded values for independent and dependent variables in experimental design models are given in table 6T-5.

Table 1.6: Highest and lowest range of selected independent variables for solvent diffusion technique.

Independent variables	**Levels**		**Dependent Variables**
	Low (-)	**High (+)**	
Polymer Concentration (A)	0.5	3	Y1 (Particle size), Y2 (Entrapment Efficiency)
Surfactant Concentration (B)	1	4	
Sonication time (C)	5	7	

The chosen independent variables considerably affect the detected responses for the particle size, % EE. 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. The design engaged for optimization process for 8 batches of Nanoparticle formulation, has been shown along with the results in table.1.7.

Table 1.7: Independent variables with their coded and actual value of 8 batches of Bendamustine loaded PLGA Nanoparticle

Nanoparticles Formulation	Independent variables [Coded values]			Independent variables [Actual values]		
	A	**B**	**C**	**A**	**B**	**C**
NPF1	-1	1	-1	0.1	4	5
NPF2	1	1	-1	3	4	5
NPF3	-1	1	-1	0.1	1	5
NPF4	1	1	1	3	4	7
NPF5	-1	1	1	0.1	4	7
NPF6	1	-1	-1	3	1	5
NPF7	-1	-1	1	0.1	1	7
NPF8	1	-1	1	3	1	7
Dependent variables						**Limits**
Y_1 = Particle size (PS)						Minimize
Y_2 = Entrapment efficiency (%EE)						Maximize

5.5 Characterization of prepared nanoparticles [110]:

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 for parameters written below:

5.5.1 Particle size distribution, PDI and Zeta potential:

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. Both the testing samples were dispersed in the adequate quantity of ultra-pure water (pH-7) before starting the experimentation. The polydispersity index can be described by particle size distribution which is defined as width or broadness of molecular mass. The zeta potential was calculated by using Helmholtz–Smoluchowski equation [111]:

$$\xi = EM \times \frac{4\pi\eta}{\varepsilon}$$ **.....equ.no.3**

Here

ξ for zeta potential (mv)

A for electrophoretic mobility

η for viscosity of dispersion medium

ε for dielectric constant

5.5.2 Percentage yield [112]:

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 was calculated using equations.no.4.

Percentage yield =Weight of nanoparticles /weight of polymer + weight of drug x 100…equation.4

5.5.3 Drug loading, entrapment efficiency:

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 [113]. The percentage of drug entrapment efficiency and drug loading was obtained by the resulting formula.

$$\%\ Entrapment\ Efficiency = \frac{Weight\ of\ drug\ in\ nanoparticle - Weight\ of\ drug\ in\ supernatant}{Weight\ o\ f\ drug\ in\ nanoparticle} X\ 100$$

% of Drug loading =
Total weight of drug entrapped/ Total weight of nanoparticle X100

5.5.4 Transmission electron microscopy:

Surface methodology and contour of nanoparticles were assessed by TEM. In Transmission electron microscopy a beam of electrons is passed on all the way through a sample to create an representation. 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 transmission electron microscopy image was captured [114].

5.5.5 Differential scanning calorimetry

DSC was done for the determination of physical nature of the drug Bendamustine and polymer interaction in nano carrier system. The DSC curves were recorded for pure drug BM with chitosan and PLGA, along with the lyophilized drug laden nanocarriers. The calibration was done by using alumina powder as the standard. 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 [115].

5.5.6 X-ray diffractions study (XRD)

This was executed for the examination of crystalline or unstructured nature of prepared nanoparticles. 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^{θ} with a specified voltage current [116].

5.5.7 In-vitro drug release

The in-vitro drug release of BM loaded chitosan as well as PLGA nanoparticles and pure drug suspension was assessed by dialysis membrane. Initially the dialysis membrane was put into

distilled water for 24 hours. After that 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 37ºC ± 0.5^0C 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 percent of cumulative release of drug was calculated [117].

5.5.8 Kinetics of Drug release [118]

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. The models studied given below in a tabulation form:

Table.1.8. Interpretation of Drug release mechanism

Release exponent (n)	**Drug release mechanism**	**Order of release**
0.5	Fickian diffusion	$t^{-0.5}$
0.45<n=0.89	Non-Fickian diffusion	t^{n-1}
0.89	Case II transport	Zero order release
More then 0.89	Super case II transport	T^{n-1}

a) **Zero order drug release kinetics** – in this study concentration did not affect the drug release rate. Here, the graph was designed among the cumulative percent drug released and time.

b) **First order drug release kinetics** – this kinetics depends on concentration.

c) **Higuchi's kinetics** – It is also known as the Higuchi's classical diffusion equation/ Higuchi matrix. In this system, the drug release is related to Fickian diffusion through insoluble matrix.

d) **Korsmeyer Peppas exponential kinetics** – This model is very valuable once the release mechanism is unidentified or when additional type of drug release mechanism is involved.

5.6 Formulation and evaluation of dosage form [119]:

5.6.1 Introduction:

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, polymorphism, pH to form the final drug product. It is a carrier system for drug to reach in the site of action[2]. 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. The conventional marketed formulation of Bendamustine is marketed as lyophilized powder which is very much suitable for intravenous administration.

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.

5.6.2 lyophilized Powder

Lyophilization technique was used for get rid of water from test sample by freezing it, then the ice form was changed directly from solid to vapor by skipping the liquid phase. It was found that the injection routes have significant influence on pharmacokinetics of drug. The lyophilized powder is best for parenteral preparation as it is easily reconstituted with water for injection.

Advantages of Lyophilized powder:

- Lyophilization is required when bulk drug ingredient is not stable in liquid or frozen form.
- Processing a liquid with ease so it is compatible with aseptic operation
- It is necessary to enhance the stability of dry powder dosage form.
- Dissolution of final product is very rapid,

5.6.3 Formulation of lyophilized powder of bendamustine loaded chitosan and PLGA nanoparticle:

Both the nanocarrier system (NPS-4, NPS-8) was formulated into dry lyophilized powder by (Heto Power Dry LL 3000 freeze dryer, Stuttgart, Germany) lyophilizer at temperature -40^0C and pressure 0.10 mbar for 48 hours for BM- PLGA nanoparticle and -35ºC for 60 hours. The lyophilized powder was prepared and stored for further study.

5.7 Evaluation of prepared lyophilized formulations:

The prepared lyophilized powder formulations were estimated for following parameter:

- Drug Content
- Mean particle size and Zeta Potential

- Entrapment efficiency
- In-vitro drug release

5.7.1 Drug Content [120]:

The drug content was calculated by 10ml of each formulation was dissolved in 10ml water and set aside for 24 hours. The sample was diluted about 10µg/ml with methanol and scanned by using UV-Spectrophotometer. The absorbance was noted at 329 for BM.

5.7.2 Drug entrapment efficacy [121]:

Percentage of entrapment efficacy of each formulation was based on this formula

Percentage of entrapment efficacy= total drug added-free non-entrapped/total drug added

5.7.3 Particle size Distribution and Zeta Potential [122]:

The particle size distribution, and zeta potential of chitosan, PLGA-based formulations were calculated by DLS using Malvern Instruments. Analysis was performed in a triplicate manner at an angle of 90^0 at the temperature of 25^0C. Both the testing samples were dispersed in the adequate quantity of pure water before experimentation.

5.7.4 In-vitro drug release [123]:

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.

5.7.5 In-vitro Cytotoxic study [124]

Cytotoxicity study has chosen as a trial model and an significant pointer for cell toxicity assessment because it is very simple, rapid and highly sensitive as it can protect experimental animals from toxicity.

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.

5.7.6 MTT Assay [125]

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. Serial dilution was done in triplicate and again incubated for another 48 hours in specified conditions. After that the cells were treated with MTT then after 4.5 hours the complete medium was drawn out from the wells. The absorbance was measured at 570 nm through 96 well microplate readers. The percentage of cell viability was estimated through the following formula:

$$Cell\ Viability\ \% = \frac{\text{Absorbance of Sample}}{\text{Absorbance of control}} \text{ x } 100$$

5.7.7 IC_{50} calculation [126]

IC_{50} was calculated according to the drug concentration at which the cell viability reduced to 50 %.

5.7.8 Stability study [127]

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. In this study product should be monitored, whether any physical, chemical, biological, and microbiological changes is occurring or not in a fixed interval of time. The formulations of bendamustine were studied as per the ICH guidelines 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.