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METHOD (RP-HPLC) DEVELOPMENT FOR BELUMOSUDIL QUANTIFICATION AND LC-MS CHARACTERISATION OF BELUMOSUDIL DEGRADANTS GENERATED IN DEGRADATIONS RESEARCH

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ABSTRACT

This article discusses the development and authentication of an isocratic mode stability-indicating RP-HPLC method for belumosudil (BLSL) measurement in bulk, Rezurock tablets, and degraded Rezurock samples. The BLSL and its degradants were separated well at room temperature using a Phenyl C18 column. The isocratic mode mobile phase comprised of a combination of 0.1% triethylamine and pure acetonitrile (40:60 v/v) delivered by 1.0 mL/min flow, and 254 nm was used for monitoring the effluents. Studies on BLSL's stress-triggered degradation revealed no impact on the suggested method's accuracy as well as specificity. During degradation investigations, four BLSL degradants were generated. They were DPA-1 (Rt – 0.734 min), DPA-2 (Rt – 0.785 min); DPO-3 (Rt - 1.749 min); DPW-4 (Rt- 1.082 min). The study also employed the LC-MS approach to characterize the generated four BLSL degradants. The DPA-1 spectra scans had *m/z* 99.042, 194.110, 372.123 and 489.173 fragment ions, DPA-2 spectra scans had *m/z* 91.042, 166.115, 238.090 and 309.163 fragment ions, DPO-3 spectra scans had *m/z* 95.042, 194.110, 254.085 and 353.153 fragment ions and DPW-4 spectra scans had *m/z* 95.042, 194.110, 263.109, 337.158 and 454.204 fragment ions. Based on scans, the molecular weight, probable structure, and likely path for their formation were proposed.

Keywords: Belumosudil, Stability, Degradation, HPLC, Degradants, Characterization, LC-MS.
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INTRODUCTION

A systemic illness referred to as "graft-versus-host disease" develops when the recipient's bodily cells become targeted by the graft's immune cells, which recognize the host as nonself-cells.[1] The major factor attributing to morbidity along with death following transplantation of hematopoietic stem cells is thought to be "graft-versus-host disease". This consequence will kill over 10% of subjects.[2,3] Kadmon Pharmaceuticals has developed belumosudil (BLSL) to treat systemic sclerosis as well as chronic "graft-versus-host disease". BLSL was very first approved in the United States in July 2021 for the therapy of chronic "graft-versus-host disease" illness in adults and also pediatric patients who were 12 years or older.[4] The production and sale of many health-related products depend heavily on quality assurance and control. These two procedures ensure that the quality standards for health-related products have been met.[5] It is advised to carry out the quantitation of BLSL for quality control along with product development. Furthermore, assessing stability is a crucial step in developing of novel pharmacological medicines.

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Verifying how a drug molecule's or drug product's quality varies over time under the influence of numerous conditions, including light, humidity, acid, reducing agent, oxidizing agent, alkaline, and temperature, is the aim of stability testing.[6,7] This makes it possible to decide on appropriate storage settings, times for retests, and shelf life. So far, just a single RP-HPLC has been established by Padmavathi and Sowmya to determine the BLSL amount in their formulations and bulk forms.[8] Padmavathi and Sowmya utilized BDS C18 analytical column fixed at 30°C, phosphoric acid: acetonitrile (ratio 45:55 *v/v*) solvent mix with a stream rate of 1.0 mL/min as mobile phase, and 225.0 nm as detection and quantifying wavelength. BLSL retention time in the Padmavathi and Sowmya techniques was reported at 2.439 min. Padmavathi and Sowmya's technique did not report peak purities for BLSL in degradation conditions, characterization of BLSL degradants, and BLSL degradation pathway. In alignment with ICH requirements, this paper details the development and authentication of an isocratic mode stability-indicating RP-HPLC technique for the measurement of BLSL in the presence of all its components of degradation. With the use of LC-MS analysis, the degradation products of BLSL were assessed, and a suggested degradation route was also included.

EXPERIMENTAL

Chemicals

Sd Chemicals Ltd., (India) provided chromatography-mark acetonitrile and triethylamine. The same firm also provided high-quality analytical chemicals including peroxide, sodium hydroxide, and hydrochloric acid. The chromatography-marked water utilized in this investigation came from Milli Q (India).

BLSL API and BLSL Formulation

The supplier of BLSL API was BLD Pharma Tech Ltd (India). Rezurock tablet manufactured by Kadmon Pharmaceuticals (US) with a 200 mg per tablet label was employed in this research study.

Mobile Phase

The isocratic mode mobile phase comprised of a combination of 0.1% triethylamine and pure acetonitrile (40:60 *v/v*). The mobile phase was subjected to degassing over 10 min employing an ultrasonic sonicator ahead of being employed in the investigation. The membrane filter used for filtration of the mobile phase was of size 0.45 µm. As a diluent, the same mixture of mobile phase solvents was deployed.

BLSL Solutions

To make the stock BLSL solution, 5 mg of BLSL API was put into a 10 mL volumetric flask along with a few volumes of diluent. The flask was well-shook before further diluent was added to arrive at a 500 µg/mL concentration. All BLSL assay procedure validation parameters were tested using the working BLSL solution at the necessary concentrations (50 µg/mL) which was prepared after stock BLSL solution (500 µg/mL) had been diluted appropriately and well mixed.

Tablet BLSL Solutions

The mean weight of twenty BLSL tablets (Rezurock) was determined by weighing them. To make a finely ground powder, the tablets were blended in a dry, clean mortar. A precise amount of BLSL (5 mg) in the form of a finely powdered tablet was taken, dissolved in a minimal volume mobile phase, completely mixed, and agitated for 5 mins in a 10 mL volumetric flask. Up to 10 mL, of diluent solvent was added to produce a volume with 500 µg/mL concentrated tablet BLSL solution. Working tablet BLSL solution at the necessary concentration (50 µg/mL) for analysis was prepared after stock tablet BLSL solution (500 µg/mL) had been diluted appropriately and well mixed.

General Procedure

The "Waters Alliance" HPLC system, which has a quaternary pump and PDA detector, was used to develop and authenticate an isocratic mode stability-indicating RP-HPLC method for BLSL measurement. The BLSL and its degradants were separated well at room temperature using a Phenyl C18 column of length 150 mm, identification of 4.6 mm and 3.5 µm particle-sized dimensions. A 10 µl BLSL sample quantity for injection was applied for each study. An isocratic 1.0 mL/min input rate of the mobile phase was pumped through the phenyl column for the elution of BLSL and its degradants. Using the peak area response of BLSL measured at 254 nm wavelength, the BLSL assay was done.

BLSL Calibration Curve

To create the BLSL calibration curve, several series of BLSL solutions were made appropriately by diluting stock BLSL solution (500 µg/mL) with diluent. Six concentrations (12.5; 25.0; 37.5; 50.0; 62.5; and 75.0 µg/mL) were selected with a concentration spanning 12.50–75.00 µg/mL. Every BLSL concentration solution was evaluated following the general procedure given. Peak area, as well as peak height of BLSL, were submitted for least squares regression calculations to assess BLSL linearity. The result was a calibration equation that included the Y-intercept, slope, plus correlation coefficient.

Analysis of BLSL in Rezurock Tablets

Working tablet Rezurock solution (50 µg/mL concentration) was evaluated following the general procedure given. Peak area, as well as peak height of BLSL, were obtained. These data allow for the evaluation of the BLSL content in the tablet.

Mass Spectral Studies on BLSL

The "Triple Quad TM 5600" LC-MS/MS System controlled using "Analyst" software was used for this. To determine the BLSL fragmentation patterning, mass spectrum measurements were performed in the positive electrospray ionization manner in the mass span of 0–800 Da. Using a syringe pump, BLSL was directly fed into the mass spectrometer at a quantity of 50 µg/mL in the diluent. The mass parameters for BLSL analysis were tweaked correctly to generate an exclusive molecular ion peak for BLSL. Nebulizer and auxiliary gas were both high-purity nitrogen.

Degradation Studies

The compelled degradation investigation of BLSL employed conditions including 30% H_2O_2 (oxidizing reagent), 1.0 N HCl (acid reagent), 30% sodium bisulfate (reducing reagent), 1.0 N NaOH (alkaline reagent), Milli Q water (hydrolytic reagent), UV light (photo condition), and temperature (thermal condition).[9,10] 1.0 mL volume of 30% H_2O_2, 1.0 N HCl, 30% sodium bisulfate, 1.0 N NaOH, and Milli Q water were applied to degrade tablet Rezurock powder (500 µg/mL BLSL). For 30 min the solutions (Rezurock powder + 7 mL diluent + 1.0 mL of degradation reagent) were refluxed at a fixed temperature of 60 °C. The solutions went through filtering and then the right volume of dilution (up to 10 mL) was made. With diluent, subsequently dilute 5 mL of the deteriorated solutions to produce 50 mL for the BLSL degradation analysis. Thermal degradation tests involved placing 50 mg of solid dry tablet Rezurock powder at 105 ºC for a whole day (24 hr). A 24-hour photolytic degradation investigation with solid dry tablet BLSL powder (50 mg) was conducted beneath UV light inside a photochamber. The thermal degradation BLSL solution and photodegradation BLSL solution for BLSL degradation analysis was made following protocol as told in the section "Tablet BLSL solutions". Each of the stressed tablet Rezurock solutions was evaluated following the general procedure given. The BLSL assay of stressed samples was contrasted with the usual untreated BLSL.

LC-MS Studies on Degradants

The samples containing the degradants were submitted to LC-MS analysis with mass spectrum measurements performed in the positive electrospray ionization manner to better comprehend the structure of the degradants generated in the degradation scenarios that were carried out and also possible paths for degradant formation. Using LC-MS fragmentation investigation, each degradant's structural verification possible paths verification was ascertained.

RESULTS AND DISCUSSION

Optimization of BLSL Assay Conditions

For column selection, two columns like Phenyl C18 column and the Aligent C18 column were investigated. Both columns have a length of 150 mm, identification of 4.6 mm, and 3.5 µm particle-sized dimensions. Several solvent combinations, such as 0.1% formic acid/acetonitrile and 0.1% trimethylamine/acetonitrile with differing ratios, have been explored regarding mobile phase optimization. With 0.1% formic acid and pure acetonitrile (20:80 *v/v*) and Aligent C18 column, a very low plate count (353) was obtained. Just 353 plate counts and higher peak tailing (2.16) were obtained using the Aligent C18 column with 0.1% formic acid and pure acetonitrile (30:70 v/v). A peak with an RT of about 1.0 min was generated using pure

acetonitrile (30% vol) and 0.1% formic acid (70% vol) in an Aligent C18 column. A disturbing baseline, a low plate count (245), and higher tailing (2.40) resulted in 0.1% formic acid, pure acetonitrile (50:50 v/v), and a Phenyl C18 column. We got the same results when 0.1% formic acid: pure acetonitrile (20:80 *v/v*), and a Phenyl C18 column was used. The same solvent blend in 30:70 (*v/v*) and the Phenyl C18 column also produced a lower plate count (1187). When 0.1% triethylamine: pure acetonitrile (40:60 *v/v*) and Phenyl C18 column were used, we got a good peak shape (tailing value of 0.98) with satisfactory plate count (6500) and more peak area (3710254) for BLSL (Fig.-1). The flow rate of solvents in column and column temperature was kept at 1.0 mL/min and room temperature, respectively, throughout all trail tests.

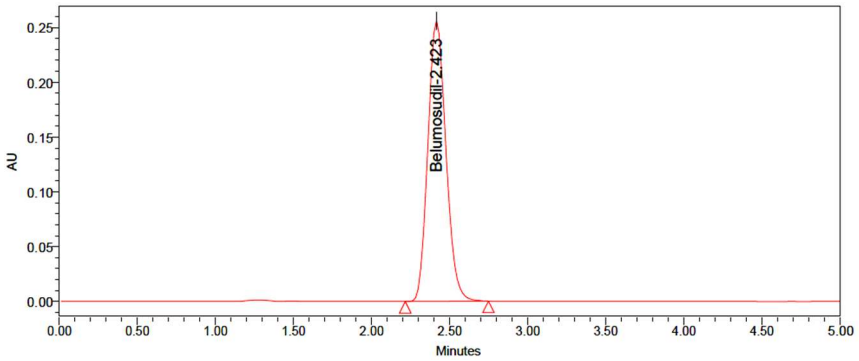


Fig.-1: BLSL Chromatogram using Phenyl C18 as the Preferred Column and 0.1% triethylamine: Pure Acetonitrile (40:60 V/V) as the Ideal Mobile Phase

The spectrum for BLSL dissolved in diluent was made. There were two determined λmax values for BLSL. One at 223.7 nm and the other at 292.4 nm. At 223.7 nm, BLSL had a greater response. Thus, 224 nm was chosen as the quantification wavelength.

Method Validation

Validated the newly established RP-HPLC BLSL assay procedure by adhering to ICH requirements.[11]

System Suitability

Six samples, each containing 50 µg/mL of BLSL, were evaluated following the aforementioned described "general procedure". In this study, the BLSL peak displayed good symmetry (mean - 0.930; %RSD – 0.130), with a satisfactory theoretical plate count (mean- 6575; %RSD – 0.545). BLSL retention time averaged 2.425 min, with a %RSD of 0.077. All parameters had RSDs substantially below 1%, signifying acceptable system suitability for BLSL analysis.

Selectivity

The "general procedure" given was followed for the assessment of the diluent blank, working BLSL solution (50 µg/mL), placebo, and tablet Rezurock solution (50 µg/mL). The chromatograms were assessed (Fig.-2). Without the intervention of the mobile phase and also excipients, the established "general procedure" demonstrated selectivity for BLSL analysis.

Linearity

Using the previously stated "general procedure," six concentrations (12.50, 25.00, 37.50, 50.00, 62.50, and 75.00 µg/mL) of BLSL were analyzed to examine the linearity. Having a correlation coefficient value of 0.9998, the linear equation for BLSL was Y = 74279.74 X + 16090.61. In this equation, 'Y' is the BLSL peak response, and 'X' is the specified working BLSL concentration in µg/mL. The strong correlation coefficient findings (>0.999) established the good linearity exhibited by the BLSL calibration graph.

Sensitivity

The following formulas were implemented to figure out both the LOD and LOQ for BLSL based on the slope and standard deviation measurements from the linearity:

LOD = SD of BLSL area response/Slope of BLSL from linearity curve × 3.3

LOQ = SD of BLSL area response /Slope of BLSL from linearity curve × 10

For BLSL, the LOD was 0.15 µg/mL and the LOQ was 0.50 µg/mL, demonstrating adequate sensitivity for BLSL analysis by the specified "general procedure".

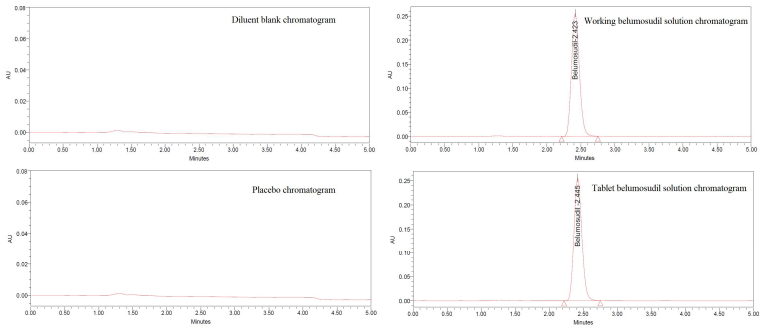


Fig.-2: Chromatograms Demonstrating Selectivity

Precision

System and method precisions for established "general procedure" were ensured by analyzing working BLSL solution (50 µg/mL) and BLSL spiked (50 µg/mL) Rezurock solution, respectively on the same day. By analyzing BLSL spiked (50 µg/mL) Rezurock solution (50 µg/mL) on a separate day employing an alternate operator and instrument, intermediate precision was verified. RSD (Table-1) is used for expressing the results. The findings demonstrated the great precision of the established "general procedure", with a deviation of fewer than 2%. This substantial level of precision is apt for QC evaluations of BLSL in the final pharmaceutical tablet Rezurock formulation.

Table-1: Precision Evaluation for BLSL Analysis

Precision →	System	Method	Intermediate
Sample ↓	BLSL Area	BLSL assay (%)	BLSL assay (%)
Mean	3738668	100.1	100.5
SD/RSD	21458.443/0.57	0.814/0.81	0.662/0.66

Accuracy

The implementation of accuracy involved producing three distinct tablet Rezurock samples that were spiked at 25 µg/mL, 50 µg/mL, and 75 µg/mL of BLSL. Three duplicates of each concentration solution were then injected followed by analysis using an established "general procedure". As indicated by Table-2, the accuracy findings (% BLSL assay) for the tested range (25 µg/mL, 50 µg/mL, and 75 µg/mL) were determined to fall between the 98–102 percent acceptance standards.

Table-2: Accuracy evaluation for BLSL analysis

Tested level ↓	Quantity Added (µg/mL)	Area Counts	Quantity Found (µg/mL)	% Quantity recovered
50 %	25	1835458	24.5	98.0
	25	1856471	24.8	99.2
	25	1869720	25.0	100.0
100 %	50	3752146	50.2	100.4
	50	3762148	50.3	100.6
	50	3726589	49.8	99.6
150 %	75	5563214	74.4	99.2
	75	5601529	74.9	99.9
	75	5598745	74.9	99.9
			Mean	99.6
			SD/RSD	0.551/0.55

Robustness

Robustness was investigated using planned little modifications, such as modest alterations to the mobile phase's composition and flow rate. The planned minor adjustments were carried out with a varied organic solvent proportion (acetonitrile) at 60% volume ± 5.0% volume and a flow speed of 1 mL/min ± 0.1 mL/min. The RDS% values for the BLSL assay (%) were used to evaluate the outcomes of deliberate

modest alterations. As stated in Table 3, the RSD% was measured to have a value of < 2% in every instance. The findings demonstrated the great robustness of the established "general procedure".

Table-3: Robustness Evaluation for BLSL Analysis

Flow plus (mL/min)	Quantity for study (µg/mL)	Area obtained	% Assay evaluated	Flow minus (mL/min)	Quantity for study (µg/mL)	Area obtained	% Assay evaluated
1.1	50	3462279	100.3	0.9	50	3960105	99.6
	50	3454826	100.1		50	3974649	100.0
	50	3453007	100.0		50	3997997	100.6
		Mean	100.1			Mean	100.1
		SD/RSD	0.153/0.15			SD/RSD	0.500/0.50
Acetonitrile proportion plus	Quantity for study (µg/mL)	Area obtained	% Assay evaluated	Acetonitrile proportion minus	Quantity for study (µg/mL)	Area obtained	% Assay evaluated
65% volume	50	3520129	99.7	55% volume	50	3939870	98.7
	50	3511307	99.5		50	3940360	98.8
	50	3531989	100.1		50	3943200	98.8
		Mean	99.8			Mean	98.8
		SD/RSD	0.306/0.31			SD/RSD	0.058/0.06

Degradation Studies

The compelled degradation investigation on tablet Rezurock solution was carried out to ascertain the stability-indicating characteristics of the established "general procedure". Additionally, to make it easier to confirm and separate the active ingredient under investigation, "BLSL", from a variety of produced catalytic degradants. Additionally, this study offers details on BLSL's stability under various degradation scenarios executed (Table-4). The order of BLSL was: thermal condition > photo condition > 30% sodium bisulphate > Milli Q water > 1.0 N NaOH > 1.0 N HCl > 30% H_2O_2. This indicates a considerable degree of oxidation labile behavior for BLSL. This information is intended to help makers of drug generic products and bulk drug molecules optimize key quality features to minimize considerable peroxide exposure throughout the BLSL production process. About the BLSL principal peak, which has peak threshold estimates higher than purity angle estimates (Table-4), the present approach gave us extremely detailed information on the resolution including the separation performance of the neighboring co-eluted peaks under all degradation scenarios executed (Fig.-3).

Table-4: Robustness Evaluation for BLSL

Stress applied ↓	Quantity for study (µg/mL)	Area obtained	Stability (%) observed	Degradation (%) observed	Angle of purity	Threshold of purity
Control	50 µg/mL	3742814	99.9	0.1	0.129	5.234
Hydrolytic stress		3358216	89.7	10.3	1.359	5.152
Alkali stress		3274723	87.4	12.6	0.149	5.217
Thermal stress		3687540	98.5	1.5	1.371	5.168
Peroxide stress		3169254	84.6	15.4	0.171	5.229
Acid stress		3236528	86.4	13.6	0.164	5.261
Reduction stress		3627451	96.9	3.1	0.182	5.253
Photo stress		3655713	97.6	2.4	1.359	5.152

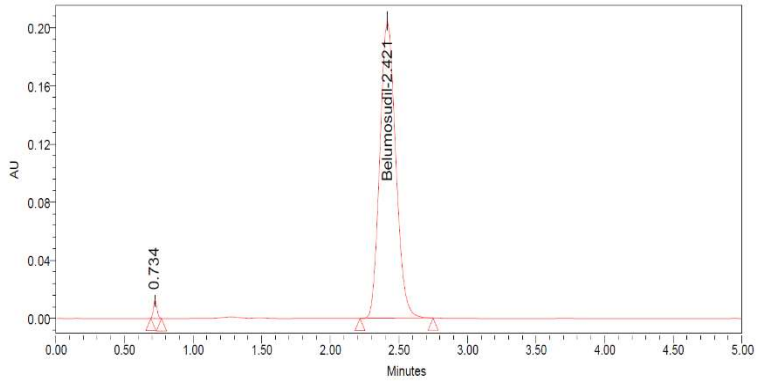
MS Spectra of BLSL

Five fragments in all were created from the BLSL, as Fig.-4 illustrates. The formula found for BLSL from mass studies was $C_{26}H_{24}N_6O_2$ and the experimental mass was 453.20.

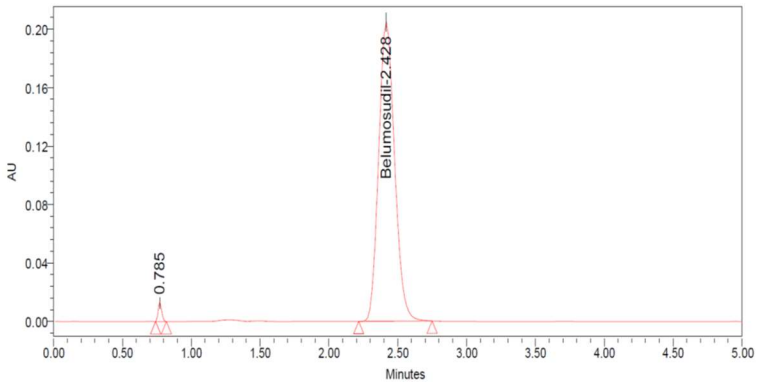
Characterization of Degradants Produced

Upon treating BLSL with 1.0 N HCl (acid reagent), a single impurity (DPA-1) with a retention time scale of 0.734 min was generated. Through LC-MS spectral characterization (Fig.-5), the mass of DPA-1 was

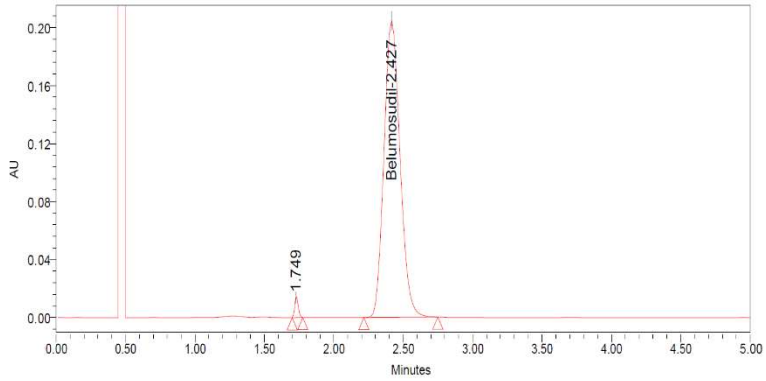
determined to be $(M+H)^+$ 489.1732, with a predicted molecular makeup of $C_{26}H_{25}N_6O_2^+$ and chemical name *N*-(1*H*-indazol-5-yl)-2-(3-(2-(isopropylamino)-2-oxoethoxy)phenyl)quinazolin-4-aminium.



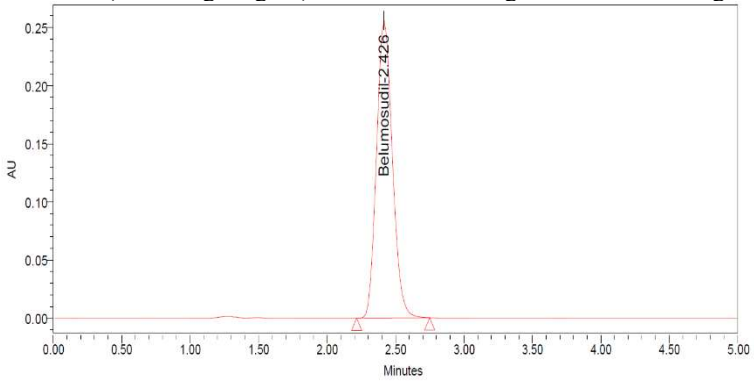
1.0 N HCl (acid reagent) induced BLSL degradation chromatogram



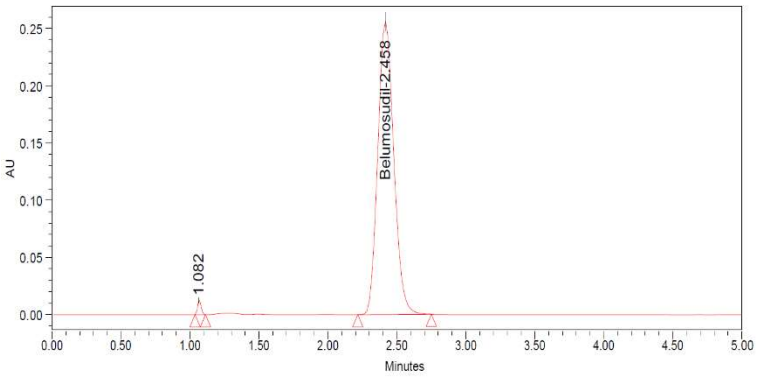
1.0 N NaOH (alkaline reagent) induced BLSL degradation chromatogram



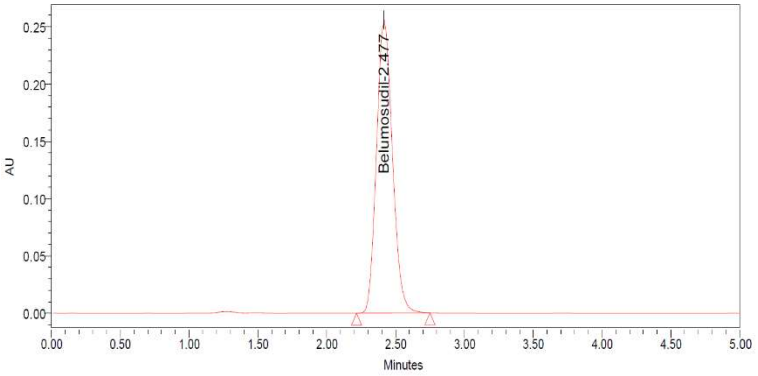
30% H_2O_2 (oxidizing reagent) induced BLSL degradation chromatogram



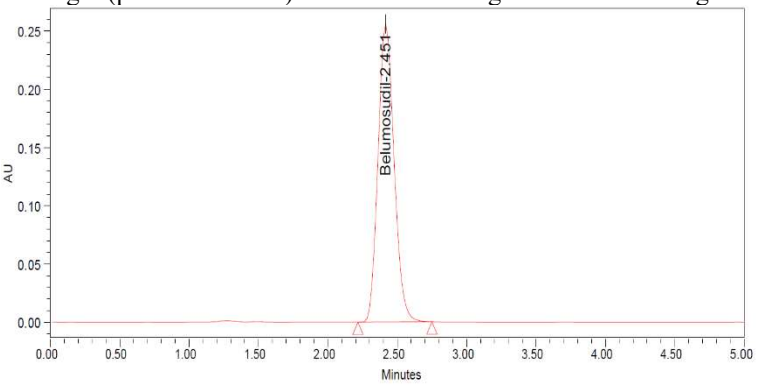
30% sodium bisulphate (reducing reagent) induced BLSL degradation chromatogram



Milli Q water (hydrolytic reagent) induced BLSL degradation chromatogram



UV light (photo condition) induced BLSL degradation chromatogram



Temperature (thermal condition) induced BLSL degradation chromatogram

Fig.-3: Chromatograms of BLSL under all Degradation Scenarios Executed

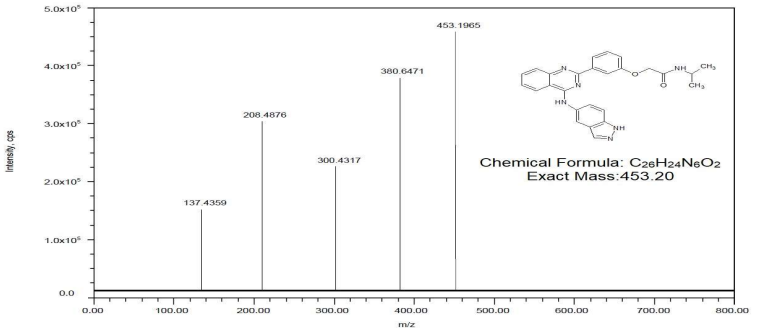


Fig.-4: BLSL Line Spectrum Obtained Through an MS Investigate

Figure-6 shows a probable route for the generation of DPA-1 from BLSL in an environment that is acidic. Upon degradation treatment with 1.0 N NaOH (an alkaline reagent), BLSL yielded one impurity (DPA-2) with a retention duration of 0.785 min. Through LC-MS spectral characterization (Fig.-5), the mass of

DPA-2 was determined to be $(M+H)^+$ 166.115, with a predicted molecular makeup of $C_{10}H_{15}NO$ and chemical name N-(phenoxymethyl)propan-2-amine. Figure-6 illustrates a likely pathway for BLSL to produce DPA-2 in an alkaline environment. After BLSL was oxidized with peroxide, one impurity (DPO-3) with a retention timescale of 1.749 min resulted. The mass of DPO-3 was ascertained to be $(M+H)^+$ 254.085 by LC-MS spectral analysis (Fig.-5), with an anticipated molecular composition of $C_{14}H_{11}N_3O_2$ and chemical name 3-(4-(hydroxyamino)quinazolin-2-yl)phenol. Figure-6 illustrates a likely pathway for BLSL to produce DPO-3 in a peroxide environment. Using Milli Q water treatment for hydrolysis, BLSL yielded an impurity (DPW-4) with a retention time scale of 1.082 min. The mass of DPW-4 was ascertained to be $(M+H)^+$ 337.158 by LC-MS spectral analysis (Fig.-5), with an anticipated molecular composition of $C_{19}H_{20}N_4O_2$ and chemical name 2-(3-(4-aminoquinazolin-2-yl)phenoxy)-N-isopropylacetamide. A plausible route for BLSL to generate DPW-4 in a neutral environment is depicted in Fig.-6.

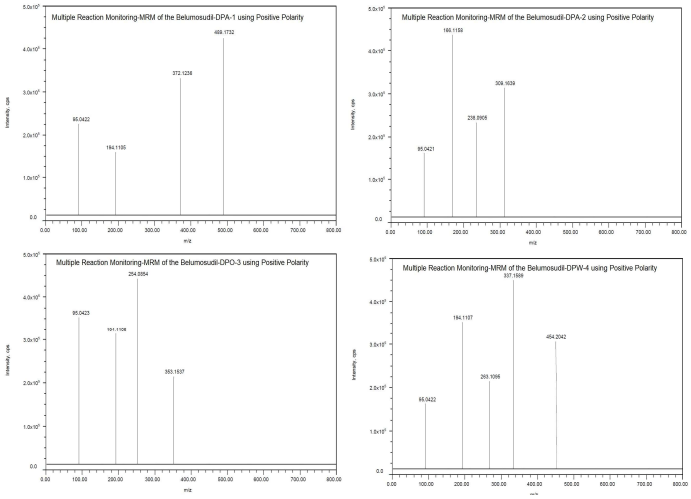


Fig.-5: Mass Spectral DPA-1, DPA–2, DPO-3 and DPW-4 Scans

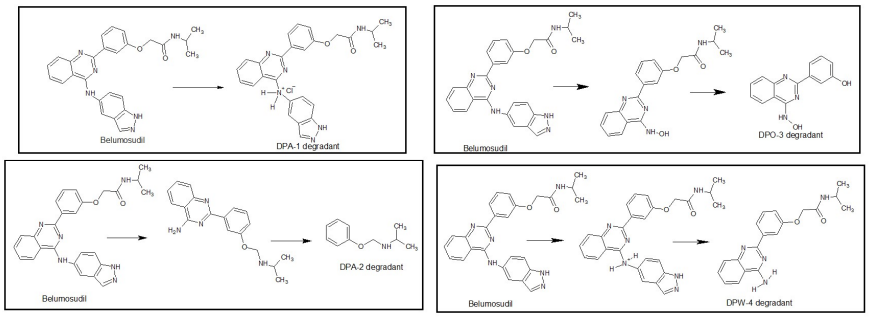


Fig.-6: Formation Path for DPA-1, DPA–2, DPO-3 and DPW-4

Method Applicability

To verify the method's applicability, the suggested "general procedure" was used with Rezurock tablets. The manufacturer claimed that each Rezurock pill included 200 mg of pure BLSL. The computed content of the 197.60 mg pure BLSL/Rezurock tablet was 98.80% of the BLSL amount as stated by the manufacturer. With recoveries ranging from 98.26% to 99.34%, the suggested "general procedure" proved to be accurate regarding BLSL determination and was deemed acceptable.

CONCLUSION

The development and authentication of an isocratic mode stability-indicating RP-HPLC technique for the measurement of BLSL was described herein for the first time. By meeting acceptance requirements, the validation guidelines supported the proposed quantitative method's validity for BLSL quantitation and detection at lower content levels in bulk and Rezurock tablets. All stressed Rezurock samples were analyzed, and the results show that the recommended “general procedure” is capable of identifying every degradant that has been produced, indicating the method's capacity for stability. The way BLSL degraded

was investigated. The drug did not exhibit any impurity development, making it stable under reduction, thermal, and photolytic conditions. However, four BLSL degradants were formed, one each in acid (DPA-1), base (DPA-2), oxidation (DPO-3), and neutral (DPW-4) stress conditions. Employed the LC-MS approach successfully to characterize the generated four BLSL degradants.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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