RJC

RASĀYAN *J. Chem.*
Vol. 17 | No. 1 |207-214| January - March | 2024
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

UPLC METHOD DETERMINATION AND QUANTIFICATION OF BELUMOSUDIL AND ITS IMPURITIES IN THE HUMAN PLASMA SAMPLES

T. Raja Reddy[1], L. A. Amarbabu Namburi[2] and M. B. Madhusudana Reddy[1,✉]

[1]Department of Chemistry, School of Applied Sciences, REVA University, Bangalore-560064, (Karnataka) India

[2]Vignan's Foundation for Sciences Technology and Research University (VFSTR), New Generation Material Lab, Department of Sciences and Humanities, Vadlamudi, Guntur-522213, (Andhra Pradesh), India

✉Corresponding Author: madhusudanareddymb@gmail.com

ABSTRACT

This current analysis demonstrates the development of the related substances (RS) method for separating and quantifying BSL and its related substances from plasma using the UPLC technique. The solid phase extraction (SPE) method was employed to extract the belumosudil (BSL) and related impurities from plasma. This SPE method has been shown to have high recovery values for BSL and its related impurities. The method employed a column from Waters, specifically the X-Bridge BEH C18 150x4.6mm, 3.5µm, with a mobile phase flow rate (0.8 mL/min) and a UV-wavelength (250 nm). In this method, BSL and its impurities show good peak shapes and high resolutions. This developed method shows high sensitivity, accuracy, and precision values. While performing the stability studies, the drug was stable and it showed less than 4.53% degradation. Six different concentration solutions (BSL (25-250µg/mL) and impurities (3-18µg/mL)) were prepared for constructing linearity plots. The obtained linearity plot shows the accepted correlation coefficient (R2) value (≥0.999). The Limit of detection (LOD) and Limit of quantification (LOQ) were determined using the signal-to-noise (S/N) ratio method. This UPLC method was validated according to ICH guidelines.

Keywords: Belumosudil, Solid Phase Extraction, Stability Studies, Validation, Related Substances, UPLC, Limit of Detection.

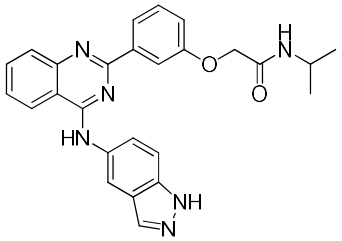
RASĀYAN *J. Chem.*, Vol. 17, No.1, 2024

INTRODUCTION

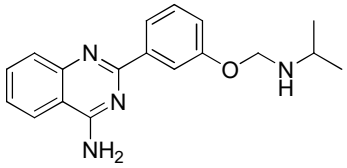
The lung-related disease of bronchiolitis obliterans syndrome or chronic graft versus host (cGVH) disease is a highly dangerous disease demonstrated with deprived consequences.[1] This results in reduced mortality, morbidity, and quality of life after allogeneic hematopoietic cell transplant.[2] USFDA has approved Belumosudil in 2021 to treat cGVH disease.[3,4] Belumosudil is a medication that works by selectively inhibiting a protein called Rho-associated coiled-coil containing protein kinase 2 (ROCK2).[5,6] It is taken orally. Belumosudil was first developed by Kadmon Pharmaceuticals its brand name is Rezurock, and it has been used to cure systematic sclerosis and cGVH disease in pediatric patients aged greater than 12 years and adults afterward the lack of success of at minimum two past appearances of systematic treatment,[7] Belumosudil (200 mg) should be taken once daily until there is a progression of cGVH disease that requires new systemic treatment. The belumosudil dose should be augmented up to 200 mg twice for a day in inpatients getting powerful associated with proton pump inhibitors or CYP3A4 inducers.[8] Considering the pharmaceutical importance of BSL, a chromatographic-related substance (RS) analytical method should be established for the detection and quantification of BSL and its four related substances. The chemical name of BSL is 2-[3-[4-(1H-Indazol-5-ylamino) quinazolin-2-yl] phenoxy]-N-propan-2-ylacetamide.The structures of BSL and its four impurities are depicted in Fig.-1. Typically SPE methods have been used to extract the compound from biological fluids. While doing the extraction of drugs and their related substances from the biological fluids by using liquid-liquid extraction methods were getting low recovery values due to the high interference of biological matrices. Compared with liquid-liquid phase extractions,

http://doi.org/10.31788/RJC.2024.1718697

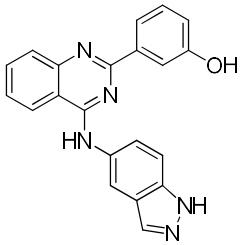
solid phase extraction has shown the following advantages high recovery values due to the low matrix effect, easy to handle, and less consumption of organic solvents. This study is primarily focused on developing a bioanalytical-relevant method that indicates stability for BSL and its related impurities. BSL and its related substances were successfully quantified after extraction from plasma using a newly developed ultra-performance liquid chromatography (UPLC) RS method.



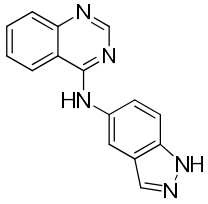
Belumosudil
2- [3- [4- (1H-Indazol -5 -ylamino) quinazolin -2-yl]phenoxy] -N-propan-2- ylacetamide



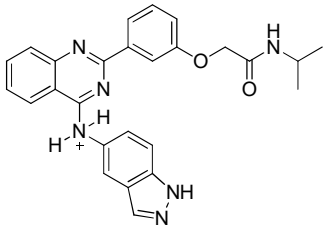
Impurity-1
2-(3-((isopropylamino) methoxy) phenyl) quinazolin-4-amine



Impurity-2
3-(4- (1H-indazole-5-ylamino) quinazolin-2-yl) phenol



Impurity-3
N-(1*H*-indazol-5-yl)quinazolin-4-amine



Impurity-4
M-(1H-indazol-5-yl)-2-(3-(2-(isopropyl amino)-2-oxoethoxy) phenyl) quinazolin-4-aminium

Fig.-1: BSL and its Related Impurities' Chemical Structures and Their Corresponding IUPAC Names

EXPERIMENTAL

Material and Methods

Analytical grade methanol, chloroform ($CHCl_3$), dichloromethane, acetonitrile, and methyl tertiary butyl (TBE) ether procured from Sigma Aldrich, USA were used for UPLC method and solid phase extraction development. The used chemicals purchased from Merck-USA (hydrogen peroxide, hydrochloric acid, KH2PO4, NaOH, and H3PO4). The development of a stability-indicating method was done using the Acquity UPLC system by Waters in the USA. Data was analyzed using Empower II software. X-Bridge C18 column was procured from the waters. Milli-Q system (Millipore) is used for water purification (USA). Elico pH meters, Sonicator (Sonica, spincotech- Italy), and 0.22µ nylon - filters (Merck-USA, Millipore) have been utilized for making the sample preparation.

Preparation of Linearity and Quality Control (QC) sample solutions

BSL and its four related impurities were dissolved in a diluent to create stock solutions with concentrations of 0.5mg/mL and 0.1mg/mL, respectively. These stock solutions were then stored in a refrigerator at -20°C.

The stock solutions of BSL and its impurities were diluted to prepare working solutions. Six various concentrations (LOQ to 150%) of BSL and its impurities were prepared in blank plasma for constructing the linearity plot. The three-level QC samples (low, middle, and high) were prepared to spike the 250µL of blank plasma. All of the samples were stored at a temperature of -20°C.

Extraction Procedure

To prepare sample solutions, 200 µL of human plasma was transferred into a centrifugal Eppendorf tube (1.5 mL) encompassing 0.3 mL of pH-6.0 sodium phosphate buffer. The samples were vortexed and transferred to Oasis HLB solid-phase cartridges (30 mg sorbent). The SPE cartridges used in this experiment were first saturated with 1.0 mL of ACN, followed by 1.0 mL of methanol, and eventually 1.0 mL of acetonitrile: water (10:90) v/v before adding the sample solutions. The cartridges containing the material were washed three times using a solution of water and methanol in a ratio of 90:10 v/v.[9] After washing, the analytes were extracted with an eluent solution (acetonitrile: water (90:10) v/v). The collected solution was then dried under a vacuum at 40 °C. After extraction, the sample was reconstituted with 100µL of blank solution, and 10µL of this mixture was straightly injected into the UPLC.

Stability studies of BSL

In order to ensure the reliability of the developed procedure, we conducted forced degradation experiments. The stability of BSL was assessed through exposure to acidic, basic, oxidative, photolytic, and thermal circumstances. For acidic degradation, 1.0 mL of 1N hydrochloric acid (HCl) solution was added into the 1.0 mL of BSL (1 mg/mL) solution and stirred for one hour at 60°C ±2. After one hour they stopped the stirring and cooled at room temperature. This solution was neutralized with NaOH solution to avoid further degradation. For basic degradation, 1.0 mL of 1N NaOH was added into the 1.0 mL of BSL (1 mg/mL) solution and stirred for one hour at 60°C ±2. After one hour they stopped the stirring and cooled at room temperature. To prevent further degradation, an HCl solution was used to neutralize this solution. The thermal stability of the sample was assessed by keeping a homogenized sample (100 mg) at 105°C ±5 for 8 hours. The sample was then prepared by diluting with the diluent. Oxidative degradation study was evaluated by adding the 1.0mL of 10% H_2O_2 solution into 1.0mL of sample solution. This solution was stirred at 30°C ±2 for one hour and afterward diluted with diluent. A photolytic degradation study was performed by placing 1.0mL of the sample in a UV chamber and then diluting it with a diluent. A neutral degradation study was conducted by adding 1.0mL of water to 1.0mL of the sample. The solution was stirred at a temperature (30°C ± 2) for one day and afterward diluted with diluent. All samples were cleaned with a 0.22µm nylon filter and 10µL of each was injected into the UPLC.

Detection Method

Prepared 10mM potassium dihydrogen orthophosphate (KH_2PO_4) into 1 L of milli-Q-water and sonicated properly for mixing. Then the pH (2.5) with diluted orthophosphoric acid. Then the solution was filtered with a 0.22µm nylon membrane filter kit. 10mM KH_2PO_4 buffer was utilized as a mobile phase (A). Pure 100% acetonitrile (ACN) was utilized as a mobile phase (B). This method of mobile phase gradient program was developed for getting good peak shapes and resolution in chromatograms. Ambient temperatures were used for column and sample temperatures. A PDA detector was used to detect and quantify the samples. Injection volume 10µL, mobile phase flow rate 0.8 mL/min, and injection run time 18 min were used. A premixed solution of Milli-Q-water and acetonitrile (ACN) (30:70) v/v is used as a diluent.

Table-1: Mobile Phase Gradient Program

Time	% of Mobile phase A	% of Mobile Phase B
00.0 min	15	85
05.0 min	15	85
10.0 min	90	10
12.0 min	15	85
18.0 min	15	85

Analytical Discussion

A UPLC method for analyzing BSL and its related impurities was developed and validated. Throughout the development of the method, various conditions such as columns and mobile phases were optimized to attain

improved peak shapes, higher resolutions, and sensitivity. The obtained results confirm that this UPLC technique is dependable and appropriate for the analysis of BSL and its impurities. Still no one has published the related substances method for BSL and its impurities. In this method, BSL and its related substances were extracted from the plasma by using the SPE method. It is easy to handle and extract the BSL and its impurities from the plasm. However, no one developed the SPE method and stability indicating the UPLC method. This developed method has been showing high sensitivity, low run time, and higher recovery values. It also shows good LOD, LOQ, precision, and accuracy values to determine the BSL and its related impurities.

RESULTS AND DISCUSSION

This work aimed to extract BSL and its impurities from plasma using solid-phase extraction (SPE). After that, the samples underwent quantification through an ultra-performance liquid chromatography (UPLC) method which is capable of detecting any instability in the samples. During the method development, various method conditions were optimized. While developing the method different mobile phases with various pH states and different columns were used. Compared with different mobile phases the mixture of 10mM potassium dihydrogen orthophosphate (pH 2.5) and acetonitrile has been shown good resolution and peak shapes. Column waters X-Bridge BEH C18 150x4.6mm, 3.5µm has been showing good resolution narrow, sharp peaks and high theoretical plate count. The mobile phase flow rate was fixed at 0.8 mL/min, injection volume (10µL), and ambient sampler temperature and column oven temperatures were optimized. Initially, for method development, poor resolutions were shown when viewed with the isocratic mode, hence a gradient method was developed to obtain better resolutions between peaks.

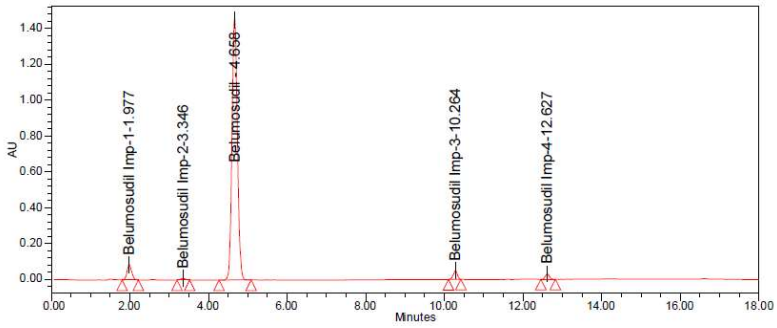


Fig.-2: UPLC Chromatogram of BSL and its Impurities after the Method Development

Generally, 5 to 20% degradation of the samples was acceptable in forced degradation stability studies and it was considered to validate the developed methods. In the current stability study, the BSL was exposed to various conditions (acidic, basic, oxidation, neutral, thermal, and photolytic). In acidic (3.68%) and oxidation (4.52%) conditions, the drug has been showing considerable degradation. In the remaining circumstances, the drug was being in stable. The purity of the dug was assessed by using the following formula. The obtained results of forced degradation studies of BSL have been shown in Table-2.

Percentage Assay = (Area of Sample/ Area of Standard) X (Standard Dilution/Sample Dilution) X(P/100) X 100

Table-2: Summary of the Results Obtained from the Stability Studies

Conditions of stress study	Time	Purity of BSL later degradation	Remarks
Unstressed sample	–	99.82	–
1.0N HCl (Acid-hydrolysis)	60 min	96.32	Significant degradation was found
1.0N NaOH (Base-hydrolysis)	60 min	98.88	It was noticed that the analytes degraded slightly
Oxidation (10% H_2O_2)	60 min	95.48	No noticeable degradation was found
Thermal (105 ° C)	8 h	99.64	No noticeable degradation was found
Photolytic-degradation (UV)	24 h	99.73	No noticeable degradation was found

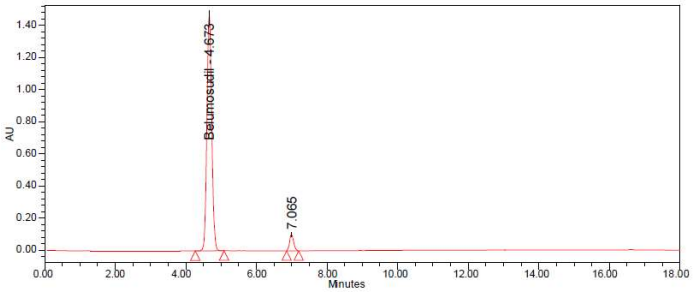


Fig.-3: UPLC chromatogram of BSL after Acidic Degradation

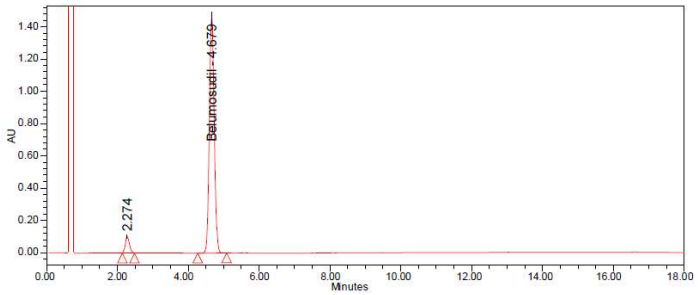


Fig.-4: UPLC Chromatogram of BSL after Oxidation Degradation

For the extraction of BSL and its related impurities SPE method has been used. For getting better recoveries various solvents (chloroform, methanol, dichloromethane, acetonitrile, and Methyl tertiary-butyl ether) and combinations were used. Compared with all of those the mixture of ACN and water (90:10) v/v has been given the high recovery values.

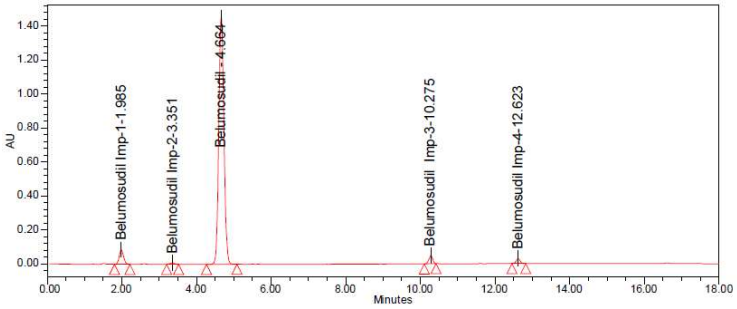


Fig.-5: After the Solid Phase Extraction, UPLC Chromatograms of BSL along with its Impurities

Validation of UPLC method

According to the ICH guidelines, the UPLC method has been statistically validated for the parameters mentioned below.[10]

Selectivity

For the extraction of BSL and its related impurities from the plasma, the SPE method was used. In this method were extracted the BSL and its impurities and the resulting chromatograms showed symmetrical peaks with good resolutions and without unknown peaks.

Specificity

Method specificity was evaluated by running the six different samples injected into the developed UPLC method. The resulting chromatograms have shown no interference with blank or unknown peaks. Hence this method was proved specific for BSL.

Calibration Curve

The linearity plots have been constructed for BSL and its related impurities by taking the six different level concentrated solutions. The calibration curves were constructed by injecting six different concentrated solutions of BSL and its related impurities into UPLC and using the obtained peak areas versus

concentrations of solutions. The resulting linearity plots have been shown accepted correlation coefficient (R^2) values for BSL and its impurities (≥ 0.999).

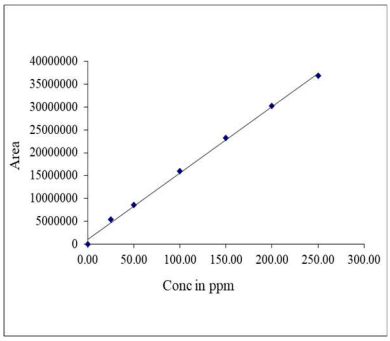


Fig.-6: Linearity diagram of BSL

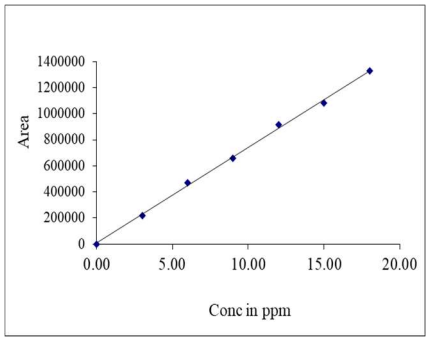


Fig.-7: Linearity diagram of Imp-1

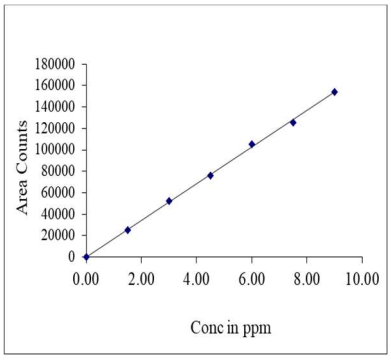


Fig.-8: Linearity diagram of Imp-2

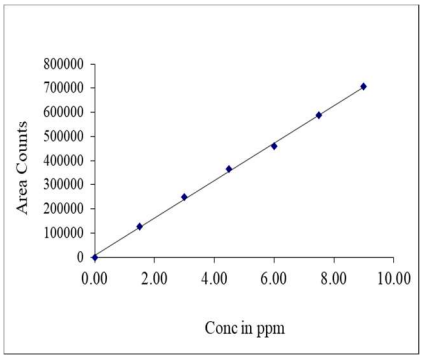


Fig.-9: Linearity diagram of Imp-3

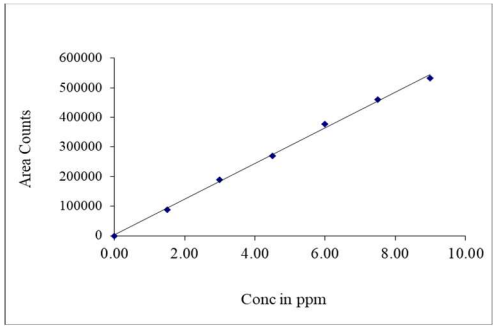


Fig.-10: Linearity diagram of Imp-4

LOD and LOQ

Table-3 presents LOD and LOQ obtained values assessed by the S/N ratio method. The obtained LOD values reflect method sensitivity and LOQ indicates its quantification limit.

Table-3: Results of Validation Parameters Linearity and LOD, LOQ

Drug	Linear range (µg/ mL)	Calibration curve equation	R^2	LOD (µg /mL)	LOQ (µg/ ml)
BSL	25.0 – 250.0	y = 145282x + 1102796	0.9990	0.2	0.6
Imp-1	3.0 – 18.0	y = 73279x + 8233	0.9992	0.06	0.2
Imp-2	1.5 – 9.0	y = 17078x + 9679	0.9995	0.02	0.06
Imp-3	1.5 – 9.0	y = 77294x + 8903	0.9994	0.01	0.025
Imp-4	1.5 – 9.0	y = 60269x + 2401	0.9991	0.01	0.025

Precision and Accuracy

Method precision (Inter and intraday) has been evaluated by preparing the three-level (low, middle, and high) QC samples. Interday precision has been analyzed by injecting the sample 8 times, for each four hours

in a single day. The calculation of interday precision has been done by injecting the samples on different days. Both results were assessed by calculating the percentages of cumulative variance (CV) of obtained peak areas. Method accuracy was examined by recovery percentages of analytes. The obtained results of accuracy and precision were tabulated in Table-4.

Table-4: Obtained Results of Accuracy and Precision

	Intra – day (%)			Inter – day (%)		
	Low	Middle	High	Low	Middle	High
Belumosudil	95.13 ± 3.21	97.55 ± 4.64	96.05 ± 3.26	94.45 ± 3.52	96.18 ± 2.95	94.61 ± 4.31
Impurity-1	96.60 ± 2.48	98.62 ± 1.83	101.21 ± 3.68	95.42 ± 4.63	97.65 ± 3.77	99.10 ± 3.24
Impurity-2	95.27 ± 4.43	94.83 ± 4.10	98.36 ± 3.45	92.97 ± 5.12	95.63 ± 3.88	100.07 ± 3.72
Impurity-3	99.27 ± 4.23	97.83 ± 4.10	98.33 ± 3.45	96.97 ± 5.12	95.63 ± 3.88	99.02 ± 3.62
Impurity-4	93.27 ± 4.43	98.83 ± 4.10	96.36 ± 3.45	101.97 ± 5.12	95.63 ± 2.88	99.16 ± 3.72

Method Robustness

The robustness of the UPLC method that was developed was tested by varying the flow rate, column temperature, wavelength, and ratios of organic solvents in the mobile phase. Flow rate was varied by ±0.02ml/min, wavelength by ±5nm, column temperature by ±5°C, and organic solvent ratio in the mobile phase by ±5%. The results obtained from Table-5 show that there were no significant variations observed when these parameters were changed. Therefore, it can be concluded that the developed UPLC method is robust.

Table-5: Robustness study

Chromatographic changes	Level	BSL		Impurity-1		Impurity-2		Impurity-3		Impurity-4	
		RT	Area	RT	Area	RT	Area	RT	Area	RT	Area
Mobile phase ratio	13 : 87	4.46	20883542	1.95	834562	3.20	112256	10.02	420875	12.54	400125
	15 : 85	4.65	19815255	1.98	816145	3.35	113524	10.26	432257	12.63	381585
	17 : 83	4.83	19254815	2.01	790485	3.52	108058	10.68	446481	12.95	372518
Mean ± SD			19984537±827454		813731±22137		111279±2861		433204±12829		384743±14072
RSD (%)			4.14		2.72		2.57		2.96		3.66
Flow rate (mL/min)	0.78	4.95	20253054	2.23	808355	3.62	112547	10.58	415410	13.02	395215
	0.80	4.65	19802546	1.98	850839	3.34	114021	10.30	432225	12.64	375864
	0.82	4.21	19752632	1.96	835995	3.05	107542	9.99	402501	12.33	362015
Mean ± SD			20269411±852226		831730±21561		111370±3396		416712±14905		377698±16676
RSD (%)			4.20		2.59		3.05		3.58		4.42
Column temperature (°C)	25°C	4.71	20166112	2.00	862844	3.61	113254	10.42	421132	12.85	358422
	30°C	4.65	19822552	1.98	840839	3.42	113321	10.25	432225	12.61	375995
	35°C	4.52	19605552	1.96	877202	3.22	120055	10.12	422595	12.24	385124
Mean ± SD			19864739±282651		860295±18315		115543±3907		425317±6027		373180±13572
RSD (%)			1.42		2.13		3.38		1.42		3.64
Wavelength	245	4.65	19254225	1.98	792548	3.35	123254	10.25	401132	12.60	408422
	250	4.64	19825855	1.98	825482	3.35	113321	10.25	432225	12.62	375995
	255	4.64	19682125	1.99	812558	9.34	112055	10.25	452595	12.62	365124
Mean ± SD			19587402±297354		810196±16594		116210±6133		428651±25917		383180±22526
RSD (%)			1.52		2.05		5.28		6.05		5.88

CONCLUSION

We have finally developed a simple and efficient UPLC method to analyze the BSL and its related impurities. The method has been validated based on the guidelines provided by the International Council for Harmonization (ICH). In addition, samples were extracted from plasma using solid phase extraction, which resulted in higher recovery values. This method has demonstrated low detection and quantification limits, as well as high accuracy and precision values. With these advantages, it can be used for clinical, bioanalytical, and routine analysis.

ACKNOWLEDGMENTS

The research is supported by scientific and technical assistance from the Laboratory of Chemistry, School of Applied Sciences at REVA University in Bangalore, Karnataka.

CONFLICT OF INTERESTS

The authors report no conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors have actively participated in the conceptualization of this research work, involved during the analysis and article writing, review, and editing. The final draft has been approved by the authors for publication. Find all author's research profiles by using their ORCID ids, given below:

T. Raja Reddy http://orchid.org/0000-0003-2396-9650
L. A. Amarbabu Namburi http://orchid.org/0000-0003-0806-4052
M. B. Madhusudana Reddy http://orchid.org/0000-0003-1415-6957

REFERENCES

1. Z. DeFilipp, H. T. Kim, Z. Yang, J. Noonan, B. R. Blazar, S. J Lee, S. Z. Pavletic, C. Cutler. *Blood Advances,* **6(24)**, 6263(2022), https://doi.org/10.1182/bloodadvances.2022008095
2. M. Jagasia, A. Lazaryan, C. R. Bachier, A. Salhotra, D. J. Weisdorf, B. Zoghi, J. Essell, L. Green, O. Schueller, J. Patel, A. Zanin-Zhorov*, Journal of Clinical Oncology*, **39(17)**, 1888(2021), https://doi.org/10.1200/JCO.20.02754
3. D. Przepiorka, R. Q. Le, A. Ionan, R. J. Li, Y. H. Wang, R. Gudi, S. Mitra, J. Vallejo, O. O. Okusanya, L. Ma, Y. Yang, *Clinical Cancer Research*, **28(12)**, 2488(2022), https://doi.org/10.1158/1078-0432.CCR-21-4176
4. D. J. Martini, Y. B. Chen, Z. DeFilipp, *The Oncologist*, **27(8)**, 685(2022), https://doi.org/10.1093/oncolo/oyac076
5. P. Brear, M. Hyvönen, *Acta Crystallographica Section F: Structural Biology Communications*, **78(10)**, 348(2022), https://doi.org/10.1107/S2053230X22008767
6. O. Schueller, A. Willson, N. Singh, L. Lohmer, A. Alabanza, J. Patel*, Clinical Pharmacology in Drug Development,* **11(7)**, 795(2022), https://doi.org/10.1002/cpdd.1082
7. H. A. Blair, *Belumosudil: first approval. Drugs*, **81(14)**, 1677(2021), https://doi.org/10.6084/m9.figshare.15183840
8. C. Cutler, S. J. Lee, S. Arai, M. Rotta, B. Zoghi, A. Lazaryan, A. Ramakrishnan, Z. DeFilipp, A. Salhotra, W. Chai-Ho, R. Mehta, *Blood, The Journal of the American Society of Hematology*, **138(22)**, 2278(2021), https://doi.org/10.1182/blood.2021012021
9. A. Fortuna, J. Sousa, G. Alves, A. Falcão, P. Soares-da-Silva, *Analytical and bioanalytical chemistry*, **397**, 1605(2010), https://doi.org/10.1007/s00216-010-3673-0
10. Guideline ICH (2005) Validation of analytical procedures: text and methodology, Q2 (R1) 1(20):05

[RJC- 8697/2023]