LYOPHILIZATION TECHNOLOGY FOR THE DESIGN, DEVELOPMENT, OPTIMIZATION AND EVALUATION OF AN ANTICANCER DRUG: DACARBAZINE

Sazib Md. Saifuzzaman[1], Talukder Parimal[2], Molla Md. Islam[3], Mrityunjoy Biswas[4]

[1]Department of Pharmacy, Jagannath University, Dhaka, 1100, Bangladesh.
[2]Research and Development Division, Beacon Pharmaceuticals Ltd, Mymensingh, 2240, Bangladesh.
[3] Department of Pharmacy, Jagannath University, Dhaka, 1100, Bangladesh.
[4] Dept. of Microbilogy And Immunology, Aichi Medical University, Aichi, Japan

Abstract

To improve the bioavailability, stability, solubility and patient compliance lyophilized injection dosage form is extensively used in pharmaceutical industry. For better patient satisfaction particularly in bed ridden patients and for achieving maximum bioavailability the lyophilized injection has been considered as substitute to oral solid dosage forms. Lyophilization or freeze drying involves the elimination of water or other solvent from a frozen product by a process called sublimation followed by desorption. Freeze drying is a multistage process in which, quite obviously, each stage is critical. The key actors of this scenario are all recognized and should be under strict to complete a successful operation. Lyophilized products for parenteral use are known as Powders for injection or infusion. Dacarbazine is an alkylating agent administered as a first-line treatment for metastatic melanoma. As the stability of Dacarbazine in aqueous solution form was unstable it was formulated as Lyophilized product. The objective of the present study was to develop a stable lyophilized formulation of drug Dacarbazine for injection (200mg/vial).

Keyword: Lyophilized injection, Bioavailability, Metastatic Melanoma, Dacarbazine.

1.INTRODUCTION

Lyophilization is an essential need in pharmaceutical modern technology where heat-sensitive drugs and biologicals are allowed to dry at low temperature under conditions that permits elimination of water by sublimation. While the most common application of pharmaceutical freeze-drying is in the production of injectable dosage forms, the process is also used in the production of diagnostics. [1] About 50% of the currently marketed biopharmaceuticals are lyophilized, representing the most common formulation strategy. [2]

1.1. Preferred Characteristics of a Freeze-Dried Pharmaceutical Dosage Form

- A freeze-dried product is expected to contain almost full recovery of the original substance or biological assay after reconstitution.
- Cake should be intact occupying the same shape and size as the original frozen mass.
- Have sufficient mechanical strength to prevent cracking, powdering or collapse.
- Uniform color and pharmaceutically elegant appearance.
- The product should be sufficiently dry to maintain stability and sufficiently porous which leads to rapid and complete dissolution.

The desired properties can be attained by appropriate formulation of the product and by employing optimum freeze-drying cycles. [3, 4]

1.2. Cancer and Anticancer Agents

Cancer is a disease of uncontrolled cell division, invasion and metastasis. It is generally considered to be due to the clonal expansion of a single neoplastic cell. However there may be additional somatic leading to heterogeneous cell population. [5] The drugs which are commonly used in treatment of cancer include Dacarbazine, carboplatin, methotrexate, bleomycin, mitomycin, oxaliplatin, gemcitabine, epirubicine etc. The main side effect of chemotherapy includes nausea and vomiting, hair loss, anaemia etc. [6] Dacarbazine is an alkylating agent approved to treat metastatic melanoma in humans. This has been administered as a first-line treatment for metastatic melanoma since the 1970s. [7] Dacarbazine (DBZ) is an anti–cancer drug used for the treatment of metastatic malignant melanoma and Hodgin's disease. [8] Dacarbazine is also sometimes used in combination with other drugs for soft tissue sarcoma. [9] Dacarbazine is supplied as a sterile, lyophilized powder that can be reconstituted for intravenous injection. [10, 11]

2.AN INNOVATIVE APPROACH: LYOPHILIZATION TECHNOLOGY

The main principle involved in Lyophilization or Freeze-Drying is a unit operation in which water or solvent is removed from a product after it is frozen and placed under a vacuum, allowing the ice to change directly from solid phase to vapor without passing through a liquid phase (sublimation) depicted in Figure-1. In this technology, the water content of the product is reduced to such a low level that does not support biological growth or chemical reactions. [3, 12-15]

A typical freeze-drying process consists of three separate, unique and inter-dependent processes namely

- Freezing (solidification)
- Primary drying (sublimation)
- Secondary drying (desorption)

2.1. Freezing

Lyophilization is a drying process in which freezing step is an important step that impacts both process performance and product quality. [16] Freezing is generally the first step in a freeze-drying process, in which nearly 90% of the water is converted to ice crystals while all solutes in the formulation are solidified into a matrix either in amorphous or crystalline state, or in a mixture. It is very important in freeze drying to refreeze the product to below the eutectic temperature before beginning the freeze-drying process. When the frozen product is a suspension that undergoes glass formation during the freezing process. In lieu of forming eutectics, the total suspension becomes gradually more viscous as the temperature is lowered. To freeze dry this type of product is really difficult. [17]

2.1.1. Process Design and Control

During freezing, the chamber pressure is slightly lower than the atmospheric pressure due to low temperature, or/and pre reduction of chamber pressure to enhance the sealing of the chamber door. The stage of the process is generally controlled by the shelf-cooling/heating rate, shelf-holding temperatures, and holding times. [3]

2.1.2. Pre-freezing Hold

In order to facilitate relatively uniform ice nucleation and ice crystal growth, the product vials on the shelf are held at a temperature lower than room temperature before cooling down. This temperature is generally the loading temperature.

2.1.3. Cooling Down to the Final Freezing Temperature

Cooling the product to a terminal (final) freezing temperature facilitates the ice nucleation/growth and solute solidification. If a super-cooling hold is applied, a relatively faster cooling is generally helpful for intra-vial uniformity of ice formation.

2.1.4. Annealing

Annealing is simply holding the product at a temperature above the final freezing temperature for a defined period to crystallize the potentially crystalline components (usually, crystalline bulking agent) in the formulation during the freezing stage. An annealing step is frequently necessary to allow efficient crystallization of the crystalline bulking agent, such as mannitol or glycine. After annealing, the product temperature is generally lowered to a final temperature and held long enough to complete solidification.[4] Annealing is performed to optimize the primary drying rate, reduce freezing-induced drying rate heterogeneity, and determined Tg in pharmaceutical lyophilization. [18]

2.2. Primary Drying

After freezing, the product is ''dried'' at relatively low temperature and low pressure in which ice can be removed from the frozen product via sublimation, resulting in a dry, structurally intact product. It is extremely important that the temperature at which a product is freeze-dried is balanced between the temperature that maintains the frozen integrity of the product and the temperature that maximizes the vapor pressure of the product. This balance is the key to optimum drying. Energy supplied to sublime a gram of water from the frozen to the gaseous state as is (2700 joules per gram of ice). [17]

2.2.1. Principle of Primary Drying Process

2.2.1.1. Heat Transfer

Heat supplies the energy sufficient for the evaporation of water by sublimation. More energy is required for the transportation of water molecules to the surface. Heat transfer to the product achieves in three ways; direct conduction, gas conduction and radiation. During freeze drying heat and mass transfer scale-up issues control the degree of super cooling. [19]

2.2.1.2. Mass Transfer

The mass transfer processes influence the rate of primary drying during lyophilization. [20] The heat and mass transfer cause the top of the product to dry first with drying proceeding downward to the bottom of the vial. As the dried layer increases, it becomes a great barrier or the source of greatest resistance to the transfer of mass out of the vials.[4]Freeze Drying deals the movement of heat and mass to and from the drug under preparation, respectively, so it is important to scale these transport phenomena properly from pilot plant to manufacturing-scale area to preserve product quality attributes. The lyophilization cycle is then successfully demonstrated at target parameter set-point values. [21]

2.2.2. Controlling Parameters for Primary Drying

2.2.2.1. Target Product Temperature and Structural Collapse

Collapse temperature for an amorphous system refers to the temperature, above which the dried region adjacent to ice loses its structure, that is; collapse temperature (Tc) can be higher than the glass transition temperature (Tg). A safety margin should be kept during primary drying, that is, the product temperature (Tp) should be 2–5ºC below Tc or Te. Thus, to gain a better understanding of collapse behavior and therefore the opportunity to further optimize formulations and freeze drying cycles and to evaluate the transferability of collapse temperatures measured by Freeze Drying Microscopy (FDM) on freeze drying processes. [22]

2.2.2.2. Chamber Pressure

A most efficient primary drying condition for the product in a given vial should be a combination of a ''high'' shelf temperature with a ''low'' chamber pressure.

2.2.2.3. Shelf Temperature

The shelf temperature is higher than the product temperature and sometimes can be much higher, up to 40ºC. [23]

2.2.3. Parameters must be monitored in Primary Drying

2.2.3.1. Determination of End Point of Primary Drying

Product temperature end of primary drying set point Frozen product will have a lower temperature than the temperature-controlled shelf. We can assume that, when the shelf temperature and product temperature is same and the temperature reaches above 0^0C there will be no ice. So, the product reaches the end point of primary drying. [3]

2.2.3.2. Ramp from Freezing to Primary Drying

After evacuation to reduce the chamber pressure to the target level, the shelf temperature is ramped up to the target value. The ramp rate should not be too high, normally less than 1ºC/min. During this initial period of sublimation, ice sublimation rate can be quite high, since the resistance in the product is nearly zero. [3]

2.2.3.3. Dew Point via Moisture Sensor (Moisture Sensor is required)

To determine residual moisture content of the product, a moisture sensor may use. Moisture sensor is recorder in dew point (deg C). It can determine presence of liquid or ice in an amount less than 1%. A sharp decrease in dew point gives the indication of change of the ice to vapor at the end point of primary drying stage. [24]

2.2.3.4. Barometric Pressure Rises (Isolation Valve is required)

Barometric pressure rise happens when the ice undergo sublimation and convert to vapor. When freeze drying chamber is isolated from the condenser and vacuum pump, the vapor pressure leads to a rise in vacuum. When ice is present in the chamber, pressure will rise faster than without ice in the chamber and this indicates that the process not yet reached the end point. The pressure rise slows down when less ice presents in the chamber. The acceptable range of pressure rise to determine the end point of primary drying is less 6mbar in 30 seconds in 3 or more readings in an hour. [24]

2.2.3.5. Duration of Primary Drying

The duration of primary drying is determined by the ice sublimation rate, the characteristics of formulation solution and can be roughly estimated theoretically by calculations based upon the mass and heat transfer equations. In practice, the duration is determined by monitoring the drying progression. [3]

2.3. Secondary Drying

When all ice crystals are removed from the product by sublimation, the dried product contains a fairly high amount of ''unfrozen water'' (5–20% in the solid content). In the secondary drying stage, the unfrozen water is further reduced to a desired, much lower level at a higher temperature. The glass transition temperature (Tg) of the dried formulation is a function of the moisture content, which is governed by the Gorden-Taylor equation. Therefore, the Tg changes sharply with the decrease of moisture during the ramp from primary drying to secondary drying, and during secondary drying. [3]

2.3.1. Design of Secondary Drying Process

2.3.1.1. Heating Rate and Chamber Pressure

Because of the fairly high residual moisture content in the amorphous product early in secondary drying and, thus, low glass transition temperature, the potential for collapse is greatest early in secondary drying. So, a ramp rate of 0.1 or 0.15°C/min for amorphous products is generally safe and appropriate.

During secondary drying, crystalline products do not have any probable for collapse, and a higher ramp rate is recommended for such products (0.3 or 0.4°C/min). [24]

2.3.1.2. Shelf Temperature and Secondary Drying Time

For a period sufficient the products should be kept at "high" temperature to permit the preferred water desorption. Usually, to run a high shelf temperature for a short time is better than a low temperature for a long period. Amorphous products are more difficult to dry

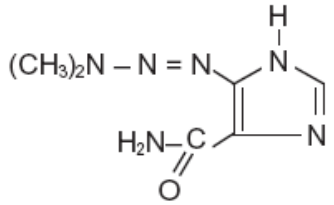
than crystalline products. Thus, higher temperatures and longer times are needed to remove the absorbed water.

The secondary drying conditions also depend on the solute concentration. The dry product has smaller specific region at higher solute concentration (i.e., >10% solids in solution) and it is more complicated to eliminate the absorbed water; thus, longer times and/or higher temperatures are required to complete secondary drying. [25] In general, drying times of 4–10 h at the range 40 to 50°C.The optimum secondary drying time can be determined by real-time residual moisture measurement using Karl Fischer titration (KF), thermal gravimetric analysis (TGA), or near IR spectroscopy. [26] At very low residual water content (about 0.5%) the product is usually freeze dried. Usually, a combination of long drying times (6 h) and low shelf temperature (about 0°C) are best, but the exact conditions must be determined by trial and error. [27]

3.MATERIALS AND METHODS

3.1. Profile of Active Substance

The molecular formula of Dacarbazine is $C_6H_{10}N_6O$. Its molecular weight is 182.2. The CAS Registry number of Dacarbazine is 4342-03-4. The structural formula of Dacarbazine appears below:



[Chemical name: 5-(3, 3-dimethyl-l-triazeno)-imidazole-4-carboxamide]

Table No.1: Physicochemical Properties of Dacarbazine

Test Parameters	Specifications	
Appearance	White or slightly yellowish, crystalline powder.	
Identification	A. Infrared absorption spectrum	
	B. UV-Vis spectroscopy	
	C. Thin-layer chromatography	
Solubility	Slightly soluble in water and in anhydrous ethanol, practically insoluble in methylene chloride.	
Appearance of Solution	Clear and not more intensely colored than reference solution.	
Water Content	Not more than 0.5%	
Sulfated Ash	Not more than 0.1%	
Related Substances	i) Impurity A	i) Not more than 0.2%
	ii) Unspecified Impurities eluting after Impurity A	ii) Not more than 0.1%
	iii) Impurity B	iii) Not more than 0.1%
	iv) Unspecified Impurities	iv) Not more than 0.1%
	v) Impurity D	v) Not more than 0.05%
	vi) Total Impurities	vi) Not more than 0.5%
Bacterial Endotoxin Limit	Not more than 0.20 EU per mg of Dacarbazine.	
Assay	98.5% to 101.0% of Dacarbazine on the anhydrous basis.	
Stability, Handling and Storage	Unstable; Wear suitable protective clothing and gloves, Respiratory protection is required when dusts are generated; Keep container tightly closed and desicated. Keep container at 2˚C to 8˚C, airtight container. Protect from light. Store in light-resistant containers.	

3.2. Excipients Profile

Substances, other than the active ingredient, which have been appropriately evaluated for safety and are included in a drug delivery system to provide support.

The excipients used must have following characteristics-

- They must be stable both physically, chemically and must be biologically inactive.
- It must be free from microbial contamination.
- Excipients used in formulation must be accepted by regulatory agencies and should meet the entire current regulatory requirement.

The excipients (Mannitol and Citric Acid Anhydrous) and solvent (Water for Injection) which are selected for the present study. In lyophilized preparations, Mannitol (20–90% w/w) has been included as a carrier to produce a stiff, homogeneous cake that improves the appearance of the lyophilized plug in a vial. It occurs as a white, odorless, crystalline powder, or free flowing granules. It is stable in the dry state and in aqueous solutions. The bulk material should be stored in a well-closed container in a cool, dry place. [28]

Citric acid (as either the monohydrate or anhydrous material) is widely used in pharmaceutical formulations and food products, primarily to adjust the pH of solutions. Citric acid monohydrate occurs as colorless or translucent crystals, or as a white crystalline, efflorescent powder. It is potentially explosive in combination with metal nitrates. On storage, sucrose may crystallize from syrups in the presence of citric acid. [28]

3.3. Critical Product Temperature Determination

Based on the outcomes of the solvent selection studies, the optimized bulk solution was subjected for freeze drying microscopic studies for determination of critical product temperature. The prepared formulation was subjected for Lyostat freeze drying microscope, equipped with Linksys32 image and data capture software. [29, 30]

3.4. Manufacturing Procedure

3.4.1. Formulation of Dacarbazine 200 mg Injection

Different formulations of pre-lyophilized Dacarbazine 200 mg injection were prepared with different concentration of Mannitol, Citric Acid Anhydrous and WFI. Take Water for Injection USP into SS Manufacturing vessel and purge Nitrogen for 1 hour at 25 °C to 30°C temperature. Dissolve Mannitol BP in freshly taken WFI into a SS beaker and transfer the solution into the SS Manufacturing vessel. Then dissolve Citric Acid Anhydrous BP in freshly taken WFI into a SS beaker and continue stirring until clear solution found and transfer the solution into the Manufacturing vessel. Collect solution from the Manufacturing vessel to dissolve Dacarbazine BP inside the isolator. Stirrer manually for 10 minutes to make slurry or clear the solution. Then transfer the solution into the Manufacturing vessel and stir at 30 frequency speed for 30 minutes. To check the pH of the solution take solution from manufacturing vessel. Finally adjust the volume with WFI USP & stir at 30 rpm for 10 min. To filter the solution use (0.45µ + 0.2µ) membrane filter under nitrogen pressure at 1 to 2 bars. The filtered solution was filled into 30 mL of amber colored USP-Type-1 vial with target fill volume of 15 mL with pre and post purging of Nitrogen half stoppered, the vials with 20 mm Lyophilized stoppers. Filled vials are loaded into the lyophilizer and lyophilized the vials as per cycle conditions.

Table No.2: Formulation of Dacarbazine 200 mg Injection

Material Name	F1	F2	F3	F4	F5
Dacarbazine	200 mg	200 mg	200 mg	200 mg	200 mg
Citric Acid Anhydrous	100 mg	150 mg	150 mg	200 mg	200 mg
Mannitol	50 mg	50 mg	75 mg	100 mg	75 mg
Water for Injection	q. s. to 10 mL	q. s. to 20 mL	q. s. to 18 mL	q. s. to 15 mL	q. s. to 15 mL

3.4.2. Development and Optimization of Lyophilization Cycles

Various lyophilization cycles were evaluated for optimizing the desired cycle to get the consistent product by modifying the different ramp, hold rates of temperature and vacuum. Several lyophilization cycles were evaluated but only four typical lyophilization cycles were presented in Table No. 3, 4, 5 & 6.

Table No.3: Lyophilization cycle for Dacarbazine 200 mg Injection (Trial-1)				
Step Type	Temperature	Vacuum Pressure	Ramp Time	Hold Time
Freezing	-10 ºC		20 min	40 min
Freezing	-45 ºC		40 min	03 hrs
Freezing	-15 ºC		40 min	03 hrs
Freezing	-45 ºC		01 hr	04 hrs
Evacuation		300 µ bar		
Drying	-10 ºC	300 µ bar	01 hr	18 hrs
Drying	+15ºC	250 µ bar	01 hr	05 hrs
Drying	+20 ºC	200 µ bar	01 hr	05 hrs
Entire Cycle Duration			44 hrs 20 min	

Table No.4: Lyophilization cycle for Dacarbazine 200 mg Injection (Trial-2)				
Step Type	Temperature	Vacuum Pressure	Ramp Time	Hold Time
Freezing	-10 ºC		20 min	40 min
Freezing	-45 ºC		40 min	03 hrs
Freezing	-15 ºC		40 min	03 hrs
Freezing	-45 ºC		01 hr	06 hrs
Evacuation		300 µ bar		
Drying	-10 ºC	250 µ bar	01 hr	18 hrs
Drying	+15 ºC	200 µ bar	01 hr	05 hrs
Drying	+20 ºC	150 µ bar	01 hr	05 hrs
Entire Cycle Duration			46 hrs 20 min	

Table No.5: Lyophilization cycle for Dacarbazine for Injection 200 mg (Trial-3)				
Step Type	Temperature	Vacuum Pressure	Ramp Time	Hold Time
Freezing	-10 ºC		20 min	01 hr
Freezing	-15 ºC		40 min	05 hrs
Freezing	-30 ºC		40 min	07 hrs
Evacuation		300 µ bar		
Drying	-10 ºC	250 µ bar	01 hr	18 hrs
Drying	+10ºC	200 µ bar	01 hr	07 hrs
Drying	+20 ºC	150 µ bar	01 hr	05 hrs
Entire Cycle Duration			47 hrs 40 min	

Table No.6: Lyophilization cycle for Dacarbazine for Injection 200 mg (Trial-4)				
Step Type	Temperature	Vacuum pressure	Ramp Time	Hold Time
Freezing	-10 ºC		40 min	02 hrs
Freezing	-15 ºC		01 hr	05 hrs
Freezing	-27 ºC		01 hr	08 hrs
Evacuation		300 µ bar		
Drying	-10 ºC	250 µ bar	30 min	14 hrs
Drying	0 ºC	200 µ bar	40 min	12hrs
Drying	+10 ºC	200 µ bar	40 min	06 hrs
Drying	+25 ºC	150 µ bar	01 hr	06 hrs
Entire Cycle Duration			58 hrs 30 min	

Finally, the optimized lyophilized cycle was stated in Table No. 7 and figure-1.

Table No.7: Optimized Lyophilization cycle for Dacarbazine for Injection 200 mg				
Step Type	Temperature	Vacuum pressure	Ramp Time	Hold Time
Freezing	-10 ºC		40 min	02 hrs
Freezing	-18 ºC		01 hr	04 hrs
Freezing	-25ºC		01 hr	08 hrs
Evacuation		300 µ bar		
Drying	-10 ºC	250 µ bar	30 min	14 hrs
Drying	0 ºC	200 µ bar	40 min	08 hrs
Drying	+10 ºC	200 µ bar	40 min	06 hrs
Drying	+20 ºC	150 µ bar	01 hr	07 hrs
Entire Cycle Duration			54 hrs 30 min	

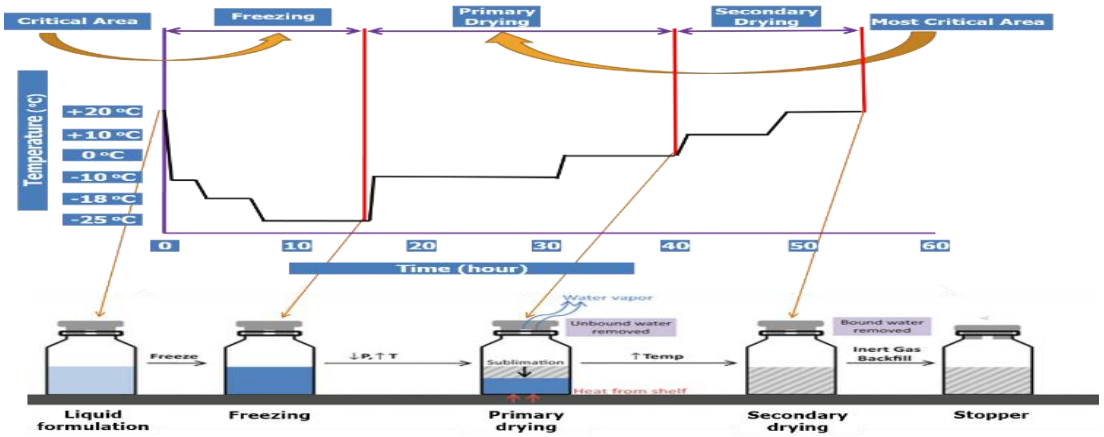


Figure No.1: Lyophilization of Dacarbazine 200 mg Injection

3.4.3. Acceptance Criteria for the Evaluation of Lyophilized Product

The lyophilized product was evaluated for these formulation characteristics such as appearance, clarity of reconstituted solution, reconstitution time, pH after reconstitution, water content, assay, related substances, sterility, particulate matter etc. Acceptance criteria for the Evaluation of Dacarbazine 200 mg Injection are presented in Table No. 8.

Table No.8: Acceptance criteria for the Evaluation of Dacarbazine 200 mg Injection

Parameter	Limit
Appearance	**Cake form:** A lyophilized mass contains in amber USP type-1 glass vial with lyophilized rubber stopper and red colored flip-off sealed down aluminium cap.
	After reconstitution: After reconstitution with 19.7 mL of Water for Injection, a clear colorless solution appears that must be free from any visible foreign particles.
Moisture Content	Not more than 1.5%
Pressure Rise Test (PRT)	10 µbar in 1 minute
pH	3.0-4.0
Assay	Content of Dacarbazine per vial: 180.0 mg to 220.0 mg
Particulate Matter	Particle size≥10 µm: 6000 Particles/ vial
	Particle size≥25 µm: 600 Particles/ vial
Sterility	Must be sterile
Bacterial Endotoxin	Not more than 0.20 EU/ mg

3.4.4. Stability Studies as per ICH guidelines

Stability studies provide evidence on how the quality of the drug product or substance varies with time under the influence of a variety of environmental factors such as temperature, humidity and light. These studies establish there-test period of the drug product, shelf life for the drug product and recommended storage conditions.

So, in the present study, the stability of the drug product is assessed by exposing the product to various temperatures and humidity conditions.

Accelerated study: The product was subjected to accelerated stability studies at 25° C±2 C/65% ±5% RH for 6 months. The frequency of testing at the Accelerated study condition includes 0, 1, 2, 3, 6 months.

Long term study: The product was subjected to long term studies at (2 – 8)° C for 12 months. The frequency of testing at the long term storage condition is for every 3 months over the first year, every 6 months over the second year. [31]

4.RESULTS AND DISCUSSION

The results of the trial batches are stated in the Table-9.

Table No.9: Result of the trial batches					
Batch No.	Description of Cake	pH	Water Content	Reconstition Time	Assay
F1	White lyophilized cake but few were collapsed	4.56	2.88%	2 min 05 sec	100.1%
F2	White lyophilized cake but few were collapsed	4.25	2.15%	1 min 12 sec	99.3%
F3	White lyophilized cake	4.11	0.79%	45 sec	102.2%
F4	White lyophilized cake	3.85	0.08%	22 sec	101.3%

From the results of Trial-1, F1 formulation was formulated as mentioned in the Table-2. The F1 formulation was then subjected to lyophilization as per the conditions shown in Tables-3. After lyophilization, the cake was found to be good but water content and reconstitution time obtained were not satisfactory.

From the results of Trial-2, F2 formulation was formulated as mentioned in the Table-2. The F2 formulation was then subjected to lyophilization as per the conditions shown in Tables-4. After lyophilization, the water content of the formulations was found to be within the prescribed limit but reconstitution time and residual solvents were out of limits. The possible reason could be due to insufficient duration of cycle, insufficient drying temperature and time.

From the results of Trial-3, F3 formulation was formulated as mentioned in the Table-2. The F3 formulation was then subjected to lyophilization as per the conditions shown in Tables-5. After lyophilization, the water content of the formulations was found to be within the prescribed limit but reconstitution time and residual solvents were out of limits.

From the results of Trial-4, F4 formulation was formulated as mentioned in the Table-2. The F4 formulation was then subjected to lyophilization as per the conditions shown in Tables-6. After lyophilization, the water content of the formulations was found within the prescribed limit, the reconstitution time was 22 sec and residual solvents were within limits.

From the results of optimization batch, F5 formulation was formulated as mentioned in the Table No. 2. The F5 formulation was then subjected to lyophilization as per the conditions shown in Tables No. 7.

Table No.10: Six Months Stability Study							
Frequency	**Initial**	**1 Month**		**3 Months**		**6 Months**	
Parameters	Initial Result	25°C/60%RH	5°C ±3°C	25°C/60%RH	5°C ±3°C	25°C/60%RH	5°C ±3°C
Appearance	Complies	Complies	Complies	Complies	Complies	Complies	Complies
Identification	Confirm	Confirm	Confirm	Confirm	Confirm	Confirm	Confirm
pH	3.71	3.69	3.69	3.78	3.78	3.75	3.77
Particulate Matter	122/Vial	Not Applicable	Not Applicable	Not Applicable	Not Applicable	141/Vial	126/Vial
	80/Vial					109/Vial	79/Vial
Sterility Test	Sterile	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Sterile	Sterile
Bacterial Endotoxin	<0.20 EU/mg	Not Applicable	Not Applicable	Not Applicable	Not Applicable	<0.20 EU/mg	<0.20 EU/mg
Assay (per Vial)	209.41 mg	206.95 mg	205.06 mg	205.48 mg	201.40 mg	201.50 mg	202.31 mg
Assay (%)	104.71%	103.47%	102.53%	102.74%	100.70%	100.75%	101.16%

From the accelerated and long term stability study of the optimized batch results confirm that the test parameters like pH after reconstitution, reconstitution time, water content, assay, osmolality and related substances were found to be within the specification limits. So, it can be concluded that the lyophilized drug was stable at accelerated and long term storage conditions of 25°C/65 % RH and (2-8)°C respectively for a period of 6 months. Six months stability study data are mentioned in the Table No. 10.

5.CONCLUSION

To put in a nut shell, our goal was to develop and optimize the Dacarbazine 200 mg injection by lyophilization technique due to its instability in liquid state. Finally, Dacarbazine 200 mg injection developed as lyophilized drug successfully to improve its stability and bioavailability. The batch was optimized and evaluated through trial error basis and six months stability studies. However, further investigation is needed to reduce the lyophilization process time.

6.ACKNOWLEDGEMENT

First of all, all praises be to the Almighty, who enabled me to undertake & complete this work & finally write up the outcome of the work. Individual efforts alone can never contribute in totality to a successful completion of any venture. I would be failing in my duty if I did not state my gratitude & appreciate to the following individuals who have made valuable contribution toward the work. I would like to express my deep gratitude & warm regards to the authorities of the Department of Pharmacy, Jagannath University and Beacon Pharmaceutcals Ltd., for extending full laboratory facilities.

REFERENCES

[1] Jose, A.V., Pamella, S.O. and Paul, H. Introduction to parenteral Preparation, pp.2-7.

[2] Nail, S.L. and Gatlin, L.A., 1993. Freeze-Drying: Principles and Practice, in Avis, K.E., Lieberman, H.A. and Lachman, L. (eds): Pharmaceutical Dosage Forms: Parenteral Medications, Marcel Dekker, New York, 2, pp.163–233.

[3] Gerald, A. The Principles of Freeze-Drying, Methods in Molecular Biology, vol. 368: Cryopreservation and Freeze-Drying Protocols, Second Edition, Edited by: Day, J.G. and Stacey, G.N., 2007, pp.15-38.

[4] Lueckel, B., Bodmer, D., Helk, B. and Leuenberger, H., 1998. Formulations of sugars with amino acids or mannitol-influence of concentration ratio on the properties of the freeze-concentrate and the lyophilizate. Pharm. Dev. Technology, pp.325–336.

[5] www.drugs.com/cancer.html (Date of Access 24/11/2010)

[6] www.answers.com/topic/antineoplastic-agents (Date of Access 19/11/2010)

[7] Gerner, R.E., Moore, G.E. and Dickey. C., 1975. Combination chemotherapy in disseminated melanoma and other solid tumors in adults. Oncology, 31, pp.22–30

[8] Bonifazi, E., Angelini, G. and Meneghini, C.L., 1981. Adverse Photo Reaction to Dacarbazine (DTIC). Contact Dermatitis, 7(3), p.161.

[9] Nussbaumer, S., Bonnabry, P., Veuthey, J. and Fleury-Souverain, S., 2011. Analysis of anticancer drugs: A review. Talanta, 85, pp.2265-2289.

[10] Tsuji, T., Ohtsubo, T., Umeyama, T., Sudou, M., Komesu, K., Matsumoto, M., Yoshida, Y., Banno, R., Mikami, T. and Kohno, T., 2014. Methods for reducing Dacarbazine photodegradation and its accompanying venous pain. Yakugaku Zasshi, 134(9), pp.981-986.

[11] Asahi, M., Matsushita, R., Kawahara, M., Ishida, T., Emoto, C., Suzuki, N., Kataoka, O., Mukai, C., Hanaoka, M., Ishizaki, J., Yokogawa, K. and Miyamoto, K., 2002. Causative agent of vascular pain among photodegradation products of Dacarbazine. Journal of Pharmacy and Pharmacololgy, 54(8), pp.1117-1122.

[12] Karger, S. and Basel Rey, L.R., 1977. International Symposium on freeze-drying of biological products, Washington, D.C. Develop. Bio. Standard, 36, p.19.

[13] Bindschaedler, C. Lyophilization process validation. In: Freeze-Drying/Lyophilization of Pharmaceutical and Biological Products, (Rey, L. and May, J.C., eds.), Marcel Dekker, New York. 1999, pp.373–408.

[14] Jinsong, L. Freeze-Drying, Pharmaceuticals, Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology, Edited by Michael, C. and Flickinger, pp.1-18.

[15] Guide to inspections of lyophilization of parenterals, Office of regulatory affairs, U.S Food and Drug Administration. Available at: http://www.fda.gov/ICECI/Inspection/InspectionGuides/ucm074909.html.

[16] Julia Christina, K. and Wolfgang, F., 2011. A Review on The Freezing Step in Lyophilization: Physico-Chemical Fundamentals, Freezing Methods and Consequences on Process Performance and Quality Attributes of Biopharmaceuticals.

[17] Rondol, A., 2006. Pharmaceutical freeze-drying - a comprehensive course reviewing freeze drying technology, Mumbai, Biopharma Technology Limited.

[18] Searles, J.A., 2004. Freezing and Annealing Phenomena in Lyophilization, in Rey, L. and May, J.C. (Eds.) Freeze-Drying/Lyophilization of Pharmaceutical and Biological Products, Marcel Dekker, Inc. USA, New York.

[19] Rambhatla, S., Ramot, R., Bhugra, C. and Pikal, M.J., 2004. Heat and mass transfer scale- up issues during freeze drying: II. Control and characterization of the degree of supercooling. American Association of Pharmaceutical Scientists (AAPS). PharmSciTech, 4, p.58.

[20] Marco, P.S. and Bryan, K.B., 1999. Simulation model for the primary drying phase of the freeze-drying cycle. International Journal of Pharmaceutics, 114, pp.159-170.

[21] Tsinontides, S.C., Hunke, W.A. and Reynolds, S.D., 2004. Freeze drying—principles and practice for successful scale-up to manufacturing. International Journal of Pharmaceutics, 280, pp.1-16.

[22] Meister, E. and Gieseler, H., 2006. Collapse Temperature Measurement by Freeze-Dry Microscopy and Transferability to Freeze Drying Processes: Influence of Solute Concentration on Collapse Behavior and Effect on Cycle Design. Process. American Association of Pharmaceutical Scientists (AAPS) Annual Meeting and Exposition, San Antonio (TX). USA.

[23] Pikal, M.J., Roy, M.L. and Shah, S., 1984. Mass and Heat transfer in vial freeze-drying of pharmaceuticals. Role of the vial. Journal of Pharmaceutical Sciences, pp.1224–1237.

[24] Sajel, M. P., Takayuki, D. and Michael, J. P., 2010. Determination of end point of primary drying in freeze-drying process control. AAPS Pharm Sci Tech, 11(1), pp.73-84

[25] Xiaolin (Charlie), T. and Michael, J. P., 2004. Design of Freeze-Drying Processes for Pharmaceuticals: A Practical Advice. Pharmaceutical Research, 21.

[26] Pikal, M.J., 2002. Heat and Mass Transfer in Low Pressure Gases: Applications to Freeze Drying, pp.610-680.

[27] Hageman, M.J., 1988. The role of moisture in protein stability, Drug Dev. Ind. Pharma, pp.2047–2070.

[28] Paul. J.S. and Raymond. C.R., 2009. Hand book of excipients sixth edition Pharmaceutical Press and American Pharmacists Association, pp.424-428.

[29] Milton, N., Pikal, M.J. and Roy, M.L., 1997. Evaluation of manometric temperature measurement as a method of monitoring product temperature during lyophilization. PDA J. Pharm. Sci. Technol., 51, pp.7-16.

[30] Victor, A.L. and Ellen, M.L., 2009. Dissolution and stability of carmustine in the absence of ethanol. Selective cancer therapeutics, 5(1), pp.33-35.

[31] ICH harmonized tripartite guideline, Q1A (R2): Stability testing of new drug substances and products.