ijlpr 2022; doi 10.22376/ijlpr.2023.13.1.SP1.P83-P93

International Journal of Life science and Pharma Research
ISSN 2250-0480

Stability Indicating Isocratic HPLC Method for Bilastine and Characterization of Forced Degradation Products by LC-MS/MS

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Abstract: The present study aims to develop and validate a simple, precise, accurate, stability indicating and isocratic reverse-phase high-performance liquid chromatography (RP-HPLC) method for estimating Bilastine in bulk and synthetic mixture. Bilastine from its degradation products were well separated and estimated on Discovery C_8 column (250 mm x 4.6 mm, 5μm) using methanol: 0.1% ortho-phosphoric acid (55:45 %v/v) as a mobile phase and detection was performed at 276 nm by PDA detector. The degradation of Bilastine was studied under different ICH recommended stress conditions. The developed stability-indicating method was validated for system suitability, linearity, accuracy, precision, robustness, detection limit and quantitation as per ICH guidelines. The method was linear over 25-150 μg/ml. Mathematically computed Limit of Detection and Limit of Quantitation were found to be 0.19μg/ml and 0.57μg/ml, respectively. It was discovered that Bilastine degrades under acid and oxidation conditions. By using LC-MS/MS analysis, the structure of Bilastine breakdown products created amid acidic and oxidative settings was elucidated. A complete fragmentation pathway of Bilastine was established to elucidate the degradation products' structures using LC–MS/MS. The elemental concentrations of oxidation products and their fragmentation products were studied using the acquired mass values. The overall data was utilized to describe the degradation of products and their fragmentation process. The developed method can be used for routine analysis of Bilastine in dosage form as it can estimate of Bilastine in presence of excipients and degradation products.

Key Words: Bilastine, Stability Indicating HPLC Method, Degradation Products, LC-MS/MS, Characterization

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Received On **18 October 2022**
Revised On **06 December 2022**
Accepted On **13 December 2022**
Published On **01 January 2023**

This research did not receive any specific grant from any funding agencies in the public, commercial or not for profit sectors.

Citation Dr. Shital Patel, Dr. T. Yunus Pasha , Stability Indicating Isocratic HPLC Method for Bilastine and Characterization of Forced Degradation Products by LC-MS/MS.(2023).Int. J. Life Sci. Pharma Res.13(1), P83-P93
http://dx.doi.org/10.22376/ijlpr.2023.13.1.SP1.P83-P93

1. INTRODUCTION

A unique second-generation H1 antihistaminic medication called bilastine was created to address the symptoms of chronic urticaria and allergic rhinitis. A potent and specific H1-antihistamine activity is achieved by bilastine [1]. It is 2-[4-(2-(4-(1-(2-ethoxyethyl)-1H-benzimidazol-2-yl) piperidin-1-yl) ethyl) phenyl]-2-methylpropionic acid chemically (Fig. 1). Bilastine has a little apparent affinity for other receptors, according to pharmacological investigations, and is extremely selective for the H1 receptor in both in vivo and in vitro experiments. The absorption of bilastine is fast, linear and dose-proportional; it appears safe and well tolerated at all dose levels in a healthy population[2]. Bilastine has been granted marketing authorization in most European countries in form of solid unit-dose preparations (tablets) with a 20 mg per unit dose.

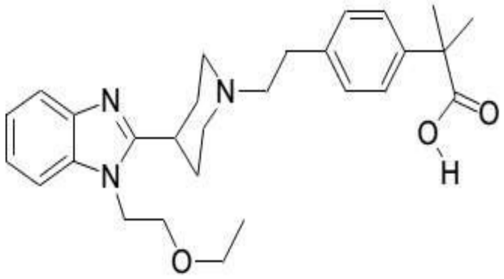


Fig. 1: Structure of Bilastine

Considering International Conference on Harmonization (ICH) recommendations Q1A (R2) [3], it is necessary to utilize a validated stability-indicating assay technique for assessing the stability of a new drug substance or new drug product. The reporting, identification and characterization of degradation products were mandated by International Conference on Harmonization (ICH) and other international agencies[3.4] stability guidelines. However, the number of degradation products produced during storage may be quite low. Hence forced degradations are advised to produce more of them [5]. The four basic processes that cause a substance to degrade are photolysis, oxidation, heat, and hydrolysis. To gather data on the degradation products likely to occur under the impact of these circumstances, the ICH advises exposing the medicine to oxidative stress of hydrolysis, oxidation, heat, and photolysis[3]. Still, as the amounts of degradation products (DPs) in the generated stressed mixture are lower, identification of DPs is very difficult. As a result, hyphenated techniques like LC–MS are currently extensively used for this purpose. However, only the UV spectrophotometric technique for quantitative measurement of bilastine[7] and hydrophilic interaction high-performance liquid chromatography technique for determination of bilastine in the presence of its impurities[6] were published, according to a survey of the literature. An inorganic salt buffer solution, including an ion agent and an organo-solvent mixture, was eluted gradientally, with a C18 column serving as the stationary phase, according to a Chinese patent[8]. Various articles have been published that describe the measurement of bilastine in pharmacokinetic investigations using tandem mass spectrometry and liquid chromatography with fluorescence detection [9, 10–13]. Recently, two publications [14, 15] addressing the oxidation reactions of bilastine were reported. However, it was not discovered that any research paper dealt with the thorough analysis of Bilastine's degradation and the characterization of its degradation products. These data serve as the basis for our study. The goal of this study was to create isocratic stability indicating the HPLC technique as recommended by ICH guidelines for the measurement of bilastine in various stress-induced materials. It also aimed to describe degradation products by LC-MS/MS analyses.

2. MATERIALS AND METHODS

2.1 *Chemicals and Reagents*

Symed Labs limited sent a complimentary sample of bilastine API; in Hyderabad (Telangana). We bought methanol and water (HPLC grade) from Merck in India. The sodium formic acid hydroxide, O-phosphoric acid (OPA), hydrochloric acid, and hydrogen peroxide utilized all of the analytical quality supplied by SD. Fine Chemicals in India.

2.2 *Instruments and apparatus*

A high-performance liquid chromatography (HPLC) system of water used for the LC investigation made up of an online degasser, a sample injector, a PDA detector, a pump, and a computer system loaded with Empower software. Reversed-phase Discovery C_8 (250 × 4.6 mm, 5-µm particle size) column was used for chromatographic separation. The LC–MS system was managed by Analyst software, which included GLscience LC-800 System and AB sciex QTRAP 4500 MS/MS system. In addition, the Inertsil C_{18} column (150 mm length x 2.1 mm i. d., 3 µm particle size) was used for LC-MS/MS study. The LC system used for LC-MS/MS study was also coupled with a UV detector.

2.3 *Chromatographic conditions*

Samples were examined between 210 - 410 nm and 276 nm - λ_{max} of Bilastine was selected for characterization. Chromatographic separation was accomplished on Discovery C_8 (250 mm x 4.6 mm i.d., 5µm) using a mobile phase system containing methanol: 0.1% OPA (55:45 v/v) pumped at a flow rate of 1.0 ml/min. The injection volume was 10 µL, and the column temperature was set at 35°C. The chromatogram ran for 7 min.

2.4 *LC/MS conditions*

Bilastine and its degradation products were separated by chromatography using an Inertsil C18 column (150 mm length x 2.1 mm i. d., 3 m particle size), mobile phase made up of 0.1% formic acid and methanol (50:50 v/v), and pumped at a rate of 0.2 ml/min. The drug injection volume was 10 L, and the column temperature was 40 °C. Utilizing a UV detector, the eluted components were found. MS/MS is also combined with the HPLC system. By conducting mass spectral analyses on the drug and its degradation products, respectively, a fragmentation profile of bilastine and its degradation products was developed. The products were ionized in the mass range of 100-540 Da using the positive channel of electron spray ionization (ESI). High intensity of molecular ions peaks and daughter ions peaks of degradation products were obtained as the parameters of MS were adequately adjusted.

2.5 Preparation of standard solution

By adequately measuring 100 mg of bilastine into a 100 ml volumetric flask, adding 50 ml of methanol, and then sonicating the mixture for 10 minutes, a 100 ml standard stock solution of bilastine was created. To acquire the working range of concentrations, methanol was diluted as needed with a standard stock solution (1000g/ml). The stock solution remained stable for at least three days when maintained at room temperature.

2.6 Preparation of test solution

In a 200 ml volumetric flask, bilastine (200 mg) and a placebo were added. Placebo has 10 mg of each excipient—magnesium stearate, colloidal anhydrous silica, microcrystalline cellulose, and sodium starch glycolate- 10mg. Subsequently, 100ml of methanol was added, and the mixture was sonicated for 40 minutes while occasionally shaken. Using methanol, dilution up to the capacity was completed. It was diluted to produce the test solution containing 100g/ml of bilastine after being filtered using a 0.45 µ PVDF Millipore Filter.

2.7 *Validation of the HPLC method*

Test for system suitability

Six replicates of 100 µg/ml of bilastine solution were analyzed to evaluate theoretical plates and tailing factor by optimized method to confirm the system's appropriateness for the planned application.

2.8 Linearity

Accurately measured volumes, 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 ml of the bilastine standard solution (1000 µg/ml), were successively transferred to 10 ml volumetric flasks. They were diluted up to volume with methanol to obtain final concentrations of 25-150µg/ml and mixed properly. Each solution's 20µL serial dilutions were chromatographed 2 - 3 times, and the analysis was carried out using an improved technique. The average area of the bilastine peak versus the Bilastine concentration was plotted to develop the regression model.

2.9 Precision

Seven injections of 100 µg/ml bilastine solution were analyzed to determine whether the system could repeat the results of the developed method. The percentage relative standard deviation for retention time and peak area of the drug was calculated. Three concentrations (50, 100 and 150 µg/ml) of bilastine were analyzed three times on the same day and for three different days to determine the intraday and interday precision, respectively.

2.10 Accuracy

In the test solution mixture having 100 µg/ml of bilastine, standard solution (100µg/ml) was added at 50%, 100% and 150% concentration levels. Then, 20 µl of each solution was injected three times and chromatographed. The amount of Bilastine was estimated using a regression equation, and the percentage recovery of bilastine was calculated.

2.11 Robustness

To assess the robustness, the experimental conditions were purposefully modified. Variations were made to the temperature of the column oven (35±1°C), mobile phase flow rate (1.0±0.1 ml/min.) and methanol content (55±2 %). In each case, the %RSD of the area of the bilastine peak and retention time was calculated. The retention time and area of the bilastine peak were also compared with that obtained under the optimized method.

2.12 Limit of Detection and Limit of Quantitation

Using the y-intercept of the linear lower concentrations (N) standard deviation and slope (S) of the calibration curve, LOD and LOQ were computed per ICH guidelines.

2.13 Forced Degradation Study

Bilastine standard solution (1 mg/ml) was stressed under thermal, photolytic, acid hydrolytic, base hydrolytic, neutral hydrolytic and oxidative stress conditions to degrade the drug partially. To induce degradation by acid, base, neutral hydrolysis and oxidation, 2 ml of the standard solution of Bilastine (1000 µg/ml) was transferred to a 20 ml volumetric flask followed by 2 ml of 2.0 M HCl, 2 ml of 2.0 M NaOH, 2 ml of distilled water and 2 ml of 10% H_2O_2 to the different flask, respectively. Then the mixture was heated in a thermostatically controlled water bath at 80°C. Samples were taken at a predetermined time interval. Then the mixtures were neutralized in acidic and basic stress conditions and diluted with methanol up to the mark. For thermal degradation, 50 mg Bilastine powder was placed in a convection oven at 70°C for 8 hr and preceded as per sample preparation. To check photolytic degradation, the Bilastine drug (50 mg) was taken in petridish was kept in a UV chamber (365 nm) for 24 hr at room temperature for photolytic degradation before being processed as directed for sample preparation.

3. RESULTS AND DISCUSSION

3.1 Development and optimization of the method

To achieve good separation of Bilastine from degradation products within a reasonable time, mobile phases of various compositions, different columns and system parameters were investigated (Table 1). In addition, a system suitability test was performed for method optimization. HPLC method using discovery C_8 (250 x 4.6 mm, 5µm) column and an isocratic solvent system consisting of methanol: 0.1% OPA (55:45 v/v) pumped at a flow rate of 1.0 ml/min. It was observed to provide a sharp and well-defined peak having a good tailing factor (1.00), theoretical plates (>6000) and low retention time (3.833 min.) (Fig.2). Hence it was selected throughout the study of Bilastine by HPLC. The optimized method separated Bilastine from acidic and oxidative degradation products.

Table 1: Optimization of the method

Stationary Phase	Mobile Phase	Flow Rate	Comments
Denali C18 (150 x 4.6 mm, 5µm)	Methanol: 0.1% OPA 50:50 v/v	1.0 mL/min.	Tailing factor -1.9 Theoretical plates - 824
Kromo C18 (250 x 4.6 mm, 5µm)	Methanol: 0.1% OPA 50:50 v/v	1.0 mL/min.	Tailing factor -1.6 Peak shape is not good
Azilen C18 (150 x 4.6 mm, 5µm)	Methanol: 0.1% OPA 55:45 v/v	1.0 mL/min.	Tailing factor -1.1 Theoretical plates - 551
Zodi C18 (150 x 4.6 mm, 5µm)	Acetonitrile: 0.1% OPA 55:45 v/v	0.8 mL/min.	Tailing factor - 0.8 Theoretical plates - 439 Splitting of peak
Discovery C18 (250 x 4.6 mm, 5µm)	Methanol: 0.1% OPA 65:35 v/v	0.8 mL/min.	Tailing factor -1.4 Theoretical plates> 2000
Discovery C18 (250 x 4.6 mm, 5µm)	Methanol: 0.1% OPA 55:45 v/v	1.0 mL/min.	Splitting of the tip of the peak
Discovery C8 (250 x 4.6 mm, 5µm)	Methanol: 0.1% OPA 55:45 v/v	1.0 mL/min.	Tailing factor-1.0 Theoretical plates- 6075 Peak shape is good.

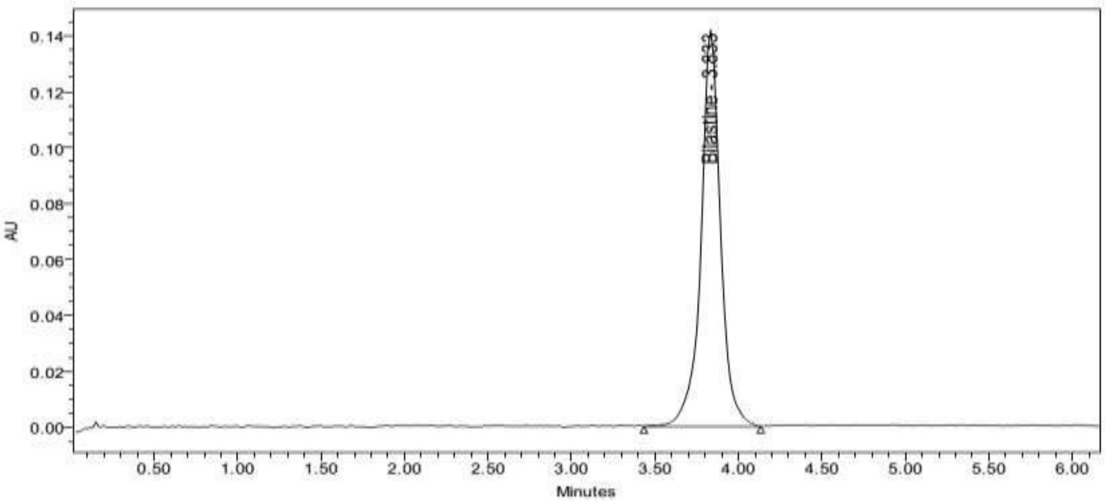


Fig. 2: RP-HPLC Chromatogram of Bilastine standard (100 µg/ml)

3.2 *Validation of the method*

The optimized method's fitness for the intended purpose was verified as per the ICH guidelines.

3.3 *System suitability Test*

The results of the system suitability test ensure the adequacy of the HPLC testing system for routine analysis of Bilastine. The number of theoretical plates for six replicate analyses found (> 5500) was more than the reference value (>2000), and the tailing factor found (1.0 ± 0.03) was as per the requirement (< 2.0)[16].

3.4 *Linearity and Range*

Over the range of concentrations of 25-150 µg/ml, the calibration curve was linear (r^2 ± SD = 0.9996 ± 0.0001). The calibration curve for the suggested HPLC technique of bilastine measurement is shown in Fig. 3. To determine the calibration equation and correlation coefficient, peak area and concentration were submitted to linear regression analysis. Over the range of concentrations of 25-150 g/ml, the regression analysis of the data showed a solid linear connection.

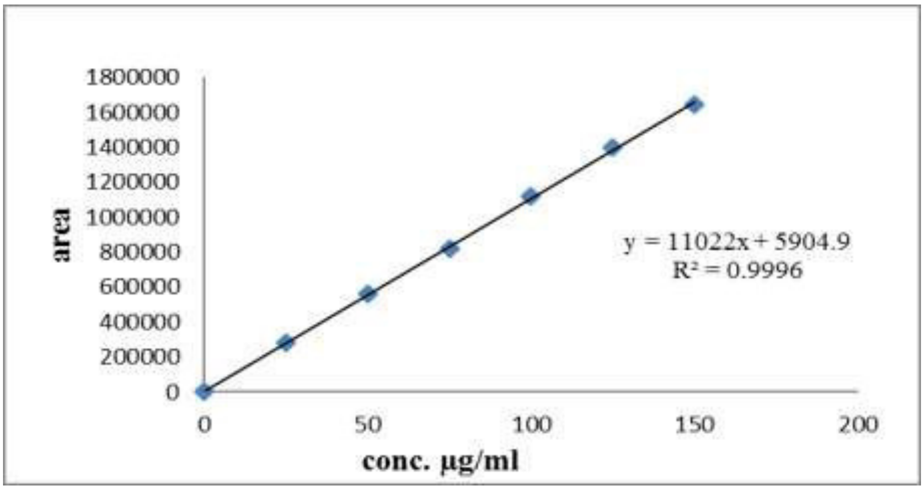


Fig. 3: Calibration curve of Bilastine for RP-HPLC

3.5 *Accuracy*

Bilastine from a previously examined laboratory mixing solution after spiking bilastine at 50, 100 and 150% levels were determined, and the % recovery calculated was between 99 -100%, as per the acceptance criteria [17]. (Table 2).

Table 2: Accuracy data

Level of recovery	Conc. (added std. sol.) µg/mL	Recovered conc.	% recovery	Mean % recovery ± S.D. (n=3)
50%	25.00	24.79	99.14	99.54 ± 0.35
		24.92	99.69	
		24.95	99.79	
100%	50.00	49.92	99.84	99.51 ± 0.37
		49.55	99.11	
		49.80	99.59	
150%	75.00	74.61	99.48	99.59 ± 0.10
		74.75	99.66	
		74.73	99.64	

3.6 *Precision*

Less than 1% RSD was discovered for retention time and the area of Bilastine measurement, demonstrating the repeatability of the established HPLC technique. Less than 1% and less than 2% are %RSD of interday and intraday precision of bilastine measurement. The small values of %RSD (acceptance criteria for repeatability ≤1% and intermediate precision ≤ 2%) [18] proved the great precision of the proposed method.

3.7 *Limit of detection (LOD) and Limit of Quantitation (LOQ)*

The limit of detection (LOD) and Limit of Quantitation (LOQ) was calculated using the following mathematical formula according to the ICH guidelines.

LOD = 3.3 x (standard deviation of y-intercept/slope)

Where y-intercept and slope were taken from the calibration curve.

3.8 *Robustness*

As shown by the consistent value of the retention time as well as the size of the Bilastine peak, it was discovered that the procedure was robust regardless of any little variation in the column temperature (35± 1°c) (Table 3). Significant changes in the retention period and area of the bilastine peak were induced by small variations in mobile phase flow rate (1.0± 0.1 ml/min) and mobile phase ratio. Table 4 shows a summary of HPLC method validation data.

Table 3: Robustness data

Parameter	Value	Retention time	% RSD	Area	% RSD
Flow rate (mL/min.)	0.9	4.117	4.960	1193463	2.363
	1.0	3.861		1138403	
	1.1	3.738		1164786	
Temperature (column) °C	34	3.975	1.985	1178901	1.971
	35	3.861		1138403	
	36	3.828		1177395	
Mobile phase ratio (methanol: 0.1% OPA v/v)	57:43	3.821	2.999	1188747	3.188
	55:45	3.861		1138403	
	53:47	4.041		1211968	

Table 4: Validation Data

Validation parameter	Bilastine
Linearity (n=3)	25-150 µg/ml
Correlation co-efficient (r^2±SD)	0.9996 ±0.0001
Precision (%RSD) Repeatability (n=6) Intraday precision (n=3) Interday precision(n=3)	 0.253 0.941 1.107
Accuracy (% recovery)	99.55±0.26
LOQ	0.57 µg/ml
LOD	0.19 µg/ml
Robustness	Robust in column temperature

3.9 *Forced degradation study*

When chromatographed after refluxing in 2M NaOH at 80°C for 2 hours and in distilled H_2O at 80°C for 2 hours, there was no appreciable change in the peak area of bilastine, and no new peak was also not seen. With high resolution (Rs=3.6), the chromatogram of an acid-stressed bilastine sample shows the degradation product at 4.824 min, and the bilastine at 3.798 min (Fig.4). The purity angle and threshold for bilastine peak are, respectively, 0.898 and 1.015, indicating the purity of bilastine at its peak. Acid-induced degradation product peak purity angle and purity threshold are 9.437 and 11.229, respectively, indicating that just one degradation product is generated.

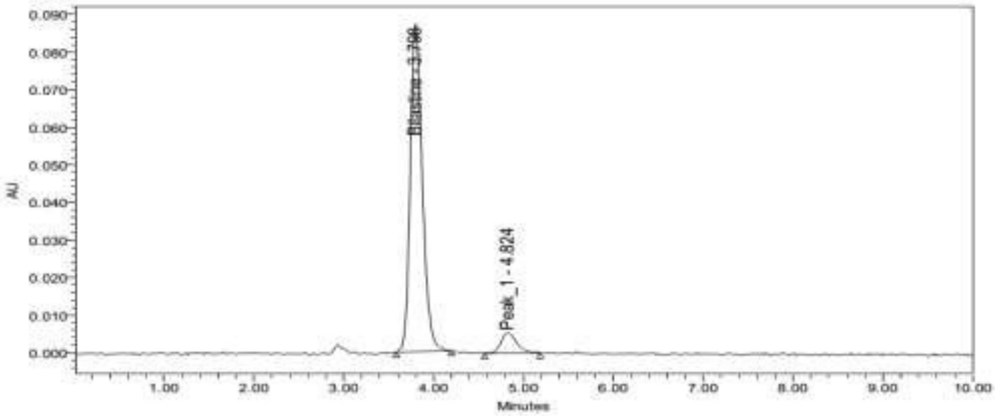
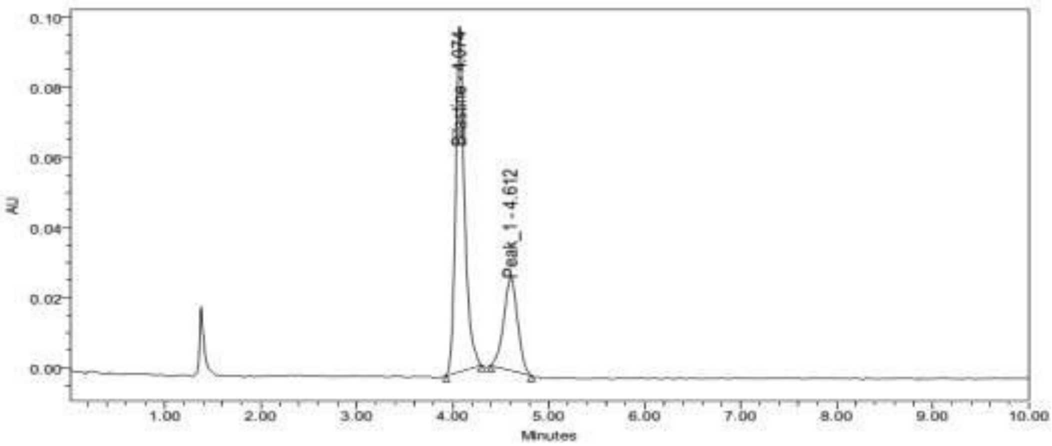


Fig. 4: Chromatogram of Bilastine after refluxing with 2 N HCl at 80° C for 30 min.

Chromatogram of a bilastine sample under H_2O_2 stress, which produced the degradation result at 4.612 min and the bilastine at 4.074 min, both with a resolution of 2.0 or higher (Rs=2.3) (Fig.5). The peak purity of bilastine is shown by the purity angle for the peak, which is 0.539, and the purity threshold for the peak, which is 0.812. For the peak of the oxidation-induced degradation product, the purity angle and threshold are 1.709 and 2.884, respectively. PDA data showed that the hydrogen peroxide retention duration is 1.4 minutes. Bilastine did not degrade when it was chromatographed after 2 hours of refluxing in 2M NaOH at 80°C, 2 hours of refluxing in distilled water at 80°C, and 2 hours of exposure to dry heat and photolysis.



Overall, forced degradation study data are presented in table 5. Forced degradation study proves that the developed method determines the Bilastine specifically in the presence of degradation products induced by acid and oxidative conditions.

Fig. 5: Chromatogram of Bilastine after refluxed with 10% hydrogen peroxide at 80° C for 30 min.

Table 5: Forced degradation study data

	Forced degradation condition	% drug after forced condition	Purity angle of drug peak	Purity threshold of drug peak	% degradation
Acid Hydrolysis	2 M HCl, 80°C for 30 min.	77.7029 %	0.898	1.015	22.2971%
Base Hydrolysis	2 M NaOH, 80°C for 3 hr	100%	0.120	0.784	Nil
Neutral Hydrolysis	Water, 80°C for 3 hr	100%	0.120	0.790	Nil
Oxidation	10% H_2O_2, 80°C for 30 min.	70.4358%	0 .539	0.812	29.5642%
Photo-degradation	UV chamber at 365 nm for 24 hr	100%	0.568	0.778	Nil
Dry heat degradation	70°C for 8 hr	100%	0.568	0.778	Nil

3.10 Characterization of degradation products using LC-MS/MS

The $[M + H]^+$ value for Bilastine observed in the positive mode of ESI was 464.3 and matched with the molecular weight of Bilastine (463.61). A mass spectrum of product ion of Bilastine produced by LC-MS/MS is presented in Fig. 6. Fragmentation pattern of Bilastine is illustrated in Fig. 7 from the interpretation of fragments by the best suitable molecular formulae to the experimental value of m/z.

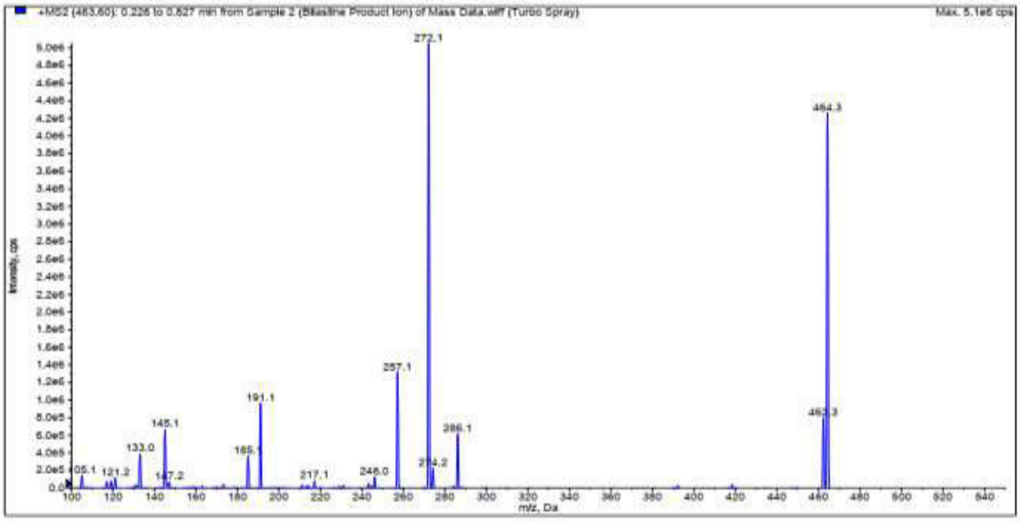


Fig. 6: Mass spectrum of Bilastine (product ion) by LC-MS/MS.

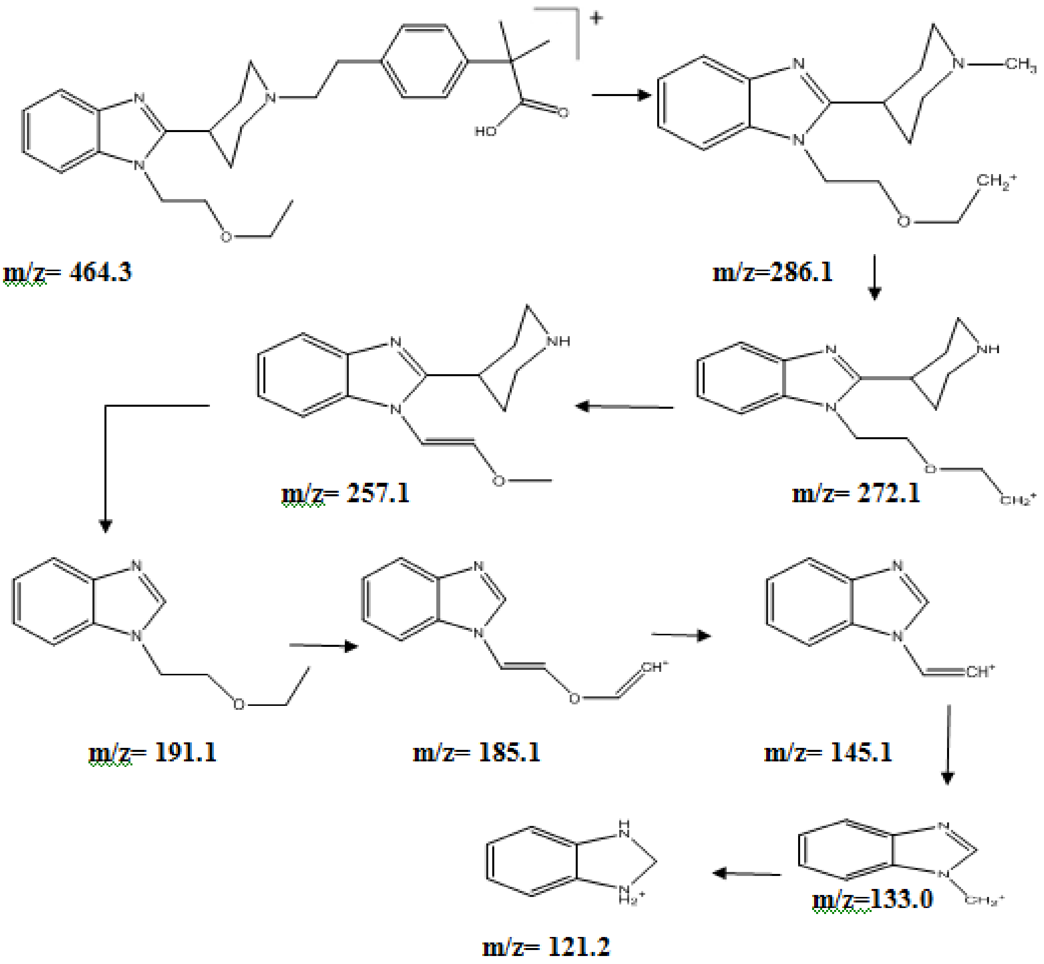


Fig. 7: Probable fragmentation pattern of Bilastine

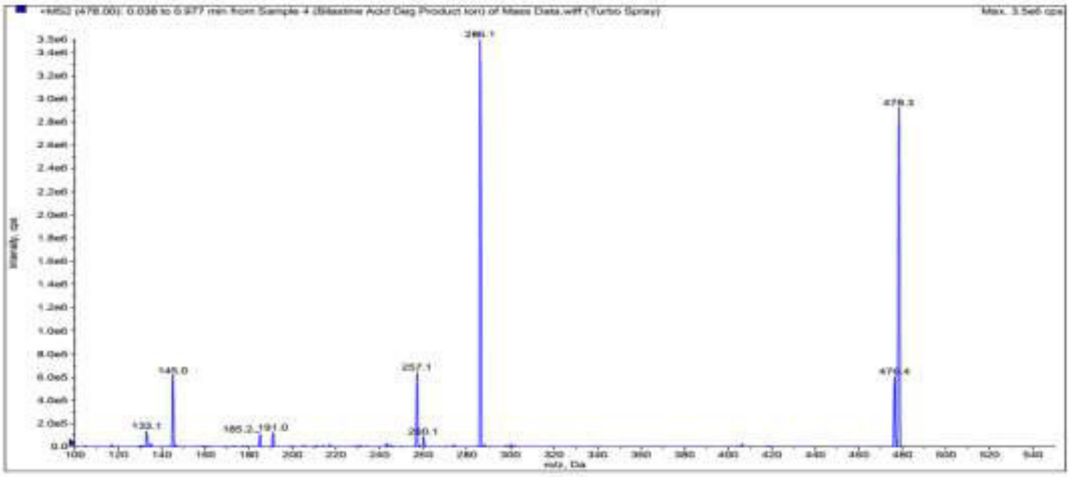


Fig. 8: Mass spectrum of degradation impurity of Bilastine by acid

Fig. 8 presents a mass spectrum of degradation impurities generated in acid-stressed samples. The results of LC–MS/MS analysis showed that the degradation product formed after acidic hydrolysis had a molecular ion peak at 478.43 Da which has 14.00 Da more value than the molecular ion peak of Bilastine (464.3 Da), which indicates the additional of methylene group. The likely Fragmentation pattern of acid stressed Bilastine is illustrated in Fig. 9. Mass spectra and the likely fragmentation pattern of acid-stressed Bilastine sample confirm that Bilastine in presence of hydrochloric acid reacts with methanol and produced methyl ester of Bilastine. This reaction suggests that methanol should be avoided as a solvent in products of Bilastine or any process of Bilastine as the accidently presence of acid produces methyl ester of Bilastine.

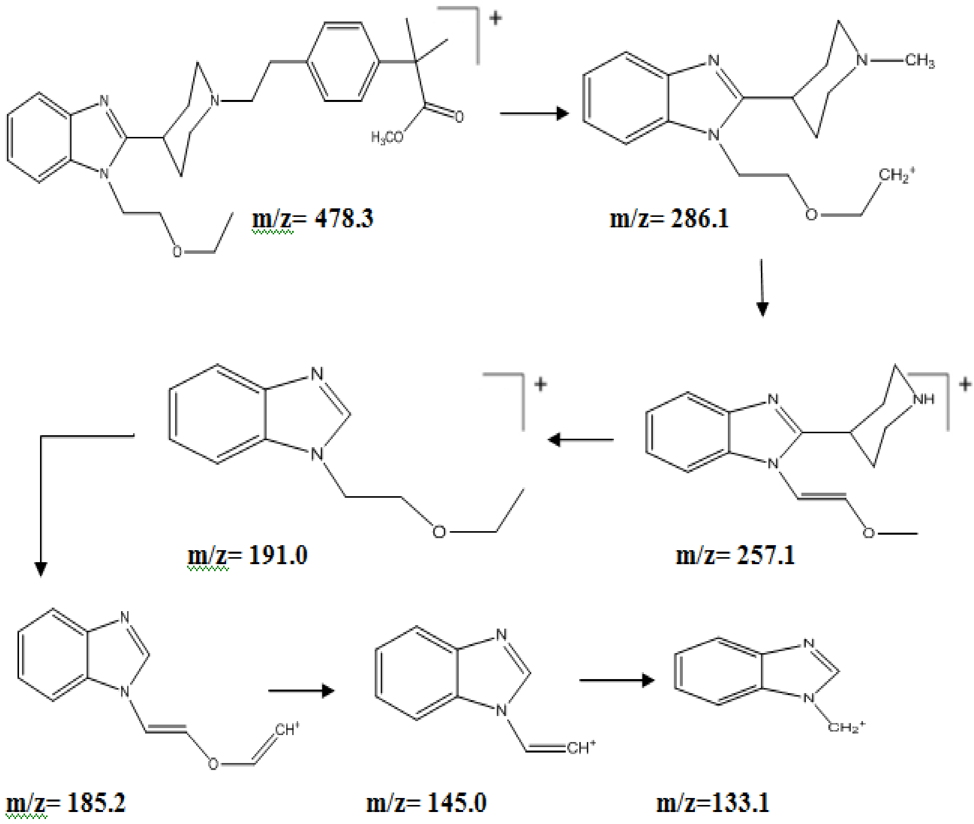


Fig. 9: Probable fragmentation pattern of acid degradation product of Bilastine

The LC-MS/MS spectrum of the oxidation degradation product of Bilastine by hydrogen peroxide showed a molecular ion peak at 480.4 Da ([M+H] $^+$) formed in positive ionization mode. The molecular ion at 480.4 Da is approx. 16 Da more than the m/z of the molecular ion of Bilastine (464.3 Da), which resembles an oxygen ion. The molecular ion peak at 480.4 Da represents the formation of N-oxide of Bilastine in the reaction of Bilastine with hydrogen peroxide. N-oxide of Bilastine is reported as a degradation product of Bilastine in the literature also [6, 13]. Similar outcomes were revealed about the genesis of N-oxide derivatives under such conditions[19-24]. Fig. 10 presents a mass spectrum of the oxidation degradation product of Bilastine. The fragmentation pattern of the degradation product of hydrogen peroxide-stressed Bilastine is illustrated in Fig. 11. Mass spectra and fragmentation pattern of oxidation degradation product affirms that Bilastine reacts with an oxidizing agent, hydrogen peroxide and produces 2-[4-(2-{4-[1-(2-Ethoxy-ethyl)-1H-benzoimidazol-2-yl]-N-oxy-piperidin-1-yl}-ethyl)-phenyl]-2-methyl propionic acid.

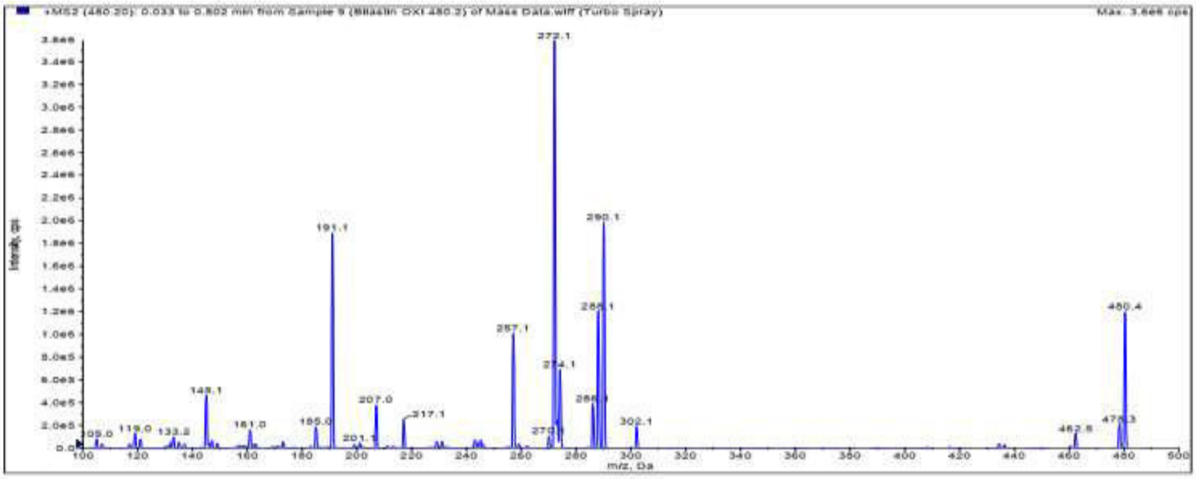


Fig. 10: Mass spectrum of Hydrogen peroxide stressed Bilastine sample

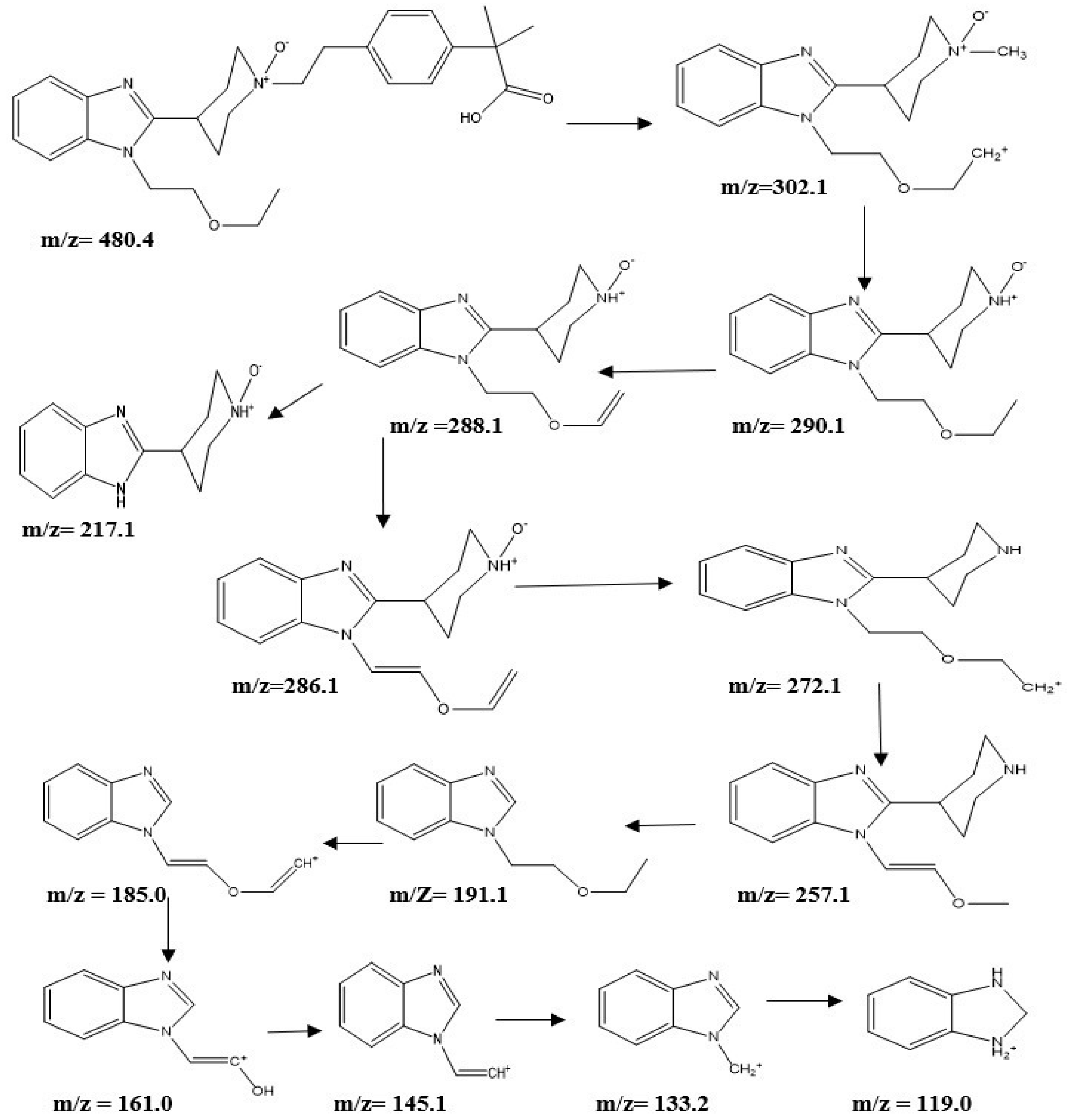


Fig. 11: Likely fragmentation pattern of oxidation degradation product of Bilastine

4. CONCLUSION

In conclusion, the present study presents accurate, precise, simple, and specific and isocratic High-Performance Liquid Chromatography method for determining Bilastine in the pharmaceutical product without interference from excipients and degradation products produced by stress study. Bilastine was determined after exposure to varied stress conditions to investigate the degradation behaviour of Bilastine, and it was observed that Bilastine is prone to acid in the presence of methanol and oxidation. All validation parameters permit the application of the suggested method in the quality control laboratory for routine assessment of Bilastine and stability testing of Bilastine. The described method is stability indicating one. Degradation products of Bilastine produced in acidic conditions, and oxidizing conditions were also identified by LC-MS/MS study.

5. ACKNOWLEDGEMENTS

The authors are grateful to Symed Labs Limited, Hyderabad, Telangana, India, for providing the gift sample of Bilastine. Research work is performed as part of PhD from Gujarat Technological University.

6. AUTHOR CONTRIBUTION STATEMENT

Dr. Shital Patel has developed and validated the analytical method. Dr. T. Yunus Pasha provided valuable inputs towards the design of the manuscript. All authors read and approved the final version of the manuscript.

7. CONFLICT OF INTEREST

Conflict of interest declared none.

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