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ORIGINAL ARTICLE

Formulation and Evaluation of an Antineoplastic Lyophilized Dosage Form

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ABSTRACT

Antineoplastic or Anticancer drugs are the drugs that prevent or inhibit the maturation and proliferation of neoplasm. Antineoplastic agents travel the body and destroy cancer cells. The aim of present research work was to formulate aqueous injection of Bleomycin Sulphate, which can be administered via intramuscular, intravenous, subcutaneous, intra-arterial, or intrapleural routes. The stability of Bleomycin sulphate aqueous injection was increased using lyophilization technology. The process involved dissolving Bleomycin sulphate in water, measuring pH, preparing the final volume, filtering, filling to vials, and subjecting the solution to varying cycles of freezing and holding times, primary and secondary drying times, and vacuum variation. The optimized lyophilization cycle of 38.86 hours was achieved, ensuring the product met all in-vitro parameters as per IP/USP specifications. A short-term accelerated stability study confirmed the formulation's stability. The research developed a stable aqueous injection of Bleomycin Sulphate using lyophilization technology, overcoming challenges like limited stability in sodium chloride solution and hygroscopic nature in dry form. This pharmaceutical formulation is suitable for treating sensitive diseases like neoplastic diseases.

Keywords: Bleomycin; Antineoplastic; Parentral; Stability; Lyophilization

INTRODUCTION

Antineoplastic drugs prevent or inhibit neoplasm maturation and proliferation, destroying cancer cells through their movement within the body[1]. Cancer is caused by uncontrolled cell proliferation, invasion, and metastasis, primarily due to the clonal expansion of a single neoplastic cell[2,3]. Parenteral dosage forms differ from other forms as they are directly injected into the human body[4]. Anticancer drugs are typically administered orally and parenterally, with decreased shelf life associated with parenteral dosage forms[5]. These forms include solutions, liposomes, emulsions, suspensions, nano-systems, microspheres, and powders for injections, which can be reconstituted as solutions[6]. Antineoplastic agents are crucial in cancer therapies and other diseases. Lyophilization, or freeze-drying, is a process where water is sublimed from a composition, allowing unstable pharmaceutical and biological agents to be stored in liquid form. However, most agents require additional ingredients to protect the active ingredient during lyophilization, and reconstituting a lyophilized antineoplastic agent into an aqueous solution can be challenging. Anti neoplastic agents often require additional ingredients to protect the active ingredient during lyophilization[7,8]. Hence there is a need for lyophilization to improve the stability and shelf life of the drug candidate. The research aims to improve the stability and shelf life of a drug candidate through lyophilization. Key objectives include conducting pre-formulation studies, formulating an injectable dosage form, lyophilizing it, developing and optimizing the lyophilization cycle, evaluating the lyophilized product, performing accelerated stability studies, and documenting the results. The study focuses on formulating a generic Bleomycin product in lyophilized injection form and optimizing its preparative lyo cycle.

METHODOLOGY

Material and their sources

Bleomycin sulphate drug and water for injection (WFI) is procured from Strides Technology and Research Centre (STAR), Bangalore

Preformulation of drug

The main purpose of preformulation study is to ascertain that the drug substance complies with the pharmacopoeia's standards.

Analytical method

The selected anticancer drug is specified in the monographs of IP/USP/BP/Ph.Eur. Hence development of new analytical method for the selected anticancer drug is not necessary. Procedure for identification, assay and related substances given in these monographs were followed for the further studies hence methods for analysing where similar in IP/USP/BP/Ph. Eur.

Description

White to off–White powder, characteristic odourless, tasteless, hygroscopic, decomposes under light.

Solubility

A small of Bleomycin sulphate was dissolved in each of the solvents water for injection, Methanol, ether, Chloroform) at 25 °C.

Solution state stability studies

The Drug candidate was dissolved in WFI and stored in vials for 48 hrs at 2-8°C and room temperature and samples were withdrawn and analyzed at regular intervals and assay was carried out.

Melting point

The melting point was determined using melting point apparatus.

PH of the solution at 250C

The pH of the solution was checked with Chemlabs pH instrument and the small quantity of drug is dissolved in 10ml of water.

Loss on drying

The drug was weighed in a bottle and dried in an oven at 60 °C at a pressure not exceeding 0.7 KPa for 3 hours.

IR spectrum

A small quantity of KBr was transferred into agate mortar, triturate evenly and served as the blank disc. About 1% Bleomycin Sulphate was triturate into powdered KBr evenly. The chromatogram of a sample blank disc and sample flake was recorded and identification was done by Jasco FTIR spectrometer. The sample spectrum of Bleomycin Sulphate was compared with standard spectrum of Bleomycin Sulphate USP.

Identification by HPLC

The chromatograms were examined for composition by HPLC. The retention time and size of the two principal peaks in the chromatogram obtained with the test solution are approximately the same as those of the two principal peaks in the chromatogram obtained with the reference solution.

Relative substances by HPLC

The flow rate was set at 1.2 ml/min, with a detection wavelength of 254 nm. An injection volume of 10 microliters is used, and the column temperature is maintained at 35°C with a tolerance of ±1°C. The total run time for the analysis is 80 minutes. A stainless-steel column 20.25m × 4.6mm packed with octadecylsilyl silica gel for chromatography, Kromasil 200 C 18 or equivalent was used. Sodium pentanesulphonate in acetic acid and sodium Edetate, with pH to 4.5 using ammonia, was used as mobile phase A and methanol was used as mobile phase 2.

Assay[9]

Microbiological assay was carried out and values were calculated based on dilution and diameter of zone of inhibition. A graph was plotted by taking natural log concentration of the standard on the X-axis and zone of inhibition in mm on Y- axis. The slope, intercept and the sample concentration (U/mL) was calculated from the graph.

Differential Scanning calorimetry (DSC studies

Differential scanning calorimetry was conducted using DSC Q2000 V24.2 Build 107 Instrument, TA instruments. The mass of empty pan and reference pan were taken into account for calculation of heat flow. The sample mass varied from 3.00-10.00 ± 0.5 mg and it were placed in sealed aluminium pans. The coolant used was liquid nitrogen. The samples were scanned at 5°C/ min from 20° C to 60°C and -60°C. DSC thermograms of pure drug candidate and lyophilized product were recorded.

Lyophilization Procedure

Method of preparation

Entire development was done in Onco dedicated facility formulation development lab under standard Lab conditions. Drug dispensing, formulation, filtration and filling activities were carried out in Isolator. Sterilization of vials, rubber plugs and disinfections of flip off aluminium seals were also carried in FDD autoclave. 80% of water for injection was keep it in a cool place till it reaches room temperature (10 – 20 °C). Drug was added under stirring until a clear solution was obtained. initial pH of the solution was found to be 5.21 (range 4.5-6.0). Then the drug solution was made up to 100% volume and the solution final pH observed was 5.23 (range 4.5 and 6.0). The solution was filtered through sterilized 0.22-micron PVDF membrane filter and filled in glass vials. The

vials were half Stoppard with lyo rubber plugs and loaded into lyophilizer. Once the lyophilization was completed the vials are sealed under nitrogen with flip off aluminium seals.

Evaluation of lyophilized product

Description

Appearance of the sample observed under visible light.

PH of the reconstituted solutions

The lyophilized formulation was reconstituted in 10 mL water for injection. And the pH of the reconstituted solutions was checked.

Assay

Microbiological assay was carried out and values were calculated based on the dilution and diameter of the zone of inhibition.

Determination of water content

Karl Fischer Titration was performed to determine the moisture content in the drug by using methanol as a solvent.

Reconstitution time

The lyophilized vials of the formulation were reconstituted by 10mL WFI.

Light transmittance and colour value

Colour value and % light transmittance of the reconstituted solutions were observed at 430 nm and 650 nm respectively by UV Spectrometer.

Identification

FTIR Spectrophotometer analysis was done for identification.

Stability Studies

The stability studies were carried out as per ICH guidelines. The accelerated study was carried out at temperatures of accelerated condition at 25 °C ± 2 °C and 60 % RH ± 5 %, for a period of three months and the sample was withdrawn at 1st, 2nd and 3rd month and analysed for parameters such as pH, assay, moisture content, reconstitution time, light transmittance and related contents.

RESULTS AND DISCUSSION

Bleomycin Sulphate is an antibiotic used for treating various cancers, including squamous cell carcinoma, Hodgkin's disease, testicular teratoma, malignant effusions, metastatic melanoma, thyroid, lung, and bladder carcinomas. It is administered via intramuscular, intravenous, subcutaneous, or intraarterial routes and is available in lyophilized injectable dosage form.

Studies showed that the potency of the drug candidate gradually decreases as the time increases. The present study found that the drug was unstable in solution form as the potency of the solution showed a significant change. Hence bleomycin aqueous injection was subjected for lyophilization in order to extend the shelf life needed for a marketable product. The potency of the antibiotic was found to be 17.626 mg (Tables 1, 2 and **??**).

Table 1: Physical, chemical and solution state stability studies of lyophilised Bleomycin sulphate

Preformulation study of the drug candidate			
Sl. No.	**Tests**	**Standards**	**Results**
1	Description	A white to off-white powder	Passes
2	Solubility	Very Soluble in water, slightly soluble in ethanol, partially insoluble in acetone	Passes
3	Identification IR Test for sulphate	Compare to reference standards Compare to reference standards	Passes Passes
5	Loss on drying	Not more than 3.0%	1.31 %
6	pH	Between 4.5-6.0	5.14± 0.02
7	Melting point	275-277 °C	275 °C
8	Assay (microbial assay)	It has potency not less than 1.5 units and not more than 2.0 units per mg	103.9 %
9	Contents of Bleomycin Bleomycin A2 Bleomycin B2 Bleomycin A2 and B2 Dimethyl and Bleomycin A2 Others	Between 55.0% and 70.0 % Between 25.0% and 32.0 % Not less than 95.0% Not more than 1.05% Not more than 0.6%	68.62 % 29.4% 96.4% 0.98% 0.23%

Solution state stability studies		
Time intervals (hrs)	**Assay values was found at Conditions**	
	5 ± 3 °C	**Room temperature**
Initial potency	103.7%	
After 24	94.6 %	89.6 %
After 36	78.4 %	58.4 %
After 48	51.2 %	42.3 %

The Bleomycin sulphate was tested for preformulation, and its IR spectrum identified it as a standard drug. The antibiotic microbial assay method revealed a 103.7% concentration of Bleomycin Sulphate, with 68.62% and 29.4% contents for Bleomycin A2 and Bleomycin B2, respectively. The Bleomycin Sulphate showed a 1.31% drying

Table 2: Preparation and evaluation of formulation of trial batches

Ingredients	Quantity taken
Drug candidate	17.626 mg
WFI	QS to 100 mL

Microbial assay readings showed in the following

Sl. No.	S.D. of standard Conc.	Conc. mcg/ml	log Conc.	S.D. of zone diameter in mm (Average)
1	Standards series S1	0.02	-3.97202	16.66
2	Standards series S2	0.03	-3.50656	18.97
3	Standards series S3	0.04	-3.21888	20.27
4	Standards series S4	0.05	-2.81341	21.41
5	Standards series S5	0.06	-2.52573	22.59
6	Sample U3	0.04	-3.218876	20.42

Evaluated results of formulated Batches 1 to 4

Evaluation parameter	Batches			
	Batch-1	Batch-2	Batch-3	Batch-4
Appearance	White to off-white powder			
Moisture Content (%)	1.72-1.84	3.2-3.56	4.3-4.56	5.4-5.95
Cake Formation	Good cake formation	Good cake formation	Good cake formation	Collapsed cake
Reconstitution Time (Sec)	12 sec	25 sec	25 sec	25 sec
Reconstituted solution pH (initial)	5.14	5.22	5.34	5.30
Reconstituted solution pH (after 24 hrs)	5.16	5.24	5.35	5.45
Assay (%)	103.20	104.0	102.0	103
Light transmittance (%)	99.86	98.64	98.8	98.34
Contents of Bleomycin (%) Bleomycin A2 Bleomycin B2 Bleomycin A2 and B2 Demethyl Bleomycin A2 Others	68.62 29.4 93.4 0.98 0.25	67.63 27.4 94.2 0.96 0.29	68.21 26.8 93.3 0.89 0.35	69.56 26.45 92.9 0.86 0.34

loss and a melting point of 275 °C, with a pH of 5.14 ± 0.03, and was used to create a parenteral dosage form using lyophilization technique.

The process involved four batches with varying cycle times, freezing, drying, ramp, and holding times while maintaining active pharmaceutical ingredient quantities. The first approach, lyo cycle 1, over 38.83 hours, produced a uniform, distinct, and intact cake without shrinkage or collapse. The process involved freezing for 3.6 hours, drying for 28.33 hours, and drying for 6.8 hours, with a vacuum gradually reduced from 250 to 150 mTorr. The resulting cake was a uniform, intact white to off-white cake with a pH of 5.14-5.19, a reconstitution time of 12 seconds, and a moisture content of 1.72-1.84%.

The product met the specified light transmittance and Bleomycin content limits. A second approach was attempted to reduce moisture content. Lyo cycle 2 was conducted for 42.8 hours, including 3.5 hours of freezing, with primary drying increased by 4 hours to 32.5 hours and secondary drying for 6.8 hours, maintaining the vacuum change protocol from cycle 1. The product was a uniform white to off-white cake with a pH range of 5.22-5.26 and a reconstitution time of 24 seconds. It had a light transmittance of 104.0% and moisture content of 3.2-3.56%. The light transmittance and Bleomycin content were within specified limits. Lyophilization was carried out for 38.82 hours in the third approach (lyo Cycle 3), with the same freezing rate and primary drying time as in the first cycle.

The secondary drying process increased the time by an hour to 7.16 hours, maintaining the same vacuum variation as in cycle 1, resulting in a white to off-white cake that appeared uniform, distinct, and intact without shrinkage or collapse. The sample had a pH range of 5.35-5.37, a reconstitution time of 25 seconds, a 102.0%

Table 3: Storage condition studies

Long term storage condition at 5 ± 3 °C											
Storage interval	**Description**	**pH (Limit 4.5-6.0)**	**Assay (90 to 120%)**	**Reconstitution time (NMT 30sec)**	**Light transmittance (650nm) (NLT 97%)**	**% Moisture Content (NMT6.0%)**	**Contents of Bleomycins (in %)**				
							Bleomycin A2 (55 to 70%)	**Bleomycin B2 (25 to 32%)**	**Bleomycin A2 & B2 (NTL 90%)**	**Demethyl bleomycin A2 (NMT1.05%)**	**Others (NMT 0.6%)**
1 Month	A white to off-white powder	5.24	103.6	12 sec	99.66	1.78	66.4	28.6	95.2	0.89	0.25
2 Month		5.24	104.6	12 sec	98.89	1.76	67.7	28.9	94.2	0.88	0.29
3 Month		5.26	104.8	12 sec	98.06	1.83	68.5	29.3	94.3	0.86	0.24
Short term storage condition at 25 ± 2 °C and 60 ± 5% RH (Accelerated condition)											
Storage interval	**Description**	**pH [Limit 4.5-6.0]**	**Assay (90 to 120%)**	**Reconstitution time (NMT 30sec)**	**Light transmittance (650nm, NLT 97%)**	**% Moisture Content (NMT6.0%)**	**Contents of Bleomycins (%)**				
							Bleomycin A2 (55 to 70%)	**Bleomycin B2 (25 to 32%)**	**Bleomycin A2 & B2 (NTL 90%)**	**Demethyl bleomycin**	**Others (NMT)**
1 Month	A white to off- white powder	5.26	103.6	13 sec	99.16	1.84	66.4	27.6	94.2	0.86	0.26
2 Month		5.27	102.6	14 sec	98.19	1.89	68.3	29.9	92.1	0.79	0.25
3 Month		5.27	102.8	14 sec	99.06	1.84	67.5	29.1	92.3	0.89	0.29

assay value, and moisture content of 4.3-4.56%. Light transmittance and Bleomycin content were within specified limits. Lyophilization was conducted for 39.6 hours in lyo cycle 4, with annealing in freezing for 5.1 hours, primary drying for 28.5 hours, and secondary drying for 6 hours. The vacuum change protocol was maintained in lyo cycle 1.

The product had a collapsed cake, pH range of 5.30-5.34, and a reconstitution time of 25 seconds. It had a 103% assay value and moisture content of 5.4-5.95%. Despite its collapsed appearance, the light transmittance and Bleomycins content were within the specified limits. Lyo cycle 1 provided the best results, especially with low moisture content and reconstitution time, as the lyophilized product cake was uniform, distinct, intact, dry, and porous. Hence the protocol of the first lyo cycle was optimum and lab scale batch for 500 mL was taken and the product was loaded for stability studies. The accelerated stability studies were conducted according to ICH guidelines at two different temperature and relative humidity conditions: 5°C ± 3°C for long-term/real-time hold studies and 25°C ± 2°C/60% RH ± 5% for accelerated studies. The lyophilized products under stability studies were analyzed at time interval of first month, second month and third month. Products were analyzed for Parameters such as Description, pH, Assay, light transmittance, reconstitution time percentage moisture content and contents of Bleomycin.

The product's description remained unchanged after reconstituted with 10ml of WFI, forming a clear, colourless solution. The pH was checked, and no significant difference was found compared to the initial value, falling within the range of 5.24 - 5.28. Antibiotic microbial assay and contents of Bleomycin were analysed by HPLC and the difference was found to be within the limits. Percentage of water content was analysed and was found to be the same at 5 °C ± 3 °C, 25 °C ± 2 °C and 60 ± 5 % RH, conditions through a slight increase of 0.2 - 0.5 % was observed. Colour value and percentage light transmittance test were carried out by UV and the results were found to be within the limits. The results of stability study concluded that the product was stable at accelerated conditions.

CONCLUSION

Lyophilization technique was used to improve the cake characteristics of a drug in lyophilized form. Three different Lyo cycles were designed and investigated to optimize product characteristics. All cycles yielded the best results in parameters like Description, Assay, pH, Moisture Content, Reconstitution Time, and Bleomycin content. Cycle 1 showed better moisture content and was chosen for stability study. The lyophilization process involved a total cycle time of 38.83 hours, with a freezing time of 3.6 hours, primary drying time of 28.33 hours, and secondary drying time of 6.8 hours.

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