FORCED DEGRADATION STUDY OF SELECTIVE H_1-ANTIHISTAMINIC DRUGS IN BULK AND FORMULATION

A Thesis submitted to Gujarat Technological University

for the Award of

Doctor of Philosophy

in

Pharmacy

by

Shital Sukhabhai Patel

Enrollment No 129990990010

under supervision of

Dr. T. Y. Pasha

GUJARAT TECHNOLOGICAL UNIVERSITY

AHMEDABAD

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I declare that the thesis entitled **FORCED DEGRADATION STUDY OF SELECTIVE H_1-ANTIHISTAMINIC DRUGS IN BULK AND FORMULATION** submitted by me for the degree of Doctor of Philosophy is the record of research work carried out by me during the period from **December 2012** to **December 2018** under the supervision of **Dr. T. Y. Pasha** and this has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other University or other institution of higher learning.

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ABSTRACT

A sensitive, precise, accurate, stability indicating and isocratic HPLC method was developed and validated for estimation of Ebastine. The separation of Ebastine from its degradation products was achieved on Phenomenex C_{18} column using acetonitrile: 0.5% phosphoric acid (68:32 %v/v) as a mobile phase and detection was performed at 254 nm. The degradation of Ebastine was studied under different ICH recommended stress conditions. The method was linear over the range of 5-120 µg/mL. Limit of Detection and Limit of Quantitation, calculated mathematically, and were found 0.30 µg/mL and 0.9 µg/mL, respectively. Ebastine was found to degrade under acid and oxidation conditions with first order reaction and zero order reaction, respectively. Half time of room temperature at room temperature in acidic and oxidative condition was calculated. The Arrhenius plot was constructed and activation energy of degradation was calculated for both conditions.

A precise, accurate, stability indicating and isocratic high performance liquid chromatographic method for determination of Azelastine Hydrochloride was developed and validated. Chromatography was performed on a 250 mm x 4.6 mm, 5-µm particle size, Waters Spherisorb CN column using 50:50 %v/v mixtures of 0.05M potassium dihydrogen phosphate buffer (pH 3.0) and acetonitrile as mobile phase and the detection was carried out at 290 nm. The degradation of Azelastine Hydrochloride was studied under different ICH recommended stress conditions and it was found stable. Linearity was established in the concentration range of 5 - 120 µg/mL. Limit of Detection (LOD) and Limit of Quantitation (LOQ) were calculated 0.81µg/mL and 2.44µg/mL respectively.

A sensitive, precise, accurate, stability indicating and isocratic HPLC method was developed and validated for estimation of Bilastine. Bilastine from its degradation products were separated 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. The degradation of Bilastine was studied under different ICH recommended stress conditions. The method was linear over the range of 25-150 µg/mL. Limit of Detection and Limit of Quantitation, calculated mathematically, and were found 0.19µg/mL and 0.57µg/mL, respectively. Bilastine was found to degrade under acid and oxidation conditions. Acid

degradation of Bilastine follows first order reaction. Half time of Bilastine in 2 N HCl at room temperature was calculated. The Arrhenius plot was constructed and activation energy of degradation of Bilastine in acidic condition was calculated.

The structure of degradation products of Ebastine and Bilastine formed under various stress conditions were elucidated by LC–MS/MS study. The previously developed high performance liquid chromatographic methods were suitably modified for LC–MS/MS studies by replacing phosphoric acid with formic acid of the same concentration. A complete fragmentation pathway of the drugs (Ebastine and Bilastine) was first established to elucidate the structures of all the degradation products using LC–MS/MS fragmentation studies. The obtained mass values were used to study elemental compositions of degradation product and fragmentation products, and the total information helped to characterize degradation products, along with its fragmentation pathway.

Acknowledgement

No research is ever the outcome of single individual's talent or efforts. I have seen and experienced the countless blessing showered on me by my parents, all family members, teachers, friends and all my well-wishers knowing the Gold's hand is there, always guiding me and leading me to greater heights. It provides me pleasure to convey my gratitude to all those who have directly or indirectly contributed to make this work a success. I must make special mention of some of the personalities and acknowledge my sincere indebtedness to them.

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Words fail me to express my gratitude to my husband **Vipul**, whose dedication, love and persistent support has taken load off my shoulder. Finally I would like to thank everybody who was important to successful realization of this Dissertation.

Shital Patel

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List of Abbreviation

API	Active pharmaceutical ingredient
R&D	Research and development
QC	Quality control
ADL	Analytical development laboratory
IVIVC	In vivo- in vitro correlation
HPLC	High performance liquid chromatography
ICH	International Conference on Harmonization
FDA	Food and Drug administration
HCl	Hydrochloric acid
OPA	Ortho phosphoric acid
NaOH	Sodium hydroxide
H_2O_2	Hydrogen peroxide
UV	Ultra violet
W-h/m^2	Watts-hour per square meter
nm	Nano meter
RP-HPLC	Reverse phase high performance liquid chromatography
PDA	Photo diode array
LC-MS/MS	Liquid chromatography- tandem mass spectrometry
DL	Detection limit
QL	Quantitation limit
RSD	Relative standard deviation
ºC	Degree Celsius
Kcal	Kilo calorie
gm	Gram
L	Littre
mL	Milliliter
Hr	Hour
Min.	Minute

DMSO	Dimethyl sulphoxide
PGD_2	Prostaglandin D_2
IL	Interleukin
TNF	Tumor necrosis factor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
HILIC	Hydrophilic interaction chromatography
mM	Millimeter
M	Molar (unit of molarity)
N	Normal (unit of normality)
NMR	Nuclear magnetic resonance
MS	Mass spectroscopy
IR	Infrared
TOF	Time of Flight
HPTLC	High performance thin layer chromatography
RP-HPLC	Reverse phase high performance liquid chromatography
GC	Gas chromatography
LC	Liquid chromatography
ACN	Acetonitrile
UVDP	Ultra violet degradation product
µm	Micrometer
µg	Microgram
i.d.	Internal diameter
ESI	Electro spray ionization
SI	Spray ionization
Da	Dalton
Conc.	Concentration
$t_{1/2}$	The time required for 50% of the drug to be changed
t_{10}	The time required for 50% of the drug to be changed

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CHAPTER 1

Introduction

1.1 Antihistaminic Drugs

Histamine, meaning 'tissue amine' is almost present in all animal tissues and in certain plants. It is present mostly within storage granules of mast cells. Skin, gastric and intestinal mucosa, lungs, liver and placenta are rich in histamine. Nonmast cell histamine occurs in brain, epidermis, gastric mucosa and growing regions. Histamine is also present in blood, most body secretions, venoms and pathological fluids. Histaminergic receptors were classified by into H_1, H_2, H_3 and H_4. H_1-antihistaminics competitively antagonize actions of histamine of the H_1 receptors. The uses of H_1-antihiataminics are based on their ability to block certain effects of histamine released endogenously. They are used as antiallergic, antipruritics, in common cold, in motion sickness and as antivertigo. [1]

Classification of H_1-antihiataminics [1]:

A) First generation H_1-antihiataminics:
   1) Highly sedative:- Diphenhydramine, Dimenhydrinate, Promethazine, Hydroxyzine
   2) Moderately sedative:- Pheniramine, Cyproheptadine, Meclizine, Buclizine, Cinnarizine
   3) Mild sedative:- Chlorpheniramine, Dexchlorpheniramine, Dimenthindene, Triprolidine, Cyclizine, Clemastine

B) Second generation H_1-antihiataminics: Fexofenadine, Loratadine, Desloratadine, Cetrizine, Levoetrizine, Azelastine, Mizolastine, Ebastine, Rupatadine, Embramine, Olopatadine, Methdilazine, Bilastine etc.

Antihistamines are medications used for the treatment of a variety of allergic conditions. Second generation H_1-receptor antagonists reduce or eliminate the sedation and anti-cholinergic side effects that produced by first generation H_1-receptor antagonists. In addition to the tighter specific binding of antihistamines to H_1-receptor, they may act on

other mediators of the allergic reactions. This tight receptor specificity results to fewer side effects of other neuronal or hormonal systems. Most of the second generation H_1-receptor antagonists are metabolized by the liver to active metabolites that play a significant role in their effect. [2]

1.2 Introduction to Drug Analysis

A Drug is termed as "A chemical substance used in the treatment, cure, prevention, diagnosis of disease or used to enhance physical or mental well-being." Normal body functions are varied by a drug. Drugs are prescribed for a fixed duration, or on a regular basis depending upon type and level of diseases. Prevention and cure of new diseases is an integral part of pharmacy, so the new drugs invented must meet the regulatory requirements and specification to prove its safety, efficacy and security towards the life of individuals during particular diseased condition. So, the development and validation of proper analytical method for each newly discovered drug has become the most important task in the field of pharmaceutical industries. [3]

Drug discovery process has been shortened owing to the introduction of new technology. The development of new chemical entities (NCEs) is comprised of two major activities: Drug discovery and drug development. The goal of the drug discovery program is to investigate a plethora of compounds employing fast screening approaches, leading to generation of lead compounds and then narrowing the selection through targeted synthesis and selective screening (lead optimization). This leads to the final selection of the most potentially viable therapeutic candidates that are taken forward to drug development. [4]

The number of new and generic drugs and drug combinations introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing drug. Generally, there is a time period from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. Analytical methods for the quantification of the new drug in biological fluids may not be available. Analytical methods for a drug in combination with other drugs may not be available in the literature. Sometimes available methods are unable to qualify and quantify impurities. The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and

these may not be reliable. Because of all these reasons it is necessary to develop and validate newer analytical methods for such drugs. [5, 6]

Analytical data are used to: [7]

- Screen potential drug candidates and its metabolites, degradants as well as impurities.
- Aid in the development of drug synthesis and its manufacturing method.
- To support formulation studies.
- To evaluate the stability of bulk pharmaceuticals and formulated products.
- Finished product testing for release. The quality of analytical data is a key factor in the success of a drug development program.

1.2.1 Analytical method development

The different activities of R&D include drug development, synthesis, analytical method development, manufacturing formulation, clinical trials, evaluation and finally launching finished products. Regulatory and quality assurance functions are closely associated with these processes. Before submitting the drug product for approval to the regulatory authorities, it is required to assure that all batches of drug products comply with predetermined criteria of specific standards, utilization of approved ingredients and production methods. It is the responsibility of pharmaceutical analysts in quality control (QC) and analytical development laboratories (ADL). The methods are generally developed in an analytical R&D and transferred to QC department or other departments. [8]

Quality assurance and quality control play a central role in estimating the safety and efficacy of medicines. A highly specific and sensitive analytical technique holds the key to design, development, standardization and quality control of medicinal products. They are equally important in pharmacokinetics and in drug metabolism studies, both of which are fundamental to the assessment of bioavailability and the duration of clinical response. The pharmaceutical analysts play a major role in assuring the identity, safety, efficacy and quality of the drug product [9].

Safety and efficacy studies require that drug substance and drug product meet following critical requirements that are: [10]

- Established identity and purity

- Established bioavailability and dissolution
- Established bioequivalence and IVIVC studies

Analytical method development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals with the objectives: [11-12]

- To qualify and quantify the active pharmaceutical ingredients in bulk as well as dosage form.
- To establish impurity profile and limit of allowable impurities in dosage form.
- The official test methods that result from analytical method development are used by quality control laboratories to ensure the identity, purity, potency, and performance of drug products.
- To ensure compliance with quality and safety standards.

Therefore, United States, Europe, Japan, and other countries have published compendia, or pharmacopeias that describe official test methods for many marketed drug products.

1.3 High Performance Liquid Chromatography (HPLC)[13-16]

"The technique through which the chemical components present in complex mixtures are separated, identified and determined is termed as Chromatography." This technique is a powerful tool not only for analytical methods but also for preparative methods. Compounds of high grade purity can be obtained by this method separating present impurities in it.

HPLC is the leading analytical techniques used in pharmaceutical industries. In HPLC and high pressure is used to force solvent through closed columns containing very fine particles that give high-resolution separations. HPLC method is most popular analytical technique because of its advantages like rapidity, specificity, accuracy, precision, ease of automation, speed, greater sensitivity, improved resolution, reusable columns and ease of sample recovery, easy handling and easy maintenance. HPLC method eliminates tedious extraction and isolation procedures, also.

1.3.1 Modes of HPLC

- **On the basis of relative polarity of the two phases:**

In Normal-phase chromatography the stationary bed is strongly polar in nature (e.g., silica gel), and the mobile phase is non polar (such as n-hexane or tetrahydrofuran). Polar samples are thus retained on the polar surface of the column packing longer than less polar materials.

Reverse-phase chromatography is the inverse of this. The stationary phase is non polar (hydrophobic) in nature (e.g. C_{18}, C_8, cyano), while the mobile phase is a polar liquid (e.g. water, buffer, methanol, acetonitrile). Here the more non polar the material is, the longer it will be retained on stationary phase.

- **On the basis of elution mode:**

In the isocratic elution, constant eluents composition is pumped through the column during the whole analysis.

In the gradient elution mode, mobile phase composition (and strength) is steadily changed during the run.

1.3.2 Components of HPLC

Various components of HPLC are

a) A solvent delivery system, including pump,
b) Sample injection system,
c) A chromatographic column,
d) A detector,
e) A strip chart recorder,
f) Data handling device and microprocessor control

1.3.3 HPLC method development

Methods for analyzing drugs in complex mixtures can be developed, provided one has knowledge about the nature of the sample, namely, its molecular weight, polarity, ionic character and the solubility parameter. An exact recipe for HPLC, however, cannot

be provided because method development involves considerable trial and error procedures. The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase. In general one begins with reversed phase chromatography, when the compounds are hydrophilic in nature with many polar groups. Method development depends on selection of best mobile phase, detector, column length and diameter, buffer, pH of buffer, type of stationary phase, gradient programming etc.

In reverse phase HPLC, the retention of analytes is related to their polarity. The more hydrophobic analyte retains for longer period. When an analyte is ionized, it becomes less hydrophobic and, therefore, its retention on stationary phase decreases. When separating mixtures containing acid and/or bases, it is necessary to control the pH of mobile phase using appropriate buffer in order to achieve reproducible results. So for separating acids & bases a buffered mobile phase is recommended to maintain consistent retention and selectivity. A buffered mobile phase, resists changes in pH so that the analytes and silica will be consistently ionized, resulting in reproducible chromatography. For neutral sample, buffers are not required in the mobile phase. Buffers play an additional role in the reproducibility of a separation.

The pH range most often used for reversed-phase HPLC is 1 - 8 and can be divided into low pH (1 - 4) and intermediate pH (4 - 8) ranges. Each range has a number of advantages. Low pH has the advantage of creating an environment in which peak tailing is minimized and method ruggedness is, maximized. For this reason, operating at low pH is recommended. At a mobile phase pH greater than 7, dissolution of silica can severely shorten the lifetime of columns packed with silica-based stationary phases.

The pKa value [acid dissociation (ionization) constant] for a compound is the pH at which equal concentrations of the acidic and basic forms of the molecule are present in aqueous solutions. Analytes may sometimes appear as broad or tailing peaks when the mobile phase pH is equal or around their pKa values. A more rugged mobile phase pH will be at least 1 pH unit different from the analyte pKa. This shifts the equilibrium so that 99% of the sample will be in one form. Dramatic changes in the retention and selectivity of basic and acidic compounds can occur when the pH of the mobile phase is changed. This is often a result of different interactions between the column and the analytes when the ionization of these compounds changes. It is important to evaluate these changes when a

method is developed in order to select the mobile phase pH that provides the most reproducible results.

1.4 Forced Degradation Study

Forced degradation study is a purposeful degradation in which the natural degradation rate of a pharmaceutical drug substance and drug product is increased by the application of an additional stress. Stress testing may be useful to determine whether accidental exposures to conditions other than normal ranges are deleterious to the product. [17]

Forced degradation studies are carried out for the following reasons: [17-19]

- To develop and validate stability indicating method to determine the degradation products formed during accelerated and long term stability studies.
- To explicate the possible degradation pathways of drug substances and drug products
- To identify the reaction that causes degradation of drug substance and drug product during storage and use.
- To identify impurities related to drug substances and drug product.
- To facilitate the improvements in the manufacturing process and formulation for generating stable formulation in parallel with accelerated pharmaceutical studies.

Regulatory requirement:

Forced degradation studies are described in various international guidelines [18, 20]. The International Committee for Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has published a set of guidelines that are applicable to forced degradation studies are:

- ICH Q1A – Stability Testing of New Drug Substances and Products [21]
- ICH Q1B – Photo stability Testing of New Drug Substances and Products [22]
- ICH Q2B – Validation of Analytical Procedures: Methodology [23]

In ICH Q1A, section 2.1.2, there are recommended conditions for performing the forced degradation studies to examine the effects of temperature (> 50°C), humidity (≥ 75% relative humidity), oxidation and photolysis on drug substances and drug products. These

samples are then used to develop stability indicating method. ICH Q1B gives the methodology to assess the photo stability of drug substances and drug products. According to it samples of drug substance and drug product, should be exposed to minimum of 1.2 million lux hours and 200 watt hours per square meter light. ICH Q2B gives guidance on validation of analytical methodology and in section B 1.2.2, there is a recommendation to use samples from forced degradation studies to prove specificity if impurities are not available.

Ragine Maheshwaran reported a clear perspective on FDA regarding the scientific considerations of forced degradation studies. A generic approach for stress testing has been proposed to achieve purposeful degradation which is predictive of long-term and accelerated storage condition. The generally 5-20% degradation is required, which covers the generally permissible 10% degradation for drug products. Over-stressing may form secondary degradants that would not be seen in formal shelf-life stability studies and under stressing may not serve the purpose of stressing. So, it is required to control the degradation to a desired level, 5-20%. If the substance does not show any degradation under any of the stress conditions then the stress testing shall be repeated to obtain adequate degradation. If degradation is not achievable then rationale shall be provided. If the conditions employed for stress study are too harsh and that most of the drug substance has degraded, stress studies should be repeated using milder conditions or short exposure of time to generate relevant degradation products [(20)].

Origin of degradation product/ degradation related impurities: [(17, 18)]

The main cause of appearance of impurities in drug substance or drug product is due its degradation or process related impurities. The chemical instability of the drug substance under the conditions of heat, humidity, solvent, pH and light encountered during manufacture, isolation, purification, drying, storage, transportation and/or formulation is main reason for its degradation. It is governed by inherent chemical stability of the drug substance. The major routes of degradation of any drug substance include hydrolysis, oxidation, heat and photolysis. The stress testing helps in generation of all possible degradation products that may form under different conditions.

- Hydrolytic condition

Hydrolysis is one of the most common degradation reactions over wide range of pH. Hydrolysis is a process in which API reacts with water to yield breakdown products of different chemical compositions. Water either as a solvent or as moisture in the air comes in contact with pharmaceutical products is responsible for degradation of the most of drugs. Hydrolytic study under acidic and basic condition involves catalyzation of ionisable functional groups present in the molecule. HCl, NaOH and water are employed for generating acidic, basic and neutral stress samples, respectively.

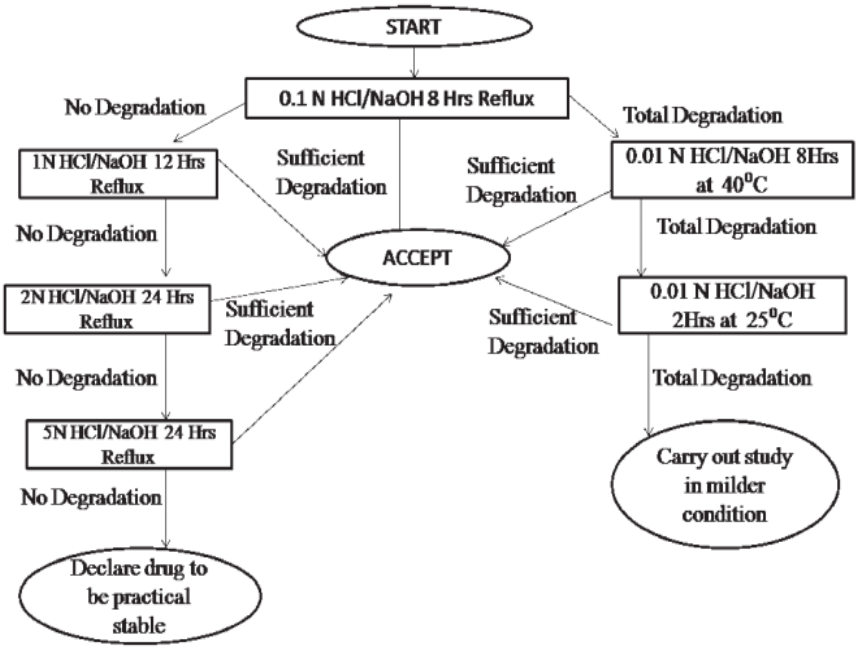


Figure 1.1 Flow chart for performing hydrolytic degradation

- Oxidative condition

Oxidation is an important degradation pathway as many drug substances undergo auto-oxidation i.e., oxidation under normal storage condition. Auto-oxidation is a free radical reaction that requires free radical initiator to begin the chain reaction. Hydrogen peroxide, metal ions, or trace level of impurities act as initiators for auto-oxidation.

Hydrogen peroxide is very common oxidant to produce oxidative degradation products which may arise as minor impurities during long term stability studies. The mechanism of oxidative degradation of drug substance involves an electron transfer mechanism to form reactive anions and cations. Amines, sulphides and phenols are

susceptible to electron transfer oxidation to give N-oxides, hydroxylamine, sulphones and sulphoxide.

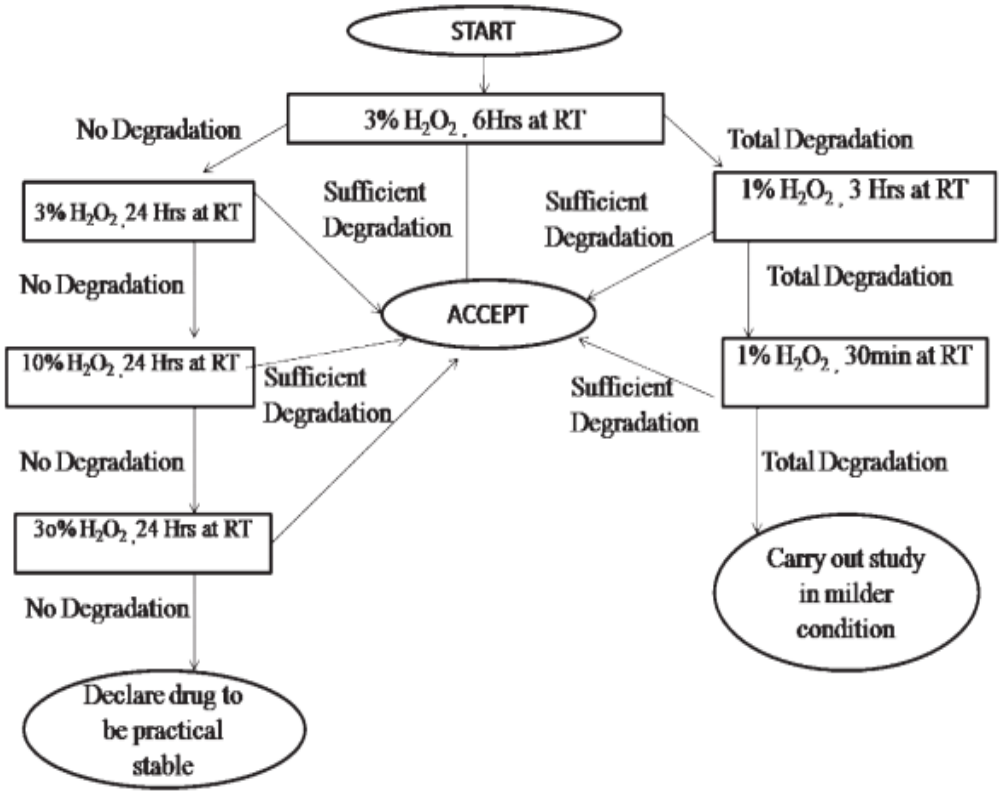


Figure 1.2 Flow Chart for performing oxidative degradation

- Thermal condition

In general, rate of a reaction increase with increase in temperature. Hence, the drugs are susceptible to degradation at higher temperature. Many APIs are sensitive to heat or tropical temperatures. Effect of temperature on thermal degradation of a substance is studied through Arrhenius equation:

$K= Ae^{-Ea}/RT$

Where k is specific reaction rate, A is frequency factor, Ea is energy of activation, R is gas constant (1.987 cal/deg mole) and T is absolute temperature.

Thermal degradation study is carried out at 40˚C to 80˚C. The most widely accepted temperature is 70˚C at low and high humidity for 1-2 months. High temperature (>80˚C) may not produce predictive degradation pathway.

- Photolytic condition

Exposure of drug molecules to light may produce photolytic degraded products. The rate of photo-degradation depends upon the intensity of incident light and quantity of light absorbed by the drug molecule. Photolytic degradation is carried out by exposing the drug substance or drug product to a combination of visible and UV light. The most commonly accepted wavelength of light is in the range of 300-800 nm to cause the photolytic degradation.

The overall illumination should not be less than 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200 W-h/m^2 as per ICH Q2B.

Identification of degradation products [17, 18]:

Detection of the impurity is the first step in impurity identification. The unknown impurity that are observed during the analysis, stress studies and formal stability studies of the drug substances and drug product by using various analytical techniques like Liquid Chromatography (LC), Thin Layer Chromatography (TLC), Gas Chromatography (GC), Capillary Electrophoresis (CE) , Capillary Electrophoresis Chromatography (CEC), Super critical Fluid Chromatography (SFC) etc. The RP-HPLC is most widely used analytical tool for separation and quantifying the impurities and it is most frequently coupled with UV detector or PDA detector. Structural information of drug related impurities (DRI) can be obtained from IR (Infrared) Spectroscopy, NMR (Nuclear Magnetic Resonance) and Mass spectra.

An excellent combination of hyphenated chromatographic and spectroscopic technique such as HPLC-DAD (High Performance Liquid Chromatography- Photodiode Array Detector), LC-MS (Liquid Chromatography-Mass Spectrometry), LC-NMR (Liquid Chromatography-Nuclear Magnetic Resonance) and GC-MS (Gas Chromatography-Mass Spectrometry) are used when DRI cannot be isolated in pure form. LC-MS/MS is particularly useful during the analysis of complex mixtures, such as degradation products, because it can separate and identify components with similar structures and close retention times.

1.4 Validation [23, 24]

Validation is a process of establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics.

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Here, various validation parameters are applied for the assessment of method. Results from method validation can be used to judge the quality, reliability and consistency of analytical results. The main advantage of method validation is that it establishes a high degree of confidence, not only for the developer but also to the user. Initially, the validation process may appear costly and time consuming but it results inexpensive, eliminates repetitions and leads to better time management in the end.

The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics which need to be evaluated. Typical validation characteristics which should be considered are listed below:

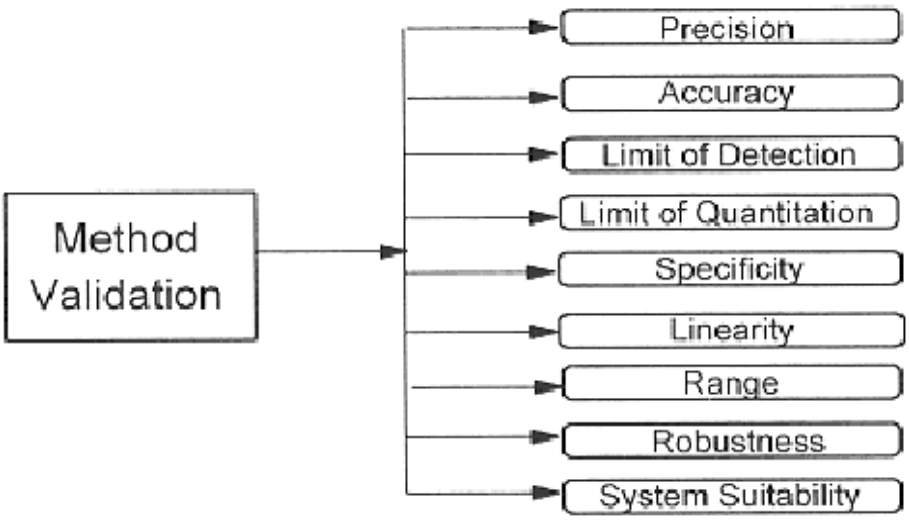


Figure 1.3 Validation parameters as per ICH Q (2) R1

- **Accuracy**

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted

reference value and the value found. This is sometimes termed trueness. Accuracy should be established across the specified range of the analytical procedure.

Following are the methods to determine Accuracy in various analytical tests.

- Drug Substance

1) Application of an analytical procedure to an analyte of known purity (e.g. reference material)
2) Comparison of the results of the proposed analytical procedure with those of a second well-characterized procedure, the accuracy of which is stated and/or defined.
3) Accuracy may be inferred once precision, linearity and specificity have been established.

- Drug Product

1) Application of the analytical procedure to synthetic mixtures of the drug product components to which known quantities of the drug substance to be analysed have been added
2) In cases where it is impossible to obtain samples of all drug product components, it may be acceptable either to add known quantities of the analyte to the drug product or to compare the results obtained from a second, well characterized procedure, the accuracy of which is stated and/or defined.
3) Accuracy may be inferred once precision, linearity and specificity have been established.

As per ICH recommendation accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g., 3 concentrations /3 replicates each of the total analytical procedure). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.

- **Precision**

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Precision may be considered at three levels:

1) Repeatability

2) Intermediate precision

3) Reproducibility

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

1) Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

Repeatability should be assessed using:

a) A minimum of 9 determinations covering the specified range for the procedure (e.g. 3 concentrations/3 replicates each); or

b) A minimum of 6 determinations at 100% of the test concentration.

2) Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

3) Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology). Reproducibility should be considered in case of the standardization of an analytical procedure, for instance, for inclusion of procedures in pharmacopoeias.

- **Linearity**

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be submitted. A plot of the data should be included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity. For the establishment of linearity, a minimum of five concentrations is recommended.

- **Range**

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

- **Specificity**

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present in the sample. Typically these might include impurities, degradants, matrix, etc.

- For chromatographic procedures, representative chromatograms should be used to demonstrate specificity and individual components should be appropriately labelled. Similar considerations should be given to other separation techniques.
- Critical separations in chromatography should be investigated at an appropriate level. For critical separations, specificity can be showed by the resolution of the two components which elute closest to each other.

- **Detection Limit**

The detection limit of an analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value.
Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental.

1) Based on Visual Evaluation:

Visual evaluation is generally used for non-instrumental methods but may also be used with instrumental methods. The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected. It is used for limit tests.

2) Based on Signal-to-Noise:

This approach can only be applied to analytical methods which exhibit baseline noise. The signal-to-noise ratio is determined by comparing measured signals from analyte samples with known low concentrations with those of blank samples and establishing the minimum

concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

3) Based on the Standard deviation of the Response and the Slope:

The detection limit (DL) may be expressed as:

$$DL = 3.3\sigma / S$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The estimate of σ may be carried out in following ways, for example:

A) Based on the Standard Deviation of the Blank Measurement: The magnitude of analytical background response is taken by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

B) Based on the Calibration Curve: The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines is used as the standard deviation.

- **Quantitation Limit**

The quantitation limit of an analytical method is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is used particularly for the determination of impurities and/or degradation products.

1) Based on Visual Evaluation:

It is used for non-instrumental methods. The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

2) Based on Signal-to-Noise Approach

This approach can only be applied to analytical procedures that exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

3) Based on the Standard Deviation of the Response and the Slope:

The Quantitation Limit (QL) may be expressed as:

$$QL = 10\sigma / S$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte or from the blank.

- **Robustness**

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure.

Determination of Robustness is performed by changing chromatographic conditions like Flow rate, column and/or auto sampler temperature, pH of mobile phase.

- **System Suitability**

System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factors, resolution and reproducibility (% RSD, retention time and area for six repetitions) are determined and compared against the specifications set for the method. "System suitability tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The test is based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such." List of the terms to be measured and their recommended limits of the system suitability samples are given in Table 1.1.

TABLE 1.1: System Suitability Parameters and Recommendations

Parameters	**Recommendation**
Capacity Factor (k')	The peak should be well-resolved from other peaks and

	the void volume, generally K' > 2
Repeatability	RSD ≤ 2% N ≥ 5 is desirable
Resolution (R_s)	R_s of > 1.5 between the peak of interest and the closest eluting potential interfere (impurity, excipients, degradation products, internal standard, etc.)
Tailing factor (T)	T ≤ 2
Theoretical plates (N)	In general it should be > 2000

1.5 Degradation kinetics [25-27]

Kinetic principles are always of great importance in stability program. The study of drug decomposition kinetics, the development of stable dosage forms and establishment of expiration dates for commercially available drug products are activities carried out mainly in laboratories of pharmaceutical industries. In spite of the importance of solid dosage forms there have been relatively few attempts to evaluate the detailed kinetics of their decomposition, where the rate processes is generally one that leads to the inactivation of a drug through either decomposition or loss of the drug by its conversion to a less favorable physical and chemical form.

Kinetics and Stability are not identical but they are different in following way:

- Chemical kinetics is studies through half lives. Stability studied down to up 85% of initial strength.
- Chemical kinetics is carried out in pure system while stability study system contains relatively many components.
- The goal of chemical kinetics is to elucidate reaction mechanism whereas that of stability studies is to establish expiration dating.

The two main purposes for conducting studies of solid decomposition in pharmaceuticals are:

- To elucidate the mechanism of reaction.
- To predict stability.

Before estimates of a drug product shelf life and expiration date can be made, stability studies on the product must be carried out. The use of kinetics and predictive studies for establishing credible expiration dates for pharmaceutical product is now accepted worldwide. However in past only qualitative and semi quantitative methods were used in

pharmaceutical studies. As these methods are deficient they have been replaced by vigorous, scientifically designed studies using reliable, meaningful and specific stability indicating assays, appropriate statistical concepts and a computer to analyze the data.

Degradation reactions in pharmaceutical formulation takes place at definite rate and are chemical in nature. They depend on such conditions as concentration of reactants, temperature, pH, radiation and catalysis. An effective and efficient study of this reaction requires the applications of chemical kinetic principles. So rate processes are fundamental concern which clearly demonstrates that the drug and dosage form is sufficiently stable that it can be stored for reasonable lengths of time without changing to inactive form or toxic form.

1.5.1 Effect of temperature on degradation of drug

In order for the rate constant or velocity of degradation to be of use in the formulation of pharmaceutical products, it is necessary to evaluate temperature dependency of the reaction. According to rule of thumb, the rate of reaction is generally said to be doubled for each 10ºC rise in temperature. Increase in temperature usually causes a very pronounced increase in hydrolysis rate of drug in solution. Some deterioration reactions are not measurably influenced over a 10ºC temperature range, while others undergo rapid degradation changes. The recommended procedures is to set up, a planned schedule of accelerated tests for each formulation in order to ascertain the temperature dependency of chemical changes in the product undergoing evaluation.

Arrhenious equation and accelerated stability testing:

$$k = AeEa/RT$$

$$\ln k = \ln A - Ea/RT$$

$$\log k = \log A - Ea/2.303RT$$

$$\ln k1/k2 = Ea\,(T1 - T2) / T1 \times T2 \times R$$

Where ***Ea*** = Activation energy (KJ/mole) is the energy barrier which has to be overcome if reaction is going to occur when two reactant molecules collide. **T1** and **T2** denote two different temperature conditions. A plot of ***log k*** as a function of ***1/T*** referred to as Arrhenious plot, is linear if ***Ea*** is independent of temperature. Thus it is possible to conduct kinetic experiments at elevated temperatures and obtain estimates of rate

constants at lower temperatures by extrapolation of the Arrhenious plot. This procedure commonly referred as accelerated stability and is most useful when the reaction at ambient temperature is too slow to be monitored conveniently and when ***Ea*** is very high. Hydrolysis reactions typically have an ***Ea*** of 10-30 KCal/mole where as oxidation and photolysis reactions have smaller energies of activation. An undergoing assumption of the Arrhenius equation is that the reaction mechanism does not change as a function of temperature (i.e. ***Ea*** is independent temperature) since accelerated stability testing of pharmaceutical products normally employs a narrow range of temperature (typically 35ºC to utmost 70ºC), it is often difficult to detect nonlinearity in the Arrhenius plot from experimental data even though such non linearity is expected from the reaction mechanism.

1.5.2 Order of reaction

Experimentally we can monitor the rate of breakdown of the drug either by its decrease in concentration with time or alternatively from the rate of appearance of one of the rate of breakdown products. Table 1.1 shows rate expressions for zero, first, second, third order of reaction, where *a* = initial concentration of reactant, x = concentration remaining after time t and k = Degradation rate constant.

TABLE 1.1 Rate expressions for order of reaction

Order of reaction	Integrated Rate Equation	Half-life Equation
0	$x = kt$	$t_{1/2} = a / 2k$
1	$\log [a/(a-x)] = kt / 2.303$	$t_{1/2} = 0.693 / k$
2	$x / [a(a-x)] = kt$	$t_{1/2} = 1 / ak$
3	$[2ax – x^2] / [a^2(a - x)^2] = 2kt$	$t_{1/2} = 3 / 2a^2k$

Determination of Order of Reaction:

The order of reaction may be determined by several methods.

- **Substitution Method:**

The data accumulated in a kinetic study may be substituted in the integrated form of the equation that describes the various orders. When the equation is found in which the calculated k values remain constant within the limits of experimental variation, the reaction is considered to be of that order.

- **Graphical Method:**

If a straight line results when concentration is plotted against *t*, the reaction is zero order. The reaction is first-order if *log (a - x)* versus *t* gives a straight line.

- **Half life ($t_{1/2}$) Method:**

Half life is the time to reach one half or 50% of the initial concentration of reactant.

1.6 Definition of the Problem

Azelastine hydrochloride and Ebastine are official in British Pharmacopoeia 2009 while Bilastine is a non-compendial substance. Literature review revealed that few stability indicating methods [28-30] are reported for determination of Azelastine HCl but it is not sufficient for complete stability protocol and kinetic data of Azelastine HCl. There is few stability indicating methods [31-35] available in literature for determination of Ebastine. Though it provides helpful information about Ebastine degradation, it is not sufficient for complete stability protocol of Ebastine and no kinetic study was reported. One hydrophilic interaction liquid chromatographic method [36] for determination of Bilastine in presence of its impurity and one patent [37] describing detection method for Bilastine and UV spectrophotometric method [38] for determination of Bilastine were reported.

Stability studies and degradation kinetics are integral parts of the quality control of a drug on an industrial scale. Degradation kinetics is the study of the rate at which degradation occur. It is useful to predict shelf life period of the medicine and it gives an insight into the mechanisms of changes involved. International Conference on Harmonization (ICH) guideline stipulates that the stability of active drug substances must be assessed. These facts initiate this research work.

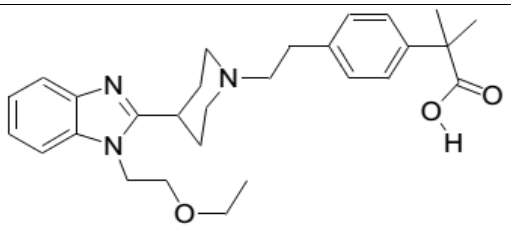
1.7 Original Contribution by the Thesis:

- A simple, precise, accurate and stability indicating reverse phase HPLC methods for determination of selected H_1-antihiataminics drugs (Bilastine, Ebastine and Azelastine) in routine analysis (different method for individual drug) are developed and validated as per ICH guideline. Forced degradation study of Bilastine, Ebastine and Azelastine hydrochloride was performed.

- The order of degradation of Bilastine in acidic medium was found and parameters related to kinetic study like order of reaction, rate constant, half time at room temperature in specific condition were calculated.
- The order of degradation of Ebastine in acidic and oxidative medium was found and half-time of the drug at room temperature was calculated and activation energy of the degradation was calculated.
- The degradation products of Ebastine and Bilastine were identified using LC-MS studies.

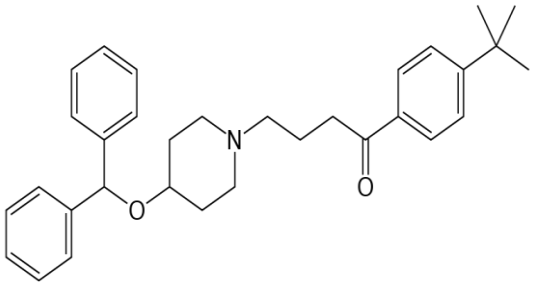
1.8 Drug Profile

1.8.1 Bilastine[39-41]

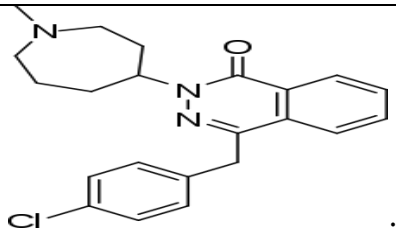
Chemical Structure	
IUPAC Name	2-[4-(2-(4-(1-(2- ethoxyethyl)- 1H benzimidazole-2-yl) piperidine-1-yl) ethyl) phenyl] -2-methyl propionic acid
Molecular formula	$C_{28}H_{37}N_3O_3$
Molecular weight	463.61 gm/mol
Description	A white to off white crystalline powder
Melting point	200.0-200.3 °C
pKa value	4.18
Solubility	Slightly soluble in methanol, ethanol. Very slightly soluble in water and acetonitrile. Freely soluble in chloroform and 1 N HCl.
Absorption	Oral bioavailability- 61% t_{max} - 1 hr. C_{max} – 220 ng/mL
Distribution	Plasma protein binding - 84-90% Volume of distribution - 59.2L for the central compartment and 30.2L for the peripheral compartment Plasma half life – 14.5 hr Apparent plasma clearance – 18.1 L/hr
Metabolism	Hepatic metabolism – not significant Bilastine is not a substrate of CYP450 family.
Excretion	95% is excreted intact in faeces (66.5%) or in urine (28.3%)

Mechanism of action	It is highly selective and specific for H_1-receptors, and has poor or no affinity for other receptors such as serotonin, bradykinin, leukotriene D_4, calcium, muscarinic M_3-receptors, α_1-adrenoceptors, β_2- adrenoceptors, and H_2- and H_3-receptors.
Indications	Allergic rhinitis and urticaria

1.8.2 Ebastine[42-45]

Chemical Structure	
IUPAC Name	1-(4-tert-butylphenyl)-4-[4-(diphenyl methoxy)piperidin-1-yl]butan-1-one
Molecular formula	$C_{32}H_{39}NO_2$
Molecular weight	469.658 g/mole
Description	White to off white crystalline powder
Melting point	80-82° C
pKa value	16.45
Solubility	Insoluble in water, soluble in DMSO and methanol and very soluble in methylene chloride.
Absorption	t_{max} of carebastine - 3 to 6 hours C_{max} of carebastine - 0.1 mg/L after a single 10mg dose. The bioavailability of carebastine is increased when ebastine is administered with food.
Distribution	Plasma protein binding of carebastine - 98% Volume of distribution - 90 to140L.
Metabolism	Ebastine is a prodrug which is extensively metabolised by first-pass metabolism to its active carboxylic acid metabolite carebastine by
Excretion	Elimination half-life is between 13-16 hours, carebastine being eliminated predominantly in the urine.
Mechanism of action	It blocks histamine H_1- receptor activity, and inhibits the release of anti-IgE-induced prostaglandin D2 (PGD2), leukotriene C4/D4 and cytokines which act as inflammatory mediators.
Indications	Allergic rhinitis and chronic idiopathic urticaria

1.8.3 Azelastine Hydrochloride[46-48]

Chemical Structure	 .HCl
IUPAC Name	4-[(4-chlorophenyl)methyl]-2-(1-methylazepan-4-yl)-1,2 dihydrophthalazin-1-one hydrochloride
Molecular formula	$C_{22}H_{23}Cl_2N_3O$
Molecular weight	418.37g/mole
Description	white, almost odorless, crystalline powder with bitter taste
Melting point	224-228 °C
pKa value	9.5
Solubility	Soluble in water (25 mg/mL), DMSO (>10 mg/mL), and ethanol.
Absorption	The systemic bioavailability is approximately 40% when administered intranasal. t_{max} - 2–3 hours.
Distribution	Plasma protein binding of azelastine – 88% and of N-desmethylazelastine - 97%. Volume of distribution – 14.5 L/kg
Metabolism	It is oxidatively metabolized by the cytochrome P450 family into its active metabolite, desmethylazelastine, and two inactive carboxylic acid metabolites.
Excretion	Approximately 75% of an oral dose of radio labeled azelastine hydrochloride was excreted in the feces with less than 10% as unchanged azelastine. Elimination half-life (intranasal administration) is 22 hours. Elimination half-life of the active metabolite, desmethylazelastine, is 54 hours (after oral administration of azelastine).
Mechanism of action	Azelastine is selective histamine H_1 antagonist. It is mast cell stabilizer. It inhibits other mediators involved in allergic reactions (e.g. leukotrienes and Platelet activation factor) It is shown to block secretion of IL-6, IL-8, and TNF alpha from mast cells by inhibiting NF-κB activation.
Indications	For symptomatic treatment of seasonal allergic rhinitis and non-allergic rhinitis, as well as for symptomatic relief of ocular itching associated with allergic conjunctivitis.

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CHAPTER 2

Literature Review

2.1 Bilastine

Sr. No.	Method	Description	Ref. No.
1.	Determination of Bilastine and its impurities by HILIC Method	• Sample matrix - tablet • Stationary phase – Luna HILIC (100 mm x 4.6 mm, 5 µm particle size) • Mobile phase – acetonitrile: 50 mM ammonium acetate pH adjusted to 5.3 with glacial acetic acid) (90.5:9.5 v/v) • Column temperature – 30°C • Flow rate – 1.0 mL/min. • Detection - 275 nm	1
2.	Bilastine detection method	• Sample matrix – powder • Stationary phase – C_{18} or C_8 • Mobile phase –inorganic salt buffer solution containing an ion pair reagent and an organic solvent. • Column temperature – 35-45°C • Flow rate – 0.8 -1.2 mL/min. • Detection - 254 nm	2
3	determination of Bilastine by UV Spectrophotometric method	• Solvent- 0.1 M HCl • Detection - 210 nm	3

2.2 Ebastine:

- Degradation study

Sr. No.	Method	Description	Ref. No.
1	stability-indicating LC method was validated for determination of Ebastine in tablet and syrup	• Sample matrix – tablet and syrup • Stationary phase – C_{18} column • Mobile phase – acetonitrile: phosphoric acid 0.1% pH 3.0 (55: 45 v/v) • Flow rate – 1.2 mL/min. • Detection - 254 nm	4
2	Stability indicating LC method after pre column derivatization of Ebastine was validated for determination of Ebastine in pharmaceutical preparation.	• Sample matrix –tablet and syrup • Stationary phase - C_{18} (150 mm ×4.6 mm i.d.), 5 µm particle size • Mobile phase - 0.025%w/v Zn^{2+} in a mixture of acetonitrile: methanol (1: 4) ;	5

		and Britton Robinson buffer (65: 35, v/v) adjusted to pH 4.2 • UV-detection - 260 nm. • Flow rate - 1 mL/min.	
3	Stability indicating UPLC method was developed using an innovative Quality-by-Design approach for estimation of Ebastine in the API and pharmaceutical formulations	• Sample matrix - API • Stationary phase - Waters Acquity UPLC BEH C_{18} (50 mm x 2.1 mm i.d.), 1.7 µm particle size • Mobile phase - gradient elution of 10 mM acetate buffer pH 6.2 and a mixture of acetonitrile: 2-propanol (1:1) as the mobile phase.	6
4	A novel UV degradation product of Ebastine was isolated and characterized using Q-TOF, NMR, IR and computational chemistry	<u>For analytical purpose</u> • Stationary phase -Sunfire C_{18}, (250 mm × 4.6 mm, 5 µm particles • Mobile phase -buffer: ACN (18:82, v/v), buffer- 5.0 mL of diethylamine into 1000 mL of milli-Q-water and adjusted to pH 6.0 using formic acid • UV-detection - 210 nm. • Flow rate – 0.8 mL/min. For Isolation of UVDP • Stationary phase - Cosmosil 5 C_{18}-MS-II, 250 mm × 20.0 mm packed column with 5 m particles. • Mobile phase –gradient elution of part A and part B. Part A- 5.0 mL of diethylamine into 1000 mL of milli-Q-water, adjusted to pH 6.0 using formic acid while B was 100% ACN • UV-detection - 210 nm. • Flow rate – 18.0 mL/min.	7
5	Stability-indicating spectrofluorimetric methods were developed for the estimation of Ebastine in pharmaceutical preparations	• Method I - Condensation of Ebastine with mixed anhydrides (citric and acetic anhydrides) producing a product with intense fluorescence, which was measured at 496 nm after excitation at 388 nm. • Method II - Decrease in the fluorescence intensity of eosin was directly proportional to the concentration of Ebastine; the fluorescence was measured at 553 nm after excitation at 457 nm.	8

➢ Chromatographic methods

Sr. No.	Method	Description	Ref. No.
1	Simultaneous Estimation of	• Sample matrix - tablet	9

	Montelukast and Ebastine by HPLC	• Stationary phase – Lichrocart C_{18} column (4.6 mm, i.d.×250 mm, 5µm particle diameter) • Mobile phase – methanol: water (80: 20 v/v) at pH 3 adjusted by ortho phosphoric acid • Flow rate – 1.0 mL/min. • Detection - 268 nm	
2	Reverse phase HPLC Method was developed and validated for Simultaneous Estimation of Ebastine and Montelukast Sodium in Tablet dosage form	• Sample matrix - tablet • Stationary phase – Phenomenex C18, column (250 mm × 4.6 mm id, 5 µm particle size) • Mobile phase – Methanol: Phosphate buffer (65:35 v/v) pH 5.0±0.05 • Flow rate – 1.0 mL/min. • Detection - 261 nm	10
3	RP-HPLC Method for Simultaneous Estimation of Phenylephrine hydrochloride and Ebastine in Tablet dosage form	• Sample matrix - tablet • Stationary phase – Thermo BDS Hypersil C_{18} column (250 mm × 4.6 mm i.d., 5 µm particle size) • Mobile phase – Methanol: Phosphate buffer (30: 70v/v), pH 4.0±0.05 • Flow rate – 1.0 mL/min. • Detection - 215 nm	11
4	Determination of Ebastine in pharmaceutical formulations (Tablet) by HPLC	• Sample matrix - tablet • Stationary phase – Phenomenex RP-C_{18} (250 mm × 4.6 mm i.d., Particle size 5 µm) • Mobile phase – Methanol: Water (90: 10 v/v) • Flow rate – 1.5 mL/min. • Detection – 262 nm	12
5	Assay of Ebastine in Ebastine Mouth Dissolving tablets by HPLC method	• Sample matrix –tablet • Stationary phase – C_{18} (250 mm × 4.6 mm i.d., Particle size 5 µm) • Mobile phase – phosphoric acid buffer adjusted to pH 6.0: acetonitrile (40:60 v/v) • Column temperature – 40°C • Flow rate –1.0 mL/min. • Detection – 255 nm	13
6	Quantification of Ebastine in tablet dosage form by HPLC and HPTLC methods.	• Sample matrix –tablet <u>HPLC method:</u> • Stationary phase – Hypersil C_{18} (250 mm × 4.6 mm i.d., Particle size 5 µm) • Mobile phase – Acetonitrile: methanol: ammonium acetate buffer pH 5.0 (65:25:10 v/v/v) • Flow rate –1.5 mL/min. • Detection – 254 nm	14

		HPTLC method: • Stationary phase – Silica-gel $60F_{254}$ HPTLC plates • Mobile phase – Toluene: ethylacetate (1:24 v/v) • Detection – 254 nm	
7	Simultaneous Estimation of Montelukast Sodium and Ebastine in Tablet Dosage form by RP-HPLC	• Stationary phase – Phenomenex C_{18} (150 mm × 4.6 mm i.d., Particle size 5 µm) • Mobile phase – Methanol: acetonitrile: ammonium acetate (80:10:10, % v/v/v), pH adjusted 5.5 using glacial acetic acid • Column temperature- 26 ±2 °C • Flow rate –1.2 mL/min. • Detection – 244 nm	15
8	Analysis of Ebastine in pharmaceutical preparations by HPTLC	• Sample matrix - tablet • Stationary phase - Aluminium backed silica gel $60F_{254}$ TLC plates • Mobile phase - Methanol: n-hexane (10:1 v/v) • Detection wavelength - 265 nm	16
9	Simultaneous estimation of Ebastine and its three metabolites in plasma using LC-MS/MS method	• Sample matrix –plasma • Stationary phase – C_{18} • Mobile phase – acetonitrile: 5 mM ammonium acetate, 50:50, v/v • Flow rate – 0.2 mL/min. • Detection – LC-MS/MS	17
10	Simultaneous determination of the Ebastine and its two metabolites, carebastine and hydroxyebastine, in human plasma using HPLC	• Sample matrix – human plasma • Stationary phase - Cyano column (250×4.0 mm i.d.) • Mobile phase – Acetonitrile: methanol: 0.012 M ammonium acetate buffer (20:30:48, v/v/v) • Column temperature – 40 °C • Flow rate – 1.2 mL/min. • Detection wavelength - 254 nm	18
11	Simultaneous Determination of Ebastine and its active metabolite, carebastine in human plasma using liquid chromatography- tendem mass spectrometry	• Sample matrix – human plasma • Stationary phase - C_{18} • Mobile phase – 10 mM ammonium formate in water/acetonitrile (40:60, v/v) • Detection – triple quadropole mass spectrometer	19

2.3 Azelastine Hydrochloride:

➢ Degradation study

Sr. No.	Method	Description	Ref. No.
1	Thermoanalytical study and	• The experiments were performed from	20

	purity determination of Azelastine Hydrochloride and Emedastine Difumarate	ambient temperature up to 1000°C with a heating rate of 8°C/min for azelastine HCl.	
2	Simultaneous estimation of Fluticasone propionate, Azelastine Hydrochloride, Phenylethyl alcohol and Benzalkonium chloride by reverse phase high performance liquid chromatography method in nasal spray preparations	• Sample matrix- nasal spray • Stationary phase - waters sphrerisorb CN column (250 x 4.6 mm, 5 µm) • Mobile phase - 55:45 (v/v) mixture of buffer (50 mM of potassium dihydrogen ortho phosphate) and acetonitrile • Flow rate - 1.0 mL/min. • Detection – 215 nm	21
3	Stability indicating densitometric TLC method for quantitative determination of Azelastine Hydrochloride and Emedastine Difumarate in their drug products	• Sample matrix- Nasal Spray and eye drop • Stationary phase- Aluminum plates precoated with silica gel F_{254} (20 x 20 cm) • Mobile phase- Methanol :10% ammonia (9.5:0.5 % v/v) • Detection- Azelastine HCl at 291 nm	22
4	Stability study of the antihistamine drug Azelastine hydrochloride along with a kinetic investigation and identification of the new degradation products	• Sample matrix-Nasal spray and Eye drop • Stationary phase – Cosmosil 5 C_{18}-MS (150 x 4.6 mm, 5 µm) • Mobile phase – Acetonitrile: 0.04 M phosphate buffer pH 3.5 (32:68 v/v) • Flow rate - 1.0 mL/min. • Detection – 210 nm	23

➢ Chromatographic methods

Sr. No.	Method	Description	Ref. No.
1	Simultaneous estimation of Mometasone Furoate and Azelastine Hydrochloride by reverse phase high performance liquid chromatography	• Stationary phase – Zorbax SB CN Column (150 X 4.6 Mm, 5 µm) • Mobile phase – Potassium dihydrogen phosphate buffer: Acetonitrile (55:45,V/V) • Column temperature - 30°C • Flow rate - 1.0 mL/min. • Detection – 239 nm	24
2	RP-HPLC determination of Azelastine in pure and in ophthalmic formulation	• Sample matrix – Eye drop • Stationary phase- Kromosil C_{18}, 150 mm X 4.8 mm i.d., 5 µm column • Mobile phase - Phosphate buffer (pH 3): Acetonitrile (50:50 v/v) • Flow rate – 1.0 mL/min. at ambient temperature.	25
3	Identification and estimation of Ketotifen hydrogen fumarate, Azelastine	• Sample matrix- Nasal spray • Stationary phase- Merck HPTLC Silica Gel 60 F_{254} Chromatographic	26

	hydrochloride, Dimetindene maleate and Promethazine hydrochloride by densitometric method	plates • Mobile phase - Diethylether: Diethylamine (40:1, V/V) • Detection- Azelastine HCl at 295 nm	
4	Quantitative analysis of Azelastine in human plasma by liquid chromatography - tandem mass spectrometry (LC-ESI/MS/ MS)	• Sample matrix – human plasma • Stationary phase- YMC Pack Pro C_8, (2.0 mm i.d. × 50 mm, 3 µm particle size • Mobile phase - Acetonitrile: 5 mM ammonium acetate solution (70:30 v/v, at pH 6.4) • Flow rate – 0.25 mL/min. at ambient temperature. • Detection – MS/MS	27
5	Analysis of Azelastine and Desmethylazelastine in human plasma by high performance liquid chromatography method	• Sample matrix – human plasma • Stationary phase- Altex Ultrashere C_{18}, (4.6 mm i.d. × 250 mm, 5 µm particle size • Mobile phase – Tetrahydrofuran: Water (30:70 v/v) with 0.3% triethylamine (relative pH 2.3-2.4 with orthophosphoric acid) • Flow rate – 0.25 mL/min. at ambient temperature. • Detection – fluorescence detection, excitation wavelength – 213 nm, emission wavelength – 360 nm	28
6	Pharmacokinetic and bioequivalence studies of Azelastine in Korean healthy volunteers using validated liquid chromatography coupled to tandem mass spectrometry method	• Sample matrix – human plasma • Stationary phase- Luna C18 Phenyl-Hexyl column (2.1×50 mm, 5µm particle size) • Mobile phase – acetonitrile and 5 mM ammonium acetate (8 : 2 v/v, pH 6.4) • Flow rate – 0.25 mL/min. • Detection – MS/MS	29
7	Simultaneous enantio-selective separation of Azelastine and three metabolites of azelastine was performed for the investigation of the enantiomeric metabolism in rats by LC-SI-MS/MS method	• Sample matrix – Rat plasma • Stationary phase- Cyclobond I 2000 (250 mm ×4.6 mm, 5µm particle size). • Mobile phase – 45 mM ammonium acetate, pH 4.7: methanol: acetonitrile (70:21:9, v/v/v) • Flow rate – 0.4 mL/min. • Detection – MS/MS	30
8	Analysis of Azelastine and desmethylazelastine (major metabolite of Azelastine) in human plasma using HPLC–tandem mass spectrometry method.	• Sample matrix – Human plasma • Stationary phase- Phenomonex Synergi Polar-RP column (50 mm × 2.0 mm, 4 µm particle size). • Mobile phase – 10 mM ammonium formate with 0.1% formic acid: acetonitrile (55:45 %v/v)	31

		• Flow rate – 0.2 mL/min. • Detection – MS/MS	
9	Azelastine and Desmethylazelastine were determined by HPLC method in guinea pig plasma and lung tissue	• Sample matrix – Guinea Pig Plasma and Lung Tissue • Stationary phase- Hypersil CPS, (250 mm × 2.0 mm, 5 µm particle size). • Mobile phase – Plasma: 0.009 M triethylammonium phosphate (pH 3.00): acetonitrile (50:50 v/v), flow rate: 0.45 mL/min at 40 °C; Lung: water: acetonitrile: triethylamine: phosphoric acid (500:500:0.4:0.2 v/v/v/v), flow rate: 0.60 mL/min at 60 °C • Detection – fluorescence detector Excitation wavelength- 215 nm Emission wavelength- 360 nm	32

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CHAPTER-3

Aim, Objectives and Rationale of Work

3.1 Aim

- To develop and validate simple, reproducible and reliable HPLC method to study decomposition of selective H_1-antihistaminic drugs under stress condition.
- To study the degradation kinetics of the selected drugs.
- To elucidate structure of degradation product using hyphenated technique LC-MS.

3.2 Objectives

- To develop simple, economic, reproducible and reliable HPLC method to determine Bilastine, Ebastine and Azelastine in pure form and formulation.
- To study forced degradation of selected drugs.
- To optimize the developed methods.
- All developed methods to be validated for specificity, linearity, accuracy, repeatability, precision, Limit of Detection, Limit of Quantification, robustness and system suitability as per ICH guideline.
- To study the degradation rate and order of degradation of the selected drugs using the developed methods.
- To apply developed methods for studying accelerated stability testing of anti-histaminic drugs in its formulation.
- To identify the degradation product produced after forced degradation using LC-MS.

3.3 Rationale

- Stability studies and degradation kinetics are fundamental parts of the quality control of a drug substance or its product. Degradation kinetics is used to determine the stability under specified conditions as well as to compare stress condition.
- Azelastine hydrochloride and Ebastine are official in BP 2009 while Bilastine is a non-compendial substance.
- Bilastine was approved by CDSCO for clinical studies in 2016. Tablet of bilastine is available in European countries, Japan, Malasia, Indonesia.
- Literature review revealed that only hydrophilic interaction liquid chromatographic method for estimation of Bilastine in presence of its impurity and UV spectrophotometric method for quantitative analysis of Bilastine were reported. There are some published papers describing the determination of Bilastine in pharmacokinetic studies by liquid chromatography-fluorescence detection and Liquid Chromatography coupled with tandem mass spectrometry. Any research article was not found to deal with degradation study of Bilastine.
- Ebastine difumarate was approved by CDSCO in 2001. Few bio-analytical methods like LC-MS/MS and HPLC have been reported for quantification of Ebastine in human plasma. There are some published papers discovered by literature survey that dealing with several spectroscopic and few chromatographic methods for quantitative analysis of Ebastine alone and in combination with other drug substances. There is few stability indicating methods available in literature for determination of Ebastine. Though it provides useful information about Ebastine decomposition, it is not adequate for complete stability protocol of Ebastine. No kinetic study was reported.
- Azelastine hydrochloride nasal spray was approved by CDSCO in July 1997. Various methods like LC, LC-ESI/MS/MS, HPLC, capillary HPLC, electro kinetic capillary HPLC had been reported in the literature for determination of Azelastine and its metabolite in bio-fluids. Literature review revealed that stability indicating methods in small numbers are reported for determination of Azelastine HCl but it is not enough for complete stability protocol and degradation kinetic data of Azelastine HCl.

- Structure elucidation of substance is required as understanding of chemical reactions of drug compounds under several stress conditions can furnish adequate guidance regarding the selection of excipients for formulation development and also encourage advancement in the manufacturing process.
- Stability studies and degradation kinetics are essential parts of the quality control of active ingredients in pharmaceutical industry. The study of the rate at which degradation occur is known as degradation kinetics. It is useful to predict shelf life period of the medicine and it gives information about the mechanisms of changes involved. International Conference on Harmonization (ICH) guideline stipulates that the stability of API must be assessed. These facts initiate this research work.

CHAPTER 4

HPLC Method For Bilastine

4.1 Materials and Methods

4.1.1 Reagents and Materials

TABLE 4.1 List of reagents and materials used in HPLC method for Bilastine

Sr. No.	Reagent/material	Grade
1	Water	HPLC
2	Hydrochloric acid	Analytical
3	Sodium hydroxide	Analytical
4	Hydrogen peroxide	Analytical
5	Methanol	HPLC
6	Acetonitrile	HPLC
7	Triethylamine	Analytical
8	Sodium dihydrogen phosphate	Analytical
9	Disodium hydrogen phosphate	Analytical
10	Ortho phosphoric acid (OPA)	Analytical
11	Bilastine procured from Symed Labs Ltd.	Reference standard

4.1.2 Instruments and Apparatus

TABLE 4.2 List of instrument and apparatus used in HPLC method for Bilastine

Sr. No.	Instrument/Apparatus	Manufacturer
1	Analytical weighing balance	Sartorius
2	FTIR instrument	Bruker
3	Glass wares	Borosil
4	pH meter	Elico
5	HPLC	Waters HPLC with EmPower software

4.1.3 Identification of API

- **Melting point determination**

Melting point was determined by open capillary method.

- **Infra Red Spectra:**

Bilastine- KBr pellet was prepared and an IR spectrum was produced by FT-IR instrument. IR spectrum of sample was compared to reference IR spectrum[1].

4.1.4 Determination of Physicochemical Properties

- **Solubility test:**

Solubility of Bilastine in distilled water, methanol and acetonitrile was checked by taking 10 mg Bilastine drug in 100 mL flask and adding the solvent till the drug dissolved.

- **Wavelength maxima:**

From PDA data, a wavelength maximum of drug was determined.

4.1.5 Preparation of solutions

- **Preparation of standard stock solution**

Bilastine standard stock solution was made by taking accurately 100 mg of Bilastine in 100mL volumetric flask, adding 50 mL methanol and was sonicated for 10 min. and making up 100 mL with methanol. Standard stock solution (1000 µg/mL) was diluted as relevant with methanol to obtain the working concentration range. Stock solution was stable for minimum 3 days when kept at ambient temperature.

- **Test solution preparation**

Bilastine (200 mg) and placebo having 10 mg of each excipient (colloidal anhydrous silica, magnesium stearate, microcrystalline cellulose, and sodium starch glycolate) were transferred in 200 mL volumetric flask, 100 mL methanol was added and sonicated for 40 min. with occasional shaking. Dilution up to the volume was done with methanol. It was filtered through 0.45µ (PVDF Millipore Filter) and diluted further to get test solution containing Bilastine (100 µg/mL).

4.1.6 Selection of chromatographic condition

Stationary phase C_{18} and C_8 column were tried (Table 4.3) with different mobile phase on the basis of physico-chemical properties of the Bilastine to develop stability indicating RP-LC method for quantitative analysis of Bilastine, and system suitability test was performed for method optimization.

TABLE 4.3 selection of chromatographic condition

Sr. No.	Stationary Phase	Mobile Phase	Problem
1	Denali C_{18} (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
2	Kromo C_{18} (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
3	Kromo C_{18} (250 x 4.6 mm, 5µm)	Methanol: 0.1% OPA 70:30 v/v, 1.0 mL/min.	Tailing factor =1.9 Theoretical plates=119 Splitting of peak
4	Azilen C_{18} (150 x 4.6 mm, 5µm)	Methanol: 0.1% OPA 50:50 v/v, 1.0 mL/min.	Tailing factor =1.4 Theoretical plates=407
5	Azilen C_{18} (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
6	Zodi C_{18} (150 x 4.6 mm, 5µm)	Acetonitrile: 0.1% OPA 50:50 v/v, 0.8 mL/min.	Tailing factor = 0.9 Fronting of peak
7	Zodi C_{18} (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
8	Discovery C_{18} (250 x 4.6 mm, 5µm)	Methanol: 0.1% OPA 65:35 v/v, 0.8 mL/min.	Tailing factor=1.4
9	Discovery C_{18} (250 x 4.6 mm, 5µm)	Methanol: 0.1% OPA 55:45 v/v, 1.0 mL/min.	Splitting of tip of peak
10	Discovery C_8 (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.

4.1.7 Validation of the method

The developed method was validated for system suitability, specificity, linearity, precision, trueness, LOD, LOQ and robustness as per ICH guideline to ensure its appropriateness for the predetermined purpose.

- **System suitability test**

To ascertain suitability of selected HPLC testing system for the expected application, the system suitability test was checked. Six replicate analyses of 100 µg/mL of Bilastine solution were chromatographed as per optimized method and theoretical plates and tailing factor were evaluated.

- **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 into six 10 mL volumetric flasks. They were diluted up to volume with methanol to obtain final concentrations of 25-150 µg/mL and mixed properly. 20 µL aliquots of each solution were chromatographed three times and analysis was performed by optimized method. The regression equation was derived from the plot of average area of Bilastine peak against the concentration of Bilastine.

- **Precision**

- **Repeatability**

To assess repeatability of the method, chromatographic analysis of six injections of 100 µg/mL Bilastine solutions was performed. The average, standard deviation and % RSD for retention time and area of Bilastine peak were estimated.

- **Intraday precision:**

Three concentrations (50, 100 and 150 µg/mL) were chromatographed for three times on same day by same analyst and area of Bilastine peak was reported to check the intraday precision. The %RSD of Bilastine peak area was calculated.

- **Interday precision:**

Three concentrations (50, 100, and 150 µg/mL) were chromatographed for three different days by same analyst and area of Bilastine peak was reported to check the interday precision. The %RSD of peak area was calculated.

- **Accuracy**

Bilastine (200 mg) and placebo having 10 mg of each excipient (colloidal anhydrous silica, magnesium stearate, microcrystalline cellulose, and sodium starch glycolate) were transferred in 200 mL volumetric flask and then to the flask about 100 mL of methanol was added and it was sonicated for 40 min. with occasional shaking. Dilution up to the volume was done with methanol. The solution was filtered through 0.45µ (PVDF Millipore Filter) and diluted further to get test solution containing Bilastine (100µg/mL). In the prepared test solution mixture, Bilastine standard solution was added at 50%, 100% and 150% concentration level. 20 µl of each solution were injected three times and were chromatographed. Actual amount of Bilastine was estimated using regression equation and percentage recovery of Bilastine was calculated.

- **Specificity**

The forced degradation studies were accomplished to determine whether the developed HPLC method was stability indicating and could uniquely determine Bilastine though impurities and degradation products are present. Bilastine standard solution was stressed in acid, base, neutral hydrolytic, oxidative, thermal and photolytic conditions to achieve partial decomposition of the Bilastine.

- Acid induced degradation

2 mL standard solution of Bilastine (1 mg/mL) was transferred in 20 mL volumetric flask and 2 mL 2.0 M HCl was added to it. For 30 min. the mixture was refluxed in a thermostatic water bath at 60°C. Then it was cooled and dilution up to the volume was done with methanol. Then the resulted solution was chromatographed as per optimized conditions.

- Base induced degradation

2 mL standard solution of Bilastine (1 mg/mL) was transferred in 20 mL volumetric flask followed by 2 mL 2.0 M NaOH. For 2 hr the mixture was refluxed in a thermostatic water bath at 80°C. Then it was cooled and dilution up to the volume was done with methanol. Then chromatography of the resulted solution was performed as per optimized conditions.

- Wet heat degradation

2 mL standard solution of Bilastine (1000µg/mL) was transferred to 20 mL volumetric flask followed by 2 mL distilled water. For 2 hr the mixture was refluxed in a thermostatic water bath at 80°C. Dilution of mixture up to the volume was done with methanol. Then chromatography of the resulted solution was performed as per optimized conditions.

- Oxidative degradation

2 mL standard solution of Bilastine (1000µg/mL) was transferred to 20 mL volumetric flask followed by 2 mL 10% H_2O_2. The mixture was refluxed in a thermostatic water bath at 80°C for 30 min. Then it was cooled and dilution up to the volume was done with methanol. Then the resulted solution was chromatographed as per optimized conditions.

- Thermal degradation

50 mg Bilastine powder in petridish was kept at 70°C in convection oven and exposed to heat for 8 hr and further proceeded as per sample preparation. Then the resulted solution was chromatographed as per optimized conditions.

- Photolytic degradation

Bilastine drug (50 mg) was transferred in petridish. It was kept in UV chamber (365 nm) for 24 hr at room temperature and further preceded as per sample preparation. Then the resulted solution was chromatographed as per optimized conditions.

➢ **Robustness**

The experimental conditions of the method were deliberately changed to determine the robustness. The mobile phase flow rate (1.0±0.1 mL/min.), temperature of column oven (35±1°C) and methanol composition (55±2 %) were varied. In each case, and %RSD of area of Bilastine peak and retention time was calculated. Comparison of the retention time and area of Bilastine peak were done with that obtained under the optimized method, also.

➢ **Limit of Detection and Limit of Quantitation**

Standard deviation of y intercept of calibration curve of linear lower concentrations (N) and slope (S) of the calibration curve were calculated and using their values LOD and LOQ were estimated as per ICH guideline.

4.2 Results and Discussion

4.2.1 Identification of API

Melting point of the Bilastine was observed in the range of 199-200°C which is almost similar to the reported value (200.3 °C) for Bilastine.[1]

An IR spectrum of Bilastine API is presented in Fig. 4.1 and a reference spectrum is presented in Fig. 4.2.

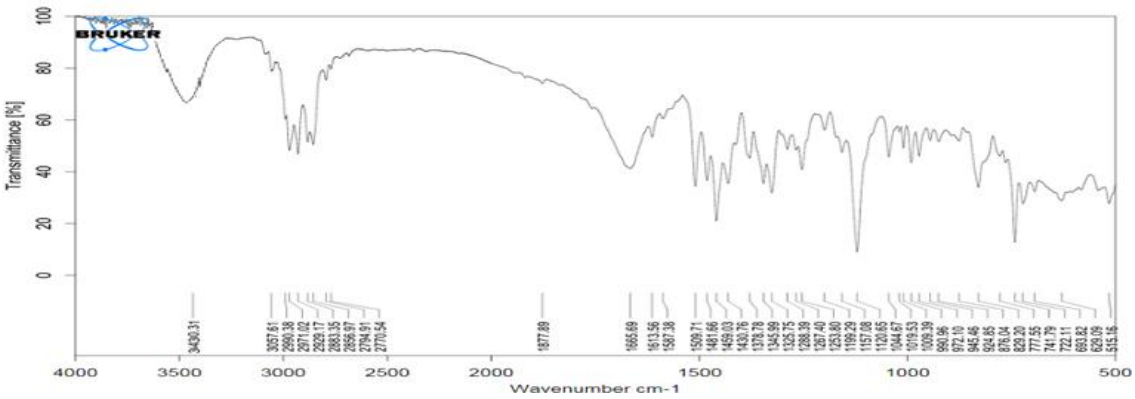

FIGURE 4.1 IR Spectrum of Bilastine API

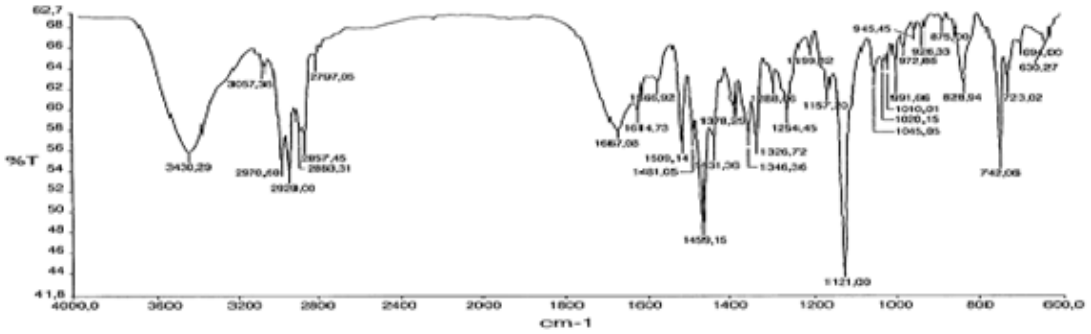

FIGURE 4.2 IR Spectrum of Bilastine Reference

As sample spectrum completely matches with the reference spectrum and practical value of melting point also matches with theoretical value, it confirms the purity of Bilastine sample.

4.2.2 Determination of physicochemical properties

Table 4.4 presents the results of solubility test.

TABLE 4.4 Solubility results for Bilastine

Solvent	mL of solvent required to dissolve 10 mg Bilastine	Solubility
Methanol	5 mL (500 mL for 1 gm)	Slightly soluble
Water	70 mL (5000 mL for 1 gm)	Very slightly soluble
Acetonitrile	80 mL (8000 mL for 1 gm)	Very slightly soluble

From PDA data, 276 nm was found λ_{max} of Bilastine and it was selected as wavelength for study.

4.2.3 Development of method

HPLC method using discovery C_8 (250 x 4.6 mm, 5µm) column and solvent system comprising of methanol: 0.1% OPA (55:45 v/v) pumped at flow rate of 1.0 mL/min. was observed to provide sharp and well defined peak having good tailing factor(1.00), theoretical plates(>6000) and low retention time (3.833 min.). Hence it was selected throughout study of Bilastine by HPLC.

- **Optimized chromatographic condition:**

Column: Discovery C_8 (250 mm x 4.6 mm i.d., 5µm particle size)

Mobile phase: 55 volumes of Methanol: 45 volumes of 0.1% OPA

Flow rate: 1.0 mL/min.

Volume of injection: 10 µL

Temperature of column: 35˚C

Run Time: 7 min.

Diluent: Methanol

Detector: PDA

Retention Time of Bilastine: 3.833 min.

Fig. 4.3 presents RP-HPLC Chromatogram of Bilastine produced by optimized method.

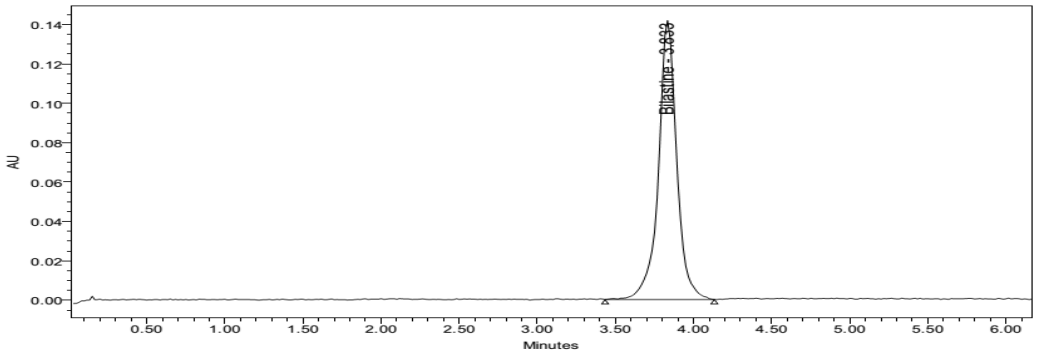

FIGURE 4.3 RP-HPLC Chromatogram of Bilastine

4.2.4 Method validation

To demonstrate appropriateness of method for the predetermined purpose, validation of the developed method was done as per ICH guideline.

- **System suitability test**

Table 4.5 presents the results of system suitability. As results of system suitability test are satisfactory it was ensured that HPLC testing system was adequate for routine analysis of Bilastine.

TABLE 4.5 Results of system suitability test for Bilastine

Sr. No.	Conc.	Theoretical plates	Tailing factor
1	100 µg/mL	6876	0.99
2		6031	1.03
3		5878	1.02
4		5848	1.02
5		6714	0.97
6		6252	1.00
Acceptance criteria		>2000	<2.0

- **Linearity**

Area of Bilastine peak and concentration were submitted to linear regression analysis to obtain the regression equation and correlation coefficient. Table 4.6 presents the data of linearity and Fig. 4.4 shows calibration curve for Bilastine. The calibration graph was linear over the concentration range 25-150 µg/mL of Bilastine (r^2 ± SD = 0.9996 ± 0.0001). The

calibration results revealed a good linear relationship of peak area of Bilastine over the concentration range of 25-150 µg/mL.

TABLE 4.6 Linearity results for Bilastine

Sr. No.	Stock solution taken in mL	Diluted to volume in mL	Concentration in µg/mL	Average area (n=3)
1	0.25	10	25	282999
2	0.50	10	50	563704
3	0.75	10	75	820846
4	1.00	10	100	1119847
5	1.25	10	125	1396424
6	1.50	10	150	1644192

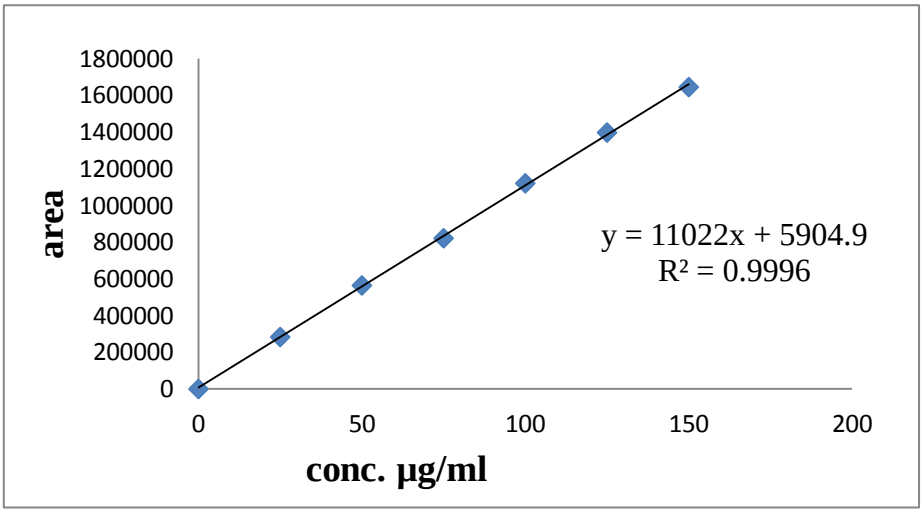

FIGURE 4.4 Calibration curve of Bilastine for RP-HPLC

➢ **Precision**

- **Repeatability**

Table 4.7 presents results of repeatability. % RSD of retention time and area of Bilastine determination was found less than 2% that proves the repeatability of the developed HPLC method.

- **Intraday and interday precision**

Table 4.8 shows the intraday precision results. % RSD of peak area of Bilastine was found less than 1% for intraday precision. Table 4.9 shows the results of interday precision. % RSD

of peak area of Bilastine was found less than 2% for interday precision. The values of %RSD were less than 2% which proved the high precision of the proposed method.

TABLE 4.7 Repeatability data of RP-HPLC for Bilastine

Sr. No.	Retention time (Min.)	Area of peak
1	3.846	1132793
2	3.856	1128885
3	3.861	1129705
4	3.866	1130324
5	3.867	1129880
6	3.874	1133794
Average	3.862	1130896.833
Std. dev.	0.001	1940.054
%RSD	0.253	0.172

TABLE 4.8 Intraday precision data of RP-HPLC for Bilastine

Conc. µg/mL	I	II	III	Avg.	Std. dev.	%RSD
50	562364	566972	561776	926153.3	9066.378	**0.505**
100	1129850	1120852	1108839	1856067	16509.83	**0.941**
150	1645610	1646039	1640927	2785878	17104.52	**0.172**

TABLE 4.9 Interday precision data of RP-HPLC for Bilastine

Conc. µg/mL	I	II	III	Avg.	Std. dev.	%RSD
50	562364	567962	560564	563630	3858.07	**0.685**
100	1129850	1130675	1108740	1123088.3	12432.87	**1.107**
150	1645610	1651005	1640139	1645584.7	5433.04	**0.330**

- **Accuracy**

When the method was used for analysis of Bilastine from previously analysed laboratory mixture solution after spiking of 50, 100 and 150% Bilastine standard, the recovery was found 99-100%. Table 4.10 presents the results of accuracy determination.

TABLE 4.10 Accuracy data of RP-HPLC for Bilastine

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

- **Specificity**

- **Acid induced degradation study**

As shown in Fig. 4.5, chromatogram of acid stressed Bilastine sample yielding the degradation product at 4.824 min. and Bilastine at 3.798 min. with good resolution (Rs=3.6). Purity angle and purity threshold for Bilastine peak are 0.898 and 1.015, respectively which indicates the peak purity of Bilastine. Purity angle and purity threshold for acid induced degradation product peak are 9.437 and 11.229., respectively which indicates the peak purity of degradation product. This study proves that the developed method determines the Bilastine specifically in presence of acid induced degradation product.

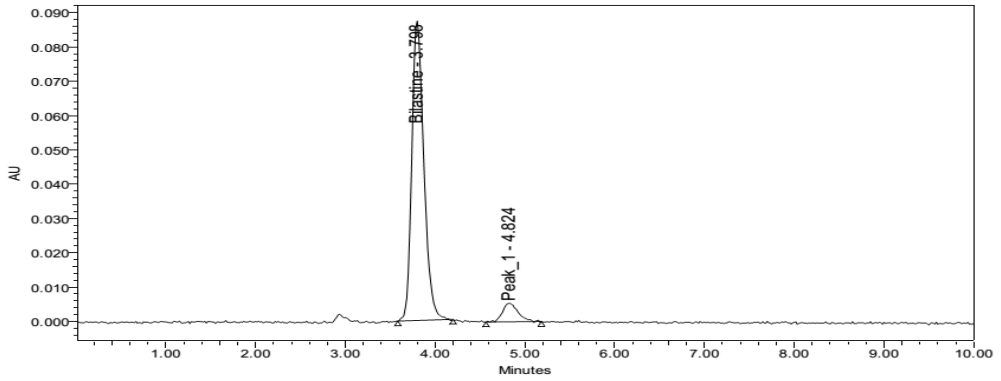

FIGURE 4.5 Chromatogram of Bilastine after refluxed with 2 N HCl at 80° C for 30 min.

- **Base induced degradation study**

Peak area of Bilastine was not changed significantly and any additional peak was not observed when chromatographed after refluxing in 2M NaOH at 80°C for 2 hr. Purity angle and purity threshold for Bilastine peak after base induced degradation are 0.120 and 0.784, respectively which shows the purity of Bilastine peak. Chromatogram of Bilastine after degradation with 2 N NaOH at 80° C for 2 hr is displayed in Fig. 4.6.

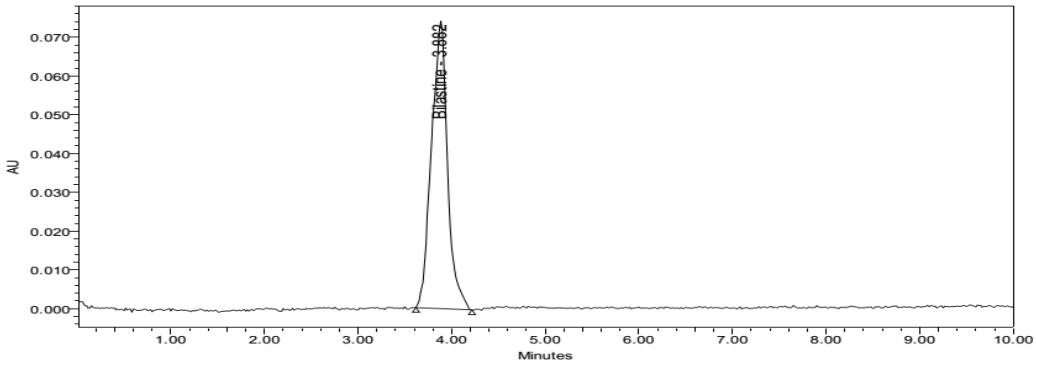

FIGURE 4.6 Chromatogram of Bilastine after refluxed with 2 N NaOH at 80° C for 2 hr

- **Wet heat degradation**

Peak area of Bilastine was not changed significantly and any additional peak was not observed when chromatographed after refluxing in distilled H_2O at 80°C for 2 hr. Purity angle and purity threshold for Bilastine peak after wet heat degradation are 0.120 and 0.790, respectively which shows the purity of Bilastine peak. Chromatogram of water stressed sample of Bilastine is displayed in Fig. 4.7.

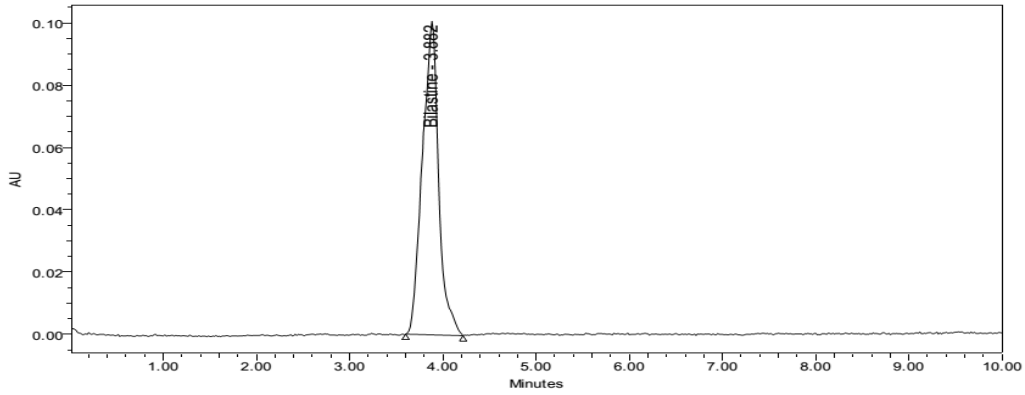

FIGURE 4.7 Chromatogram of Bilastine water stressed sample of Bilastine

- **Oxidative degradation study**

As shown in Fig. 4.8 chromatogram of H_2O_2 stressed Bilastine sample yielding the degradation product at 4.612 min. and Bilastine at 4.074 min. with resolution greater than 2.0 (Rs=2.3). Purity angle and purity threshold for Bilastine peak are 0.539 and 0.812, respectively which indicates the peak purity of Bilastine. Purity angle and purity threshold for oxidation induced degradation product peak are 1.709 and 2.884, respectively. Hydrogen peroxide retention time is 1.4 min. which was confirmed from PDA data.

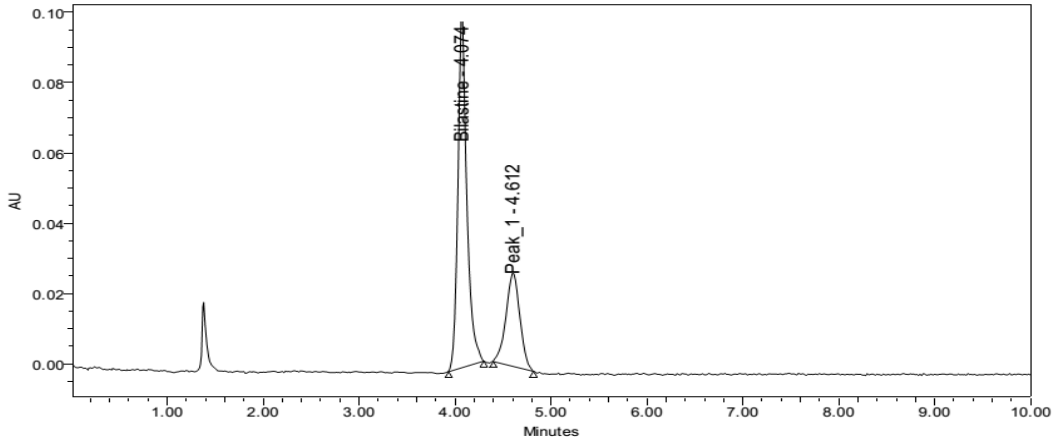

FIGURE 4.8 Chromatogram of Bilastine after degradation with 10% hydrogen peroxide at 80° C for 30 min.

- **Thermal degradation**

Peak area of Bilastine was not changed significantly and any additional peak was not observed when chromatographed after exposing at 70°C for 8 hr. Purity angle and purity threshold for Bilastine peak after degradation are 0.568 and 0.778, respectively. Chromatogram of Bilastine after thermal degradation is displayed in Fig. 4.9.

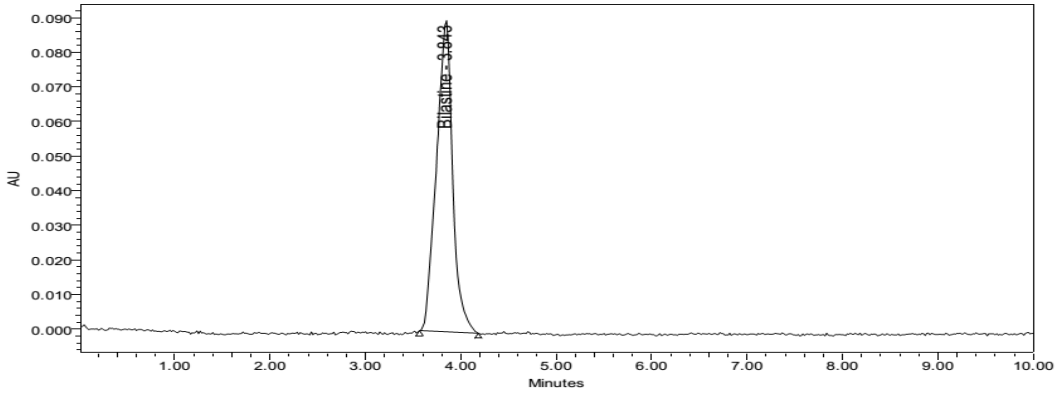

FIGURE 4.9 Chromatogram of Bilastine after thermal degradation

- **Photolytic degradation**

Fig. 4.10 presents chromatogram of Bilastine after photolytic stress. Peak area of Bilastine was not changed significantly and any additional peak was not observed when chromatographed after exposing at 365 nm for 24 hr. Purity angle and purity threshold for Bilastine peak after degradation are 0.568 and 0.778, respectively.

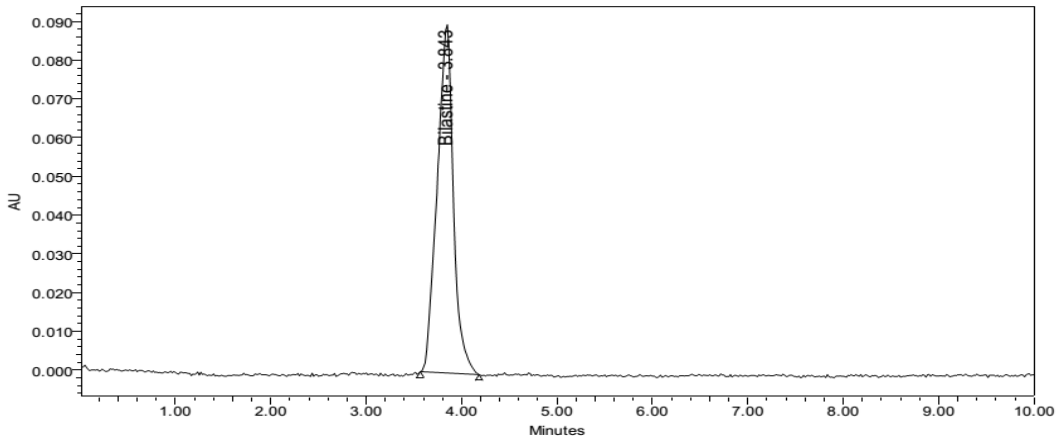

FIGURE 4.10 Chromatogram of Bilastine after photolytic stress.

The results of HPLC method for stress samples, including separation of the degradation products produced from acid degradation of Bilastine and degradation of Bilastine in oxidizing condition and quantification of Bilastine in presence of degradation impurities indicates the proposed method is stability-indicating.

- **Robustness**

It was found that method was robust regarding any minor deviation in the column temperature (35 ± 1°c) as confirmed by the constant value of the retention time and the area of Bilastine peak. Small change in mobile phase flow rate (1.0 ± 0.1 mL/min.) and mobile phase ratio led considerable change in the retention time and area of the Bilastine peak. Table 4.11 shows the results of the robustness.

TABLE 4.11 Robustness data of RP-HPLC for Bilastine.

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	

- **Limit of Detection and Limit of Quantitation**

LOD and LOQ were estimated as per ICH guideline using mathematical formula. Obtained value for LOD was 0.19µg/mL and obtained value for LOQ was 0.57µg/mL. Small value of LOD and LOQ expressed that the developed method can be used for detection and quantification of Bilastine in QC samples.

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

= 3.3 * (631.3/11022.3)

= 0.19 µg/mL

LOQ = 10 *(standard deviation of y-intercept/slope)

= 10* (631.3/11022.3)

= 0.57µg/mL

4.3 References

1. Orjales Venero et al., 2005, Polymorph of 4-2-4-1-(2-ethoxyethyl)-1h Benzimidazole-2-yl-1-piperidinyl Ethyl-αα-dimethyl-benzene acetic Acid, US 7,612,095 B2

CHAPTER 5

Degradation Kinetic Study Of Bilastine

5.1 Materials and Methods

5.1.1 Reagents and Materials

TABLE 5.1 List of reagents and materials used in HPLC method for Bilastine

Sr. No.	Reagent/material	Grade
1	Water	HPLC
2	Hydrochloric acid (HCl)	Analytical
3	Hydrogen Peroxide	Analytical
4	Methanol	HPLC
5	Ortho phosphoric acid (OPA)	Analytical
6	Bilastine procured from Symed Labs Ltd.	Reference standard

5.1.2 Instruments and Apparatus

TABLE 5.2 List of instruments and apparatus used in HPLC method for Bilastine

Sr. No.	Instrument/Apparatus	Manufacturer
1	Analytical weighing balance	Sartorius
2	Glass wares	Borosil
3	HPLC	Waters HPLC with EmPower software

5.1.3 Preparation of solutions

- **Preparation of standard solution (1mg/mL)**

Bilastine standard solution was made by taking accurately 100 mg of Bilastine in 100mL volumetric flask, adding 50 mL methanol and was sonicated for 10 min. and making 100 mL with methanol.

5.1.4 HPLC method

Waters HPLC system furnished with quaternary solvent manager, auto sampler, and PDA detector controlled by EmPower software was used for quantitative analysis of Bilastine. Discovery C_8 (250 x 4.6 mm, 5µm) column and mobile phase comprising of Methanol: 0.1% ortho phosphoric acid (55: 45 %v/v) with flow rate of 1.0 mL/min was employed for separation. The column temperature was set at 35 °C, and volume injection was 10 µL. 276 nm (λ_{max} of Bilastine) was used for characterization.

5.1.5 Degradation kinetic study of Bilastine in acid

To study order of reaction and degradation rate of Bilastine in acidic media, 2 mL of 1 mg/mL Bilastine standard solution was transferred in 20 mL volumetric flasks. To each volumetric flask 2 ml of 2 N HCl was added and the mixture was heated under reflux at 50, 60, 70, or 80 °C. Five volumetric flasks were placed at each temperature. After specific time interval volumetric flask was taken out from water bath, cooled it and dilution up to the volume was done with methanol. They were analyzed by the developed and validated HPLC method. The concentration of the undegraded Bilastine was estimated for each time period by putting the value of peak area in regression equation and the data were further treated to find order of reaction and degradation rate constant. Data got from order kinetics treatment were submitted to fitting to the Arrhenius equation and activation energy for the reaction was calculated. By processing the data further degradation rate, half time and t_{10} of the drug in 2 N HCl at 25 °C were obtained.

5.2 Results and Discussion

5.2.1 Degradation kinetic study of Bilastine in 2 N HCl

The concentration of the Bilastine in acidic condition was found to decrease with time. Fig.5.1 to Fig.5.5 displays chromatograms of Bilastine in acidic medium at 70 °C for different time interval.

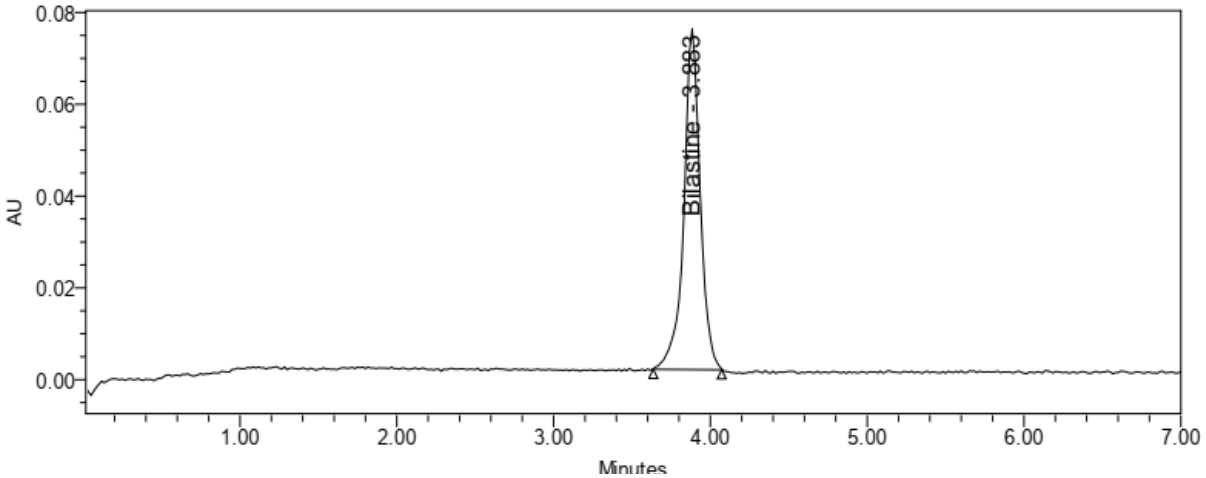

FIGURE 5.1 Chromatogram of Bilastine in 2 N HCl at 70° C at 0 min.

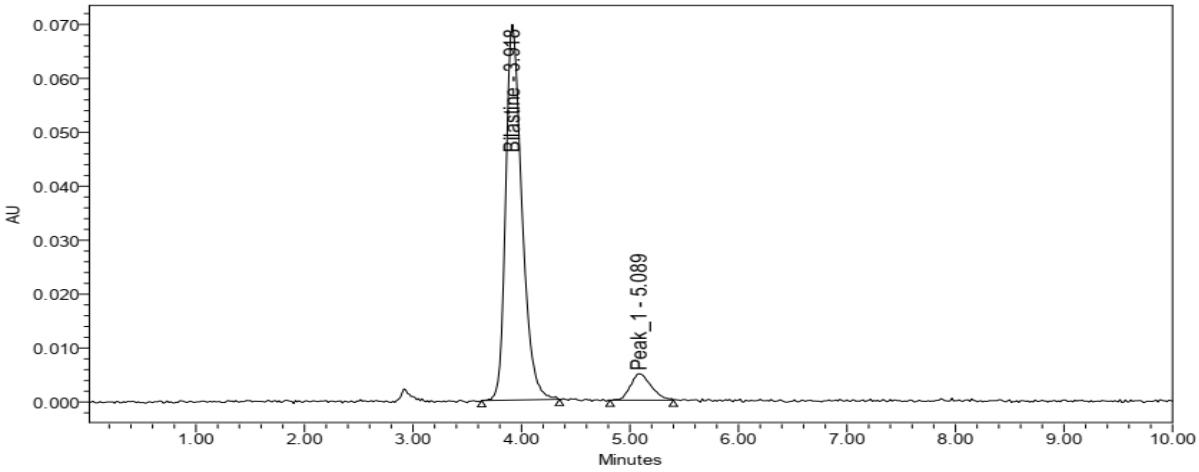

FIGURE 5.2 Chromatogram of Bilastine in 2 N HCl at 70° C after 20 min.

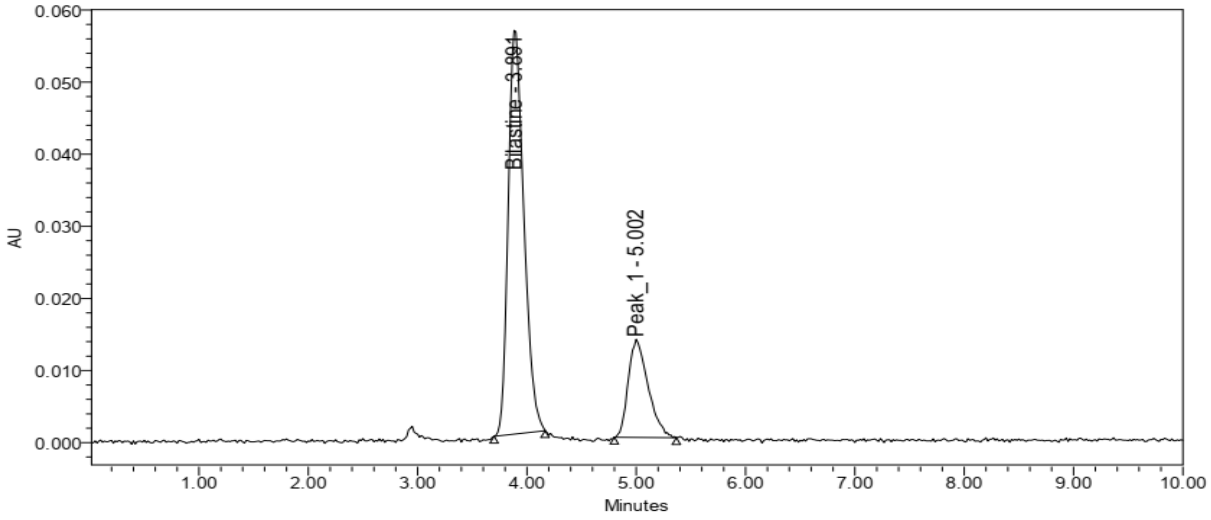

FIGURE 5.3 Chromatogram of Bilastine in 2 N HCl at 70° C after 60 min.

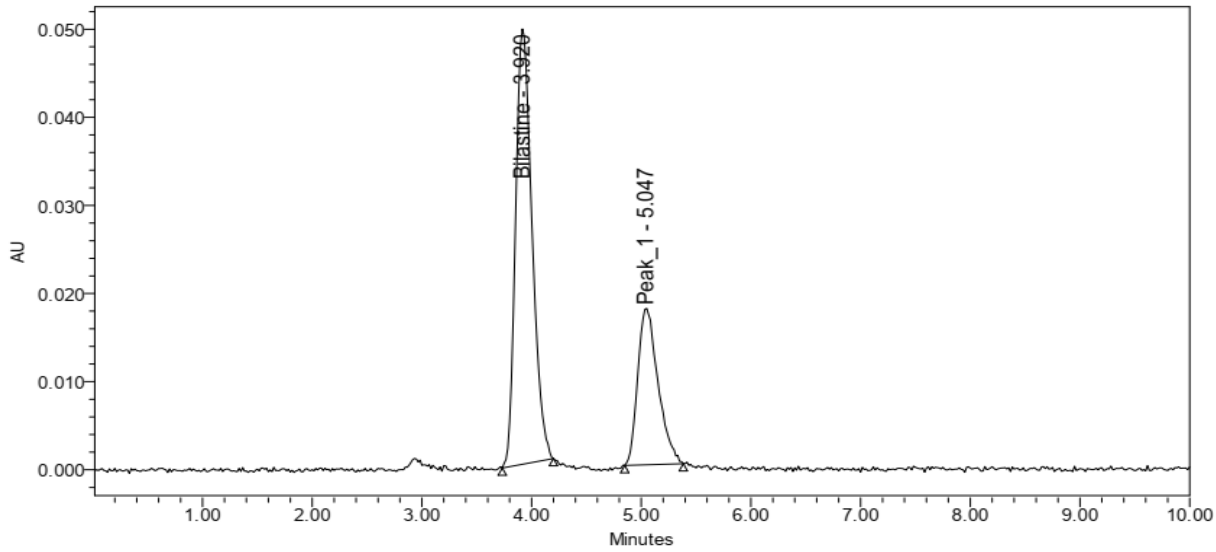

FIGURE 5.4 Chromatogram of Bilastine in 2 N HCl at 70° C after 80 min.

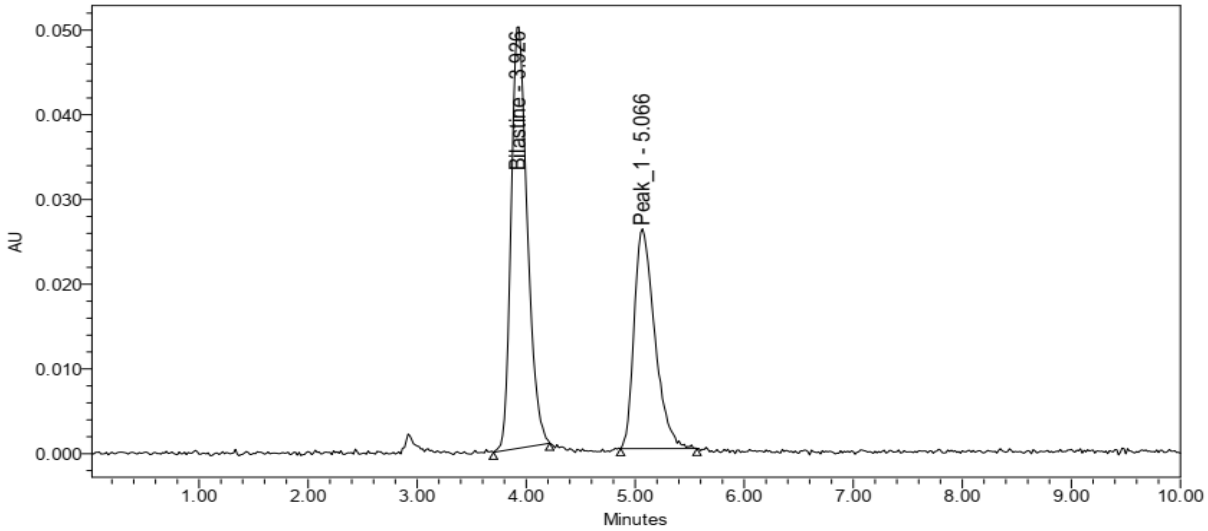

FIGURE 5.5 Chromatogram of Bilastine in 2 N HCl at 70° C after 100 min.

Results of the percentage degradation of Bilastine at 50, 60, 70, or 80 °C at specific time interval are presented in Table 5.3 to Table 5.6 respectively. When drug concentration is plotted against time, if a straight line results then the reaction is zero order. If plot of log concentration against time is linear then it is first order reaction. If plot of inverse of drug concentration against time is linear then it is second order. The regression co-efficient (rate constants) for 50, 60, 70, and 80 °C were calculated for zero order, first order and second order from graphical method and they are presented in Table 5.7. From regression co-efficient, it was found that degradation of Bilastine in acidic medium was most fit to the first order degradation.

TABLE 5.3 Degradation of Bilastine in 2 N HCl at 50°C

Time (min.)	Peak Area of Bilastine	%drug	% degradation
0	727567	100	0
45	683308	93.91685	6.083151
90	650772	89.44496	10.55504
135	574778	79.00001	20.99999

TABLE 5.4 Degradation of Bilastine in 2 N HCl at 60°C

Time (min.)	Peak Area of Bilastine	%drug	% degradation
0	727567	100	0
25	679505	93.39415	6.60585
50	644513	88.58469	11.41531
80	587229	80.71133	19.28867
140	477098	65.57444	34.42556

TABLE 5.5 Degradation of Bilastine 2 N HCl at 70°C

Time (min.)	Peak Area of Bilastine	%drug	% degradation
0	784586	100	0
20	720014	91.76993	8.230073
60	557635	71.07379	28.92621
80	513918	65.50181	34.49819
100	458568	58.44713	41.55287

TABLE 5.6 Degradation of Bilastine in 2 N HCl at 80°C

Time (min.)	Peak Area of Bilastine	%drug	% degradation
0	784586	100	0
18	675161	86.05315	13.94685
30	609646	77.70289	22.29711
46	566313	72.17985	27.82015
60	510568	65.07483	34.92517
75	465221	59.29509	40.70491

TABLE 5.7 Order of reaction for Bilastine in 2 N HCl

Temperature	Zero order	First order	Second order
50	0.9676	0.9551	0.9405
60	0.9986	0.9922	0.9785
70	0.9909	0.9961	0.9936
80	0.9747	0.9928	0.9974

The effect of temperature (50, 60, 70 and 80° C) on the degradation of Bilastine in acidic condition is shown in Fig. 5.6. (Graph of log %undegraded drug vs time in min.)

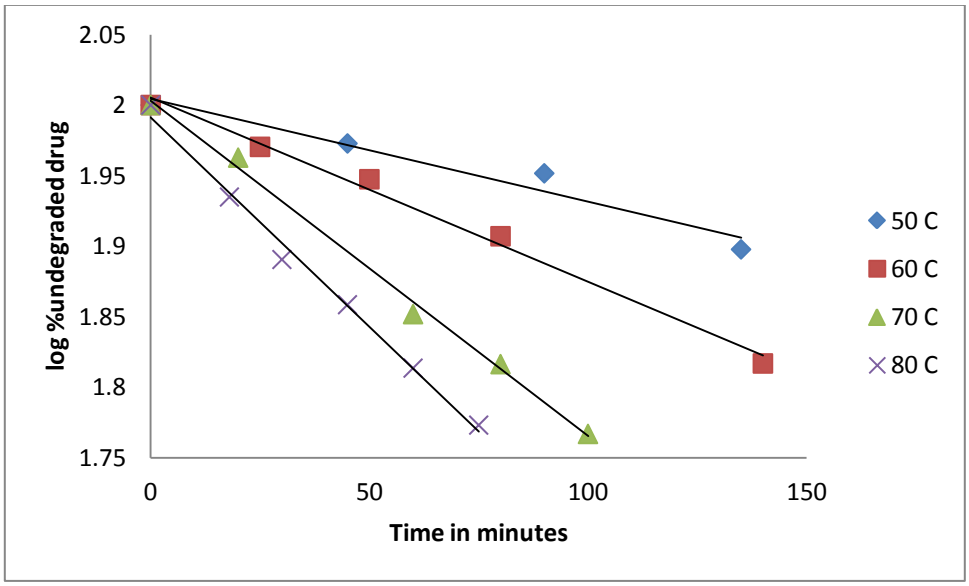

FIGURE 5.6 Temperature effect on degradation of Bilastine in 2N HCl

The degradation under acidic condition follows first order kinetics. In accordance with equation (1) from the slopes of linear graphs of log (a/a-x) versus time t, the first order rate constants (K) were determined.

$Kt = 2.303 \log (a/a-x)$ ……..(1)

Here, "a" is the initial Bilastine concentration and "a-x" is the undegraded Bilastine concentration.

$t_{1/2}$ and t_{10} for the first order degradation was determined using equation (2) and (3), respectively.

$t_{1/2}$= 0.693/K……(2)

t_{10} = 0.104/K…… (3)

Results received from order kinetics were further submitted to fit the Arrhenius equation (equation 4)

log K = log A – Ea/2.303RT……(4)

Where K is the rate constant, A is the frequency factor, Ea is the energy of activation (cal mol^{-1}), R is the gas constant (1.987 cal/deg· mol), and T is absolute temperature. Fig. 5.7 presents Arrhenius plot, a plot of K against 1000/T, gave straight line in the range of 50-80 °C for degradation of Bilastine in acid medium. The activation energy for degradation of Bilastine in 2 N HCl was estimated to be 11.340 Kcal/mol. The degradation rate constant in 2 N HCl (acidic condition) at room temperature (K), was found when Arrhenius plot was extrapolated to 25°C (where 1,000/T =3.354). K at 25 °C was obtained 0.00039 min^{-1}, and calculated $t_{1/2}$ and t_{10} were 1776.92 min. (29.62 hr) and 266.67 min.(4.44 hr), respectively. Degradation kinetic data for Bilastine in 2 N HCl is presented in Table 5.8

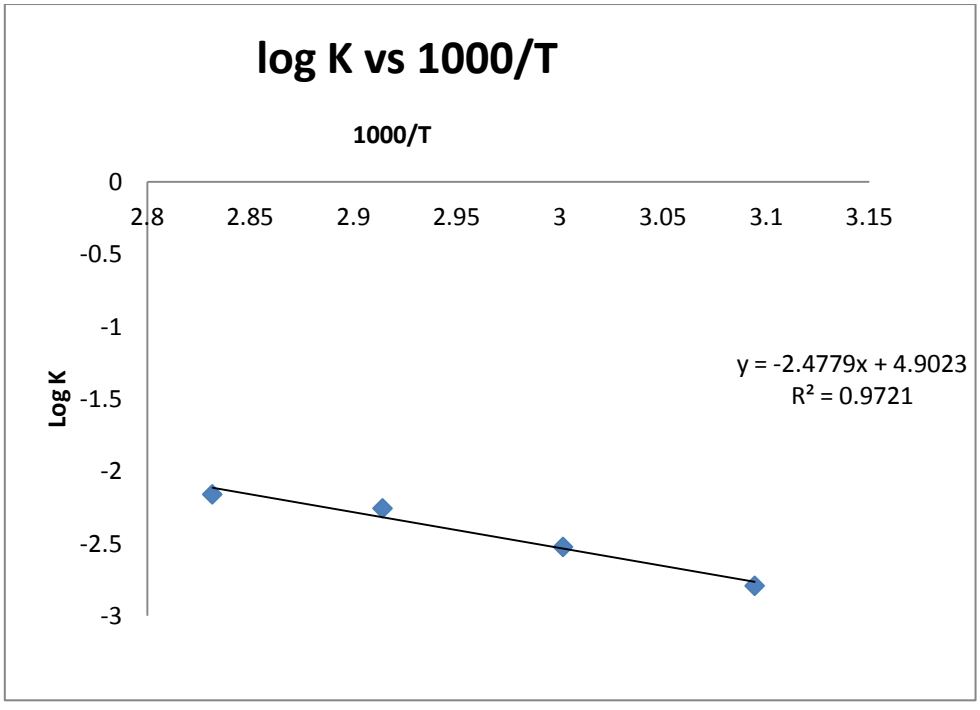

FIGURE 5.7 Arrhenius plot for Bilastine degradation in 2 N HCl

TABLE 5.8 Degradation kinetic data for Bilastine in the presence of 2 N HCl

Temperature (°C)	Degradation rate constant K (min^{-1})	Half time (min.)	t_{10} (min.)
50 °C (323.15 K)	0.001612	429.8741	64.51213
60 °C (333.15 K)	0.002994	231.4707	34.7373
70 °C (343.15 K)	0.005527	130.8312	19.63413
80 °C (353.15 K)	0.006909	100.304	15.05283
25 °C (calculated)	0.00039	1776.923	266.6667

CHAPTER 6

Identification of Degradation Products of Bilastine by LC-MS

6.1 Materials and Methods

6.1.1 Reagents and Materials

TABLE 6.1 List of reagents and materials used in LC-MS method for Bilastine

Sr. No.	Reagent/material	Grade
1	Water	HPLC
2	Hydrochloric acid (HCl)	Analytical
3	Hydrogen Peroxide	Analytical
4	Methanol	HPLC
5	Formic acid	Analytical
6	Bilastine procured from Symed Labs Ltd.	Reference standard

6.1.2 Instruments and Apparatus

TABLE 6.2 List of instruments and apparatus used in LC-MS method for Bilastine

Sr. No.	Instrument/Apparatus	Manufacturer
1	Analytical weighing balance	Mettler Toledo
2	Glass wares	Borosil
3	LC-800	Make by GL science with Analyst software
4	MS/ MS	AB sciex QTRAP 4500

6.1.3 Preparation of solutions

- **Preparation of standard solution**

Bilastine standard solution was made by taking accurately 100 mg of Bilastine in 100mL volumetric flask, adding 50 mL methanol and was sonicated for 10 min. and making 100 mL with methanol. The resulted solution was further diluted to have 10 µg/mL of Bilastine.

- **Acid induced degradation solution of Bilastine**

2 mL standard solution of Bilastine (1 mg/mL) was taken in 20 mL volumetric flask and 2 mL 2.0 M HCl was added to it. For 30 min. the mixture was refluxed in a thermostatic water bath at 60°C. Dilution up to the volume was done with methanol. 1 mL of resulted solution was diluted to 10 mL with methanol.

- **Oxidised degradation solution of Bilastine**

2 mL standard solution of Bilastine (1000µg/mL) was taken to 20 mL volumetric flask followed by 2 mL 10% H_2O_2. The mixture was refluxed in a thermostatic water bath at 80°C for 30 min. Then it was cooled and dilution up to the volume was done with methanol. 1 mL of resulted solution was further diluted to 10 mL with methanol.

6.1.4 LC-MS method

Inertsil C_{18} column (150 mm length x 2.1 mm i. d., 3 µm particle size) and the mobile phase comprised of 0.1% formic acid and methanol (50:50 v/v) pumped at rate of 0.2 mL/min. was employed for chromatographic separation of Bilastine and its degradation products. The injection volume of drug was 10 µL and temperature of column was 40 °C. The eluted components were detected using UV detector. HPLC system is coupled with MS/MS, also. Fragmentation profile of Bilastine and its degradation products was established by performing mass spectral studies on Bilastine and its degradation products, respectively. The products were ionized by positive mode of electron spray ionization (ESI) for their mass data. The data was processed and monitored using Analyst software. The parameters MS were adequately adjusted that high intensity of molecular ions peaks and daughter ions peaks of degradation products were obtained.

6.2 Results and Discussion

6.2.1 Chromatographic analysis

For determination of Bilastine in presence of degradation products a novel HPLC method was developed and validated as described in chapter 4. From the study it was found that Bilastine produced one degradation product from acidic degradation and one degradation product from oxidizing degradation. This method was transferred to LC-MS with some modification to elucidate the structures of degradation products of Bilastine. 0.1 % ortho-phosphoric acid was replaced by 0.1% formic acid. Inertsil C_{18} column (150 mm length x 2.1 mm i. d., 3 µm particle size) and the mobile phase comprised of 0.1% formic acid and methanol (50:50 v/v) pumped at rate of 0.2 mL/min. was employed for chromatographic separation. Chromatograms of Bilastine standard, acid stressed sample and hydrogen peroxide stressed sample are presented in Fig. 6.1 to Fig. 6.3, respectively. Retention time of Bilastine was found around 2.93 min. Retention time for acid degradation product was found at 2.41 min. Degradation product of acidic condition and Bilastine were separated well. Degradation product of oxidized condition and Bilastine were also separated well.

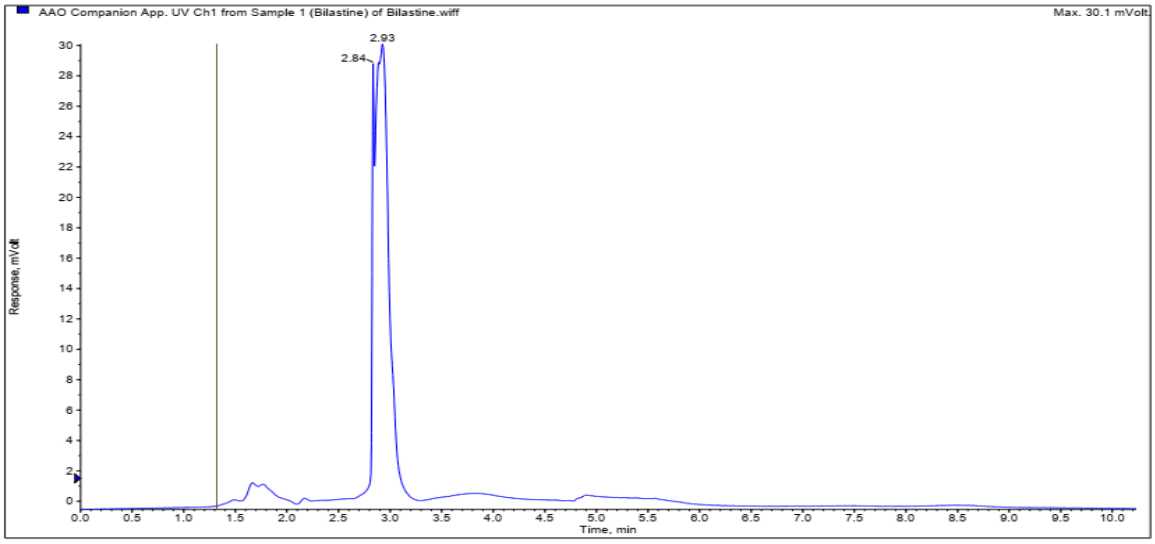

FIGURE 6.1 Chromatogram of Bilastine standard

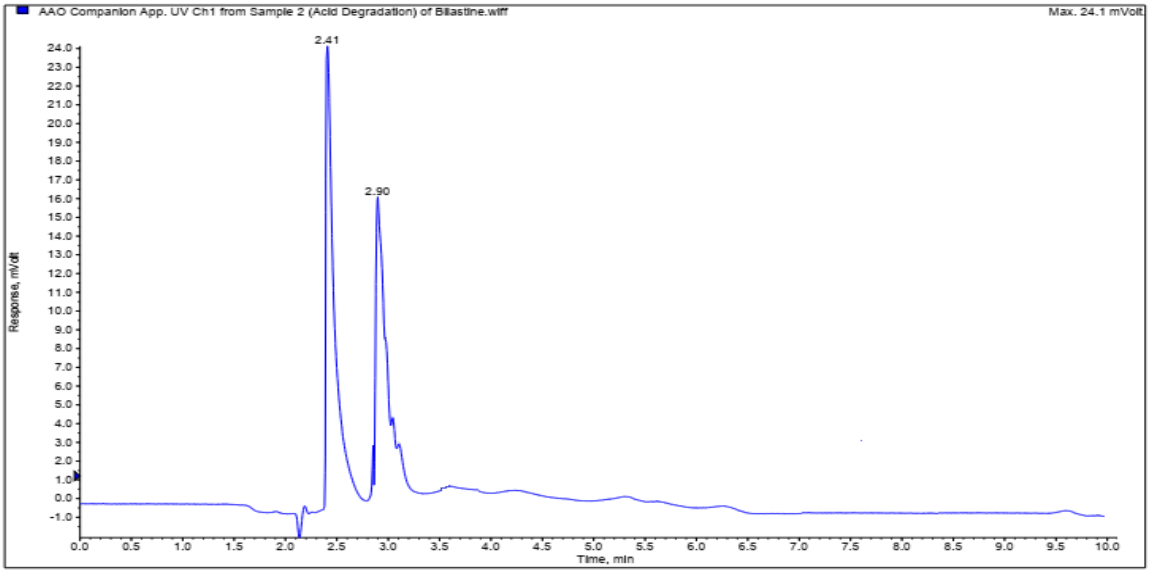

FIGURE 6.2 Chromatogram of acid stressed Bilastine sample

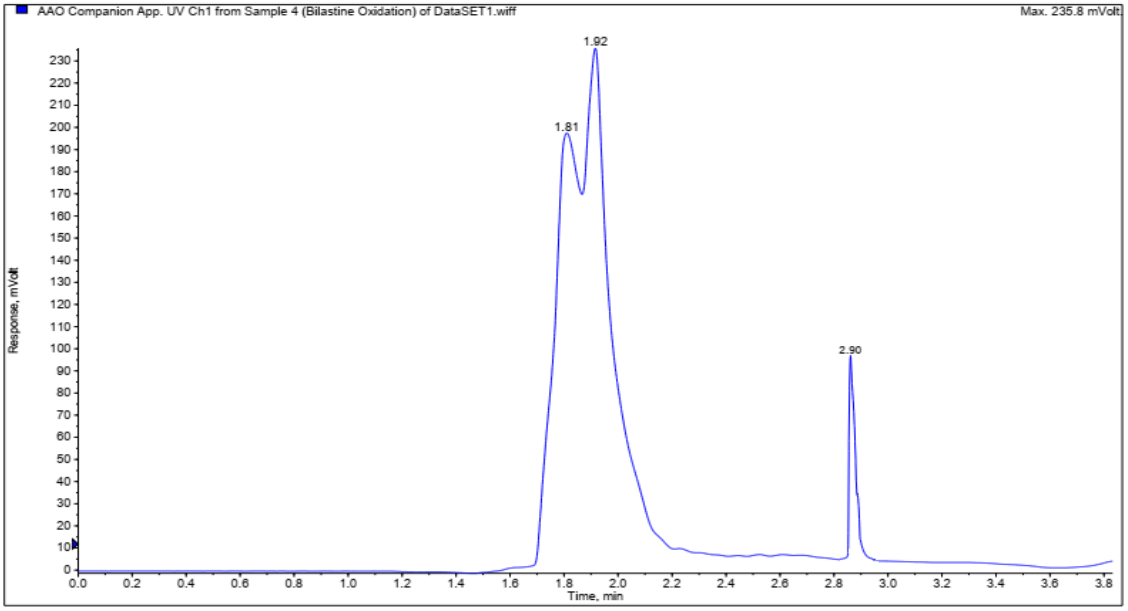

FIGURE 6.3 Chromatogram of hydrogen peroxide stressed Bilastine sample

6.2.2 LC-MS study of Bilastine

The optimization of the instrument conditions started from the tuning of detector and electron spray ionization in a positive mode. 10 µg/mL of Bilastine in methanol was submitted to MS system in positive mode of ESI in the mass range of 100-540 Da. The following instrument settings were applied: de-clustering potential of 90 eV, entrance potential of 10 eV, cell exit potential of 10 eV and collision energy of 50 eV. The $[M + H]^+$ value observed in ESI+ mode

was 464.3 and matched with molecular weight of Bilastine (463.61). Fragmentation pattern of Bilastine is illustrated in Fig. 6.4 from the interpretation of fragments by the best suitable molecular formulae to the experimental value of m/z. A mass spectrum of product ion of Bilastine produced by LC-MS/MS is presented in Fig. 6.5.

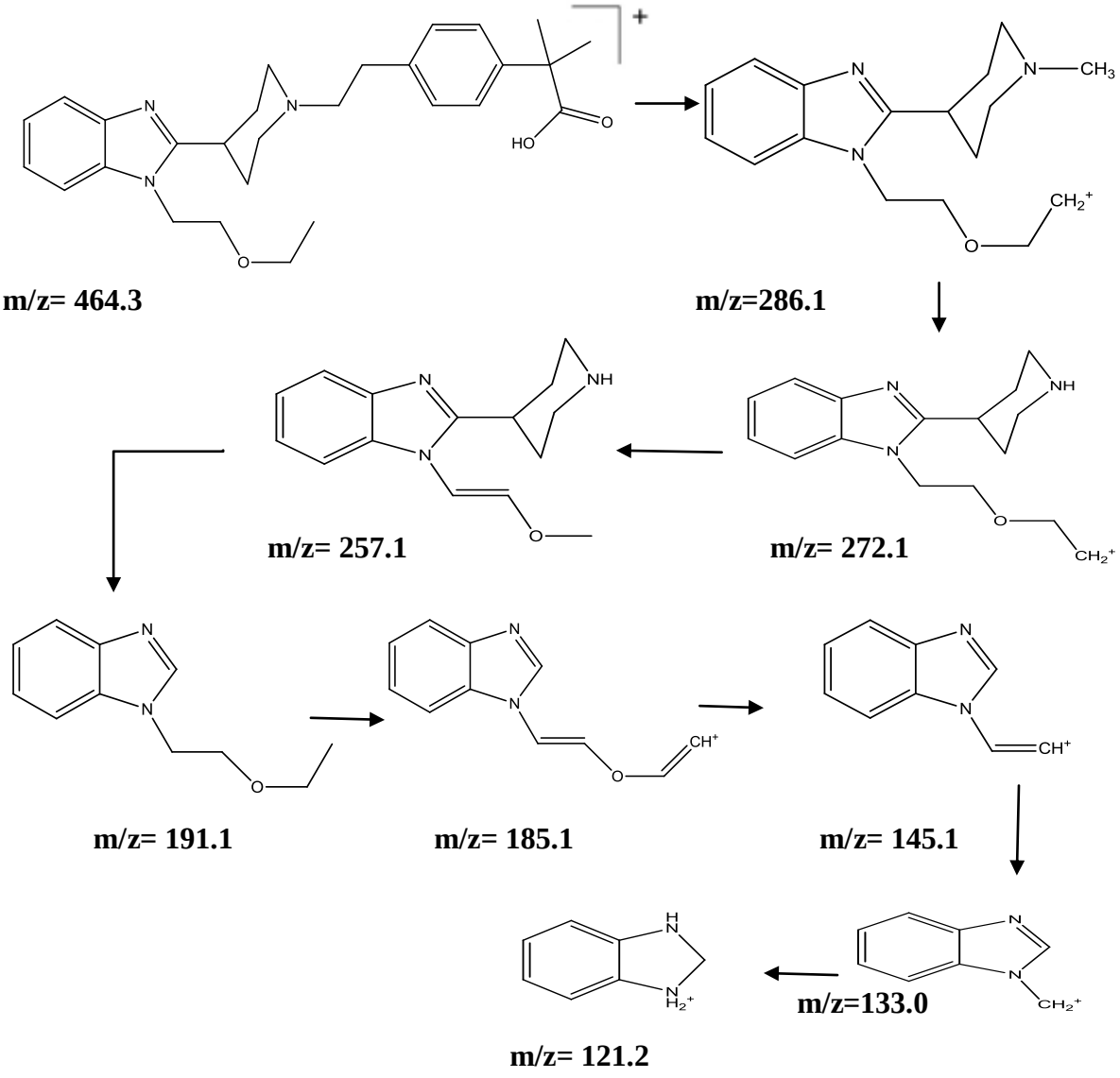

FIGURE 6.4 Probable fragmentation pattern of Bilastine

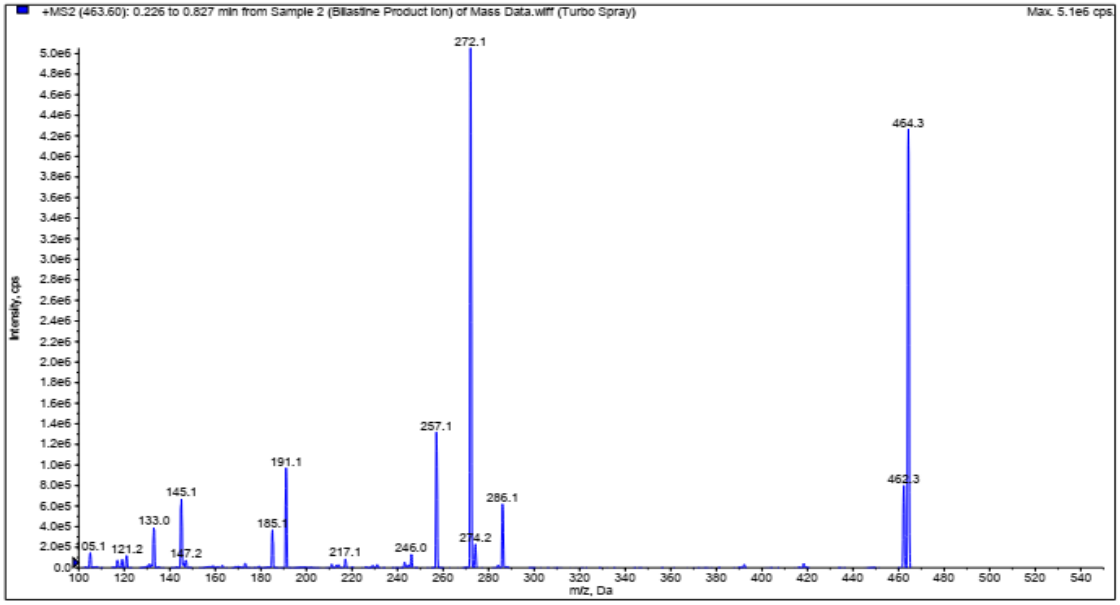

FIGURE 6.5 Mass spectrum of Bilastine (product ion) by LC-MS/MS.

6.2.3 Characterization of degradation product of acid stressed sample

Fig. 6.6 presents s mass spectrum of degradation impurity generated in acid stressed sample.

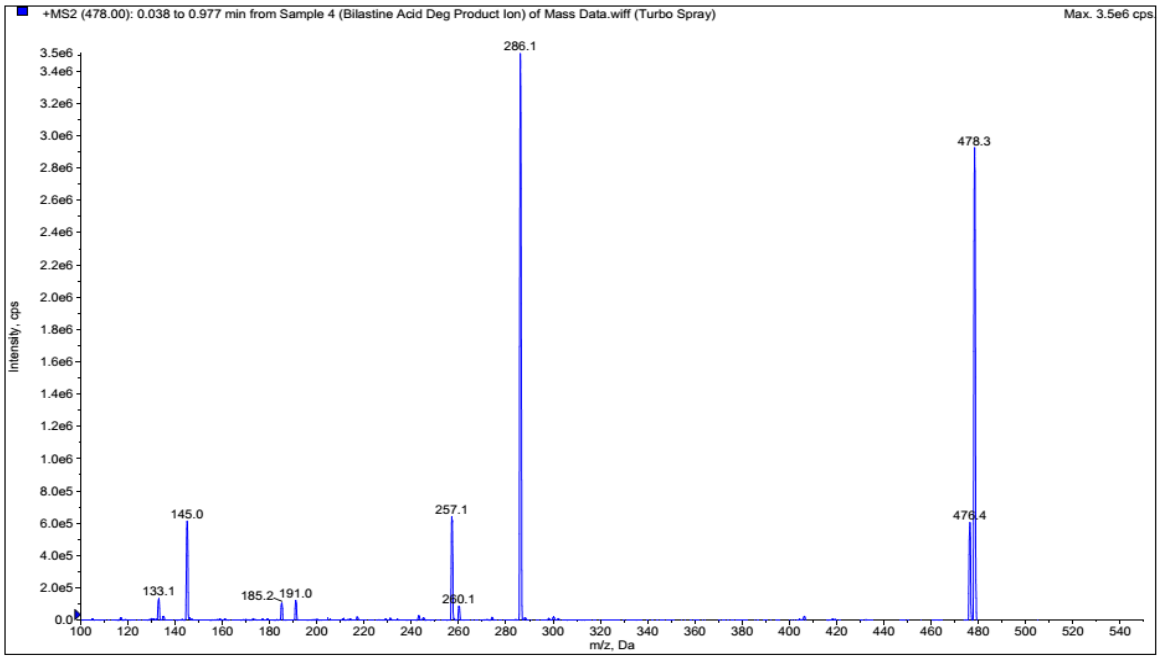

FIGURE 6.6 Mass spectrum of degradation impurity of Bilastine by acid

The results of LC–MS/MS analysis showed that the degradation product formed after acidic hydrolysis had molecular ion peak at 478.43 Da which has 14.00 Da more value than molecular ion peak of Bilastine (464.3 Da). 14.0 Da value is equal to methylene group. Likely

Fragmentation pattern of degradation product of acid stressed Bilastine is illustrated in Fig. 6.7. Mass spectra and likely fragmentation pattern of degradation product of acid stressed Bilastine sample confirms that Bilastine in presence of hydrochloric acid reacts with methanol and produced methyl ester of Bilastine.

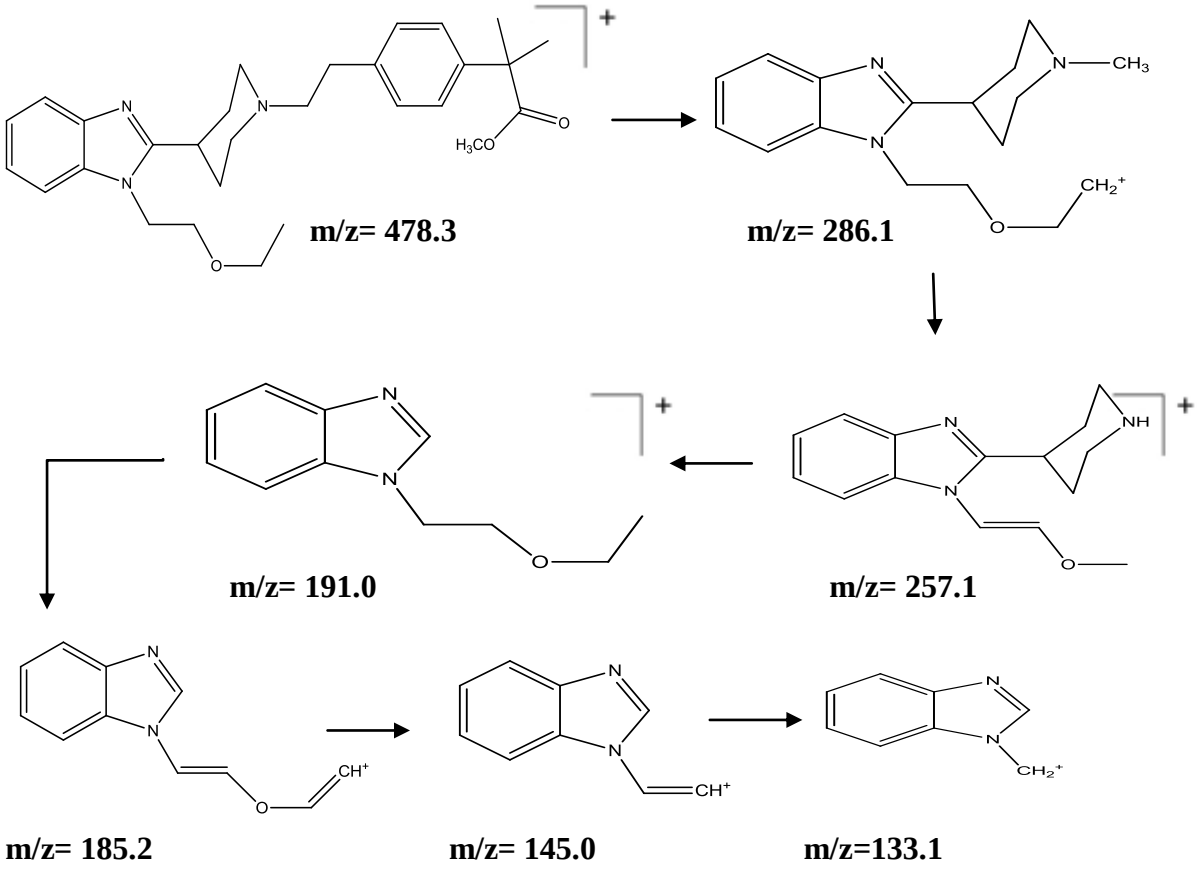

FIGURE 6.7 Probable fragmentation pattern of acid degradation product of Bilastine

6.2.4 Characterization of degradation product of oxidation stressed sample

The LC-MS/MS spectrum of degradation product after oxidation of Bilastine by hydrogen peroxide showed molecular ion peak at 480.4 Da ($[M+H]^+$) formed in positive ionization mode. Molecular ion at 480.4 Da shows the addition of approx. 16 Da to the m/z of molecular ion of Bilastine (464.3 Da) which resembles to ion of oxygen. Molecular ion peak at 480.4 Da represents the formation of N-oxide of Bilastine on reaction of Bilastine with hydrogen peroxide. N-oxide of Bilastine is reported as degradation product of Bilastine in the literature also[1-2]. A mass spectrum of degradation product of hydrogen peroxide stressed bilastine

sample is presented in Fig. 6.8. Fragmentation pattern of degradation product of hydrogen peroxide stressed Bilastine is illustrated in Fig. 6.9. Mass spectra and fragmentation pattern of degradation product of hydrogen peroxide stressed Bilastine sample confirms that Bilastine reacts with 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. Table 6.3 presents data of Mass spectra of Bilastine and its degradation products and their major fragments along with molecular formula.

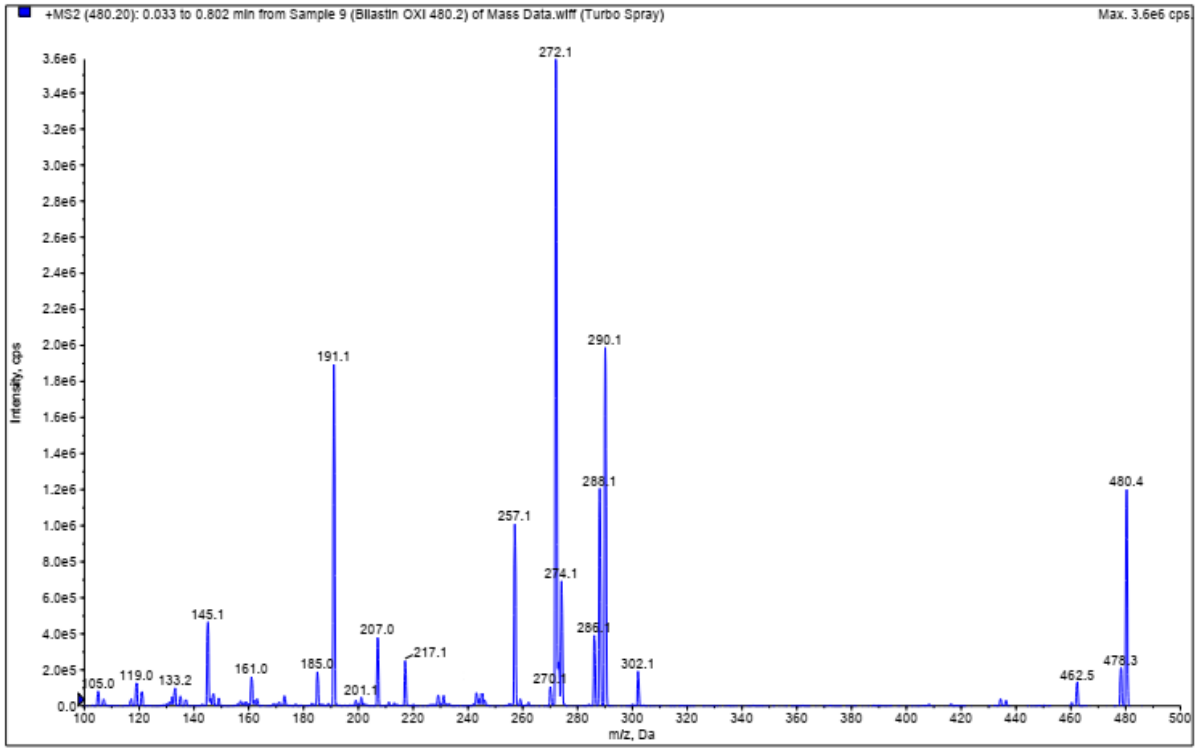

FIGURE 6.8 Mass spectrum of Hydrogen peroxide stressed Bilastine sample

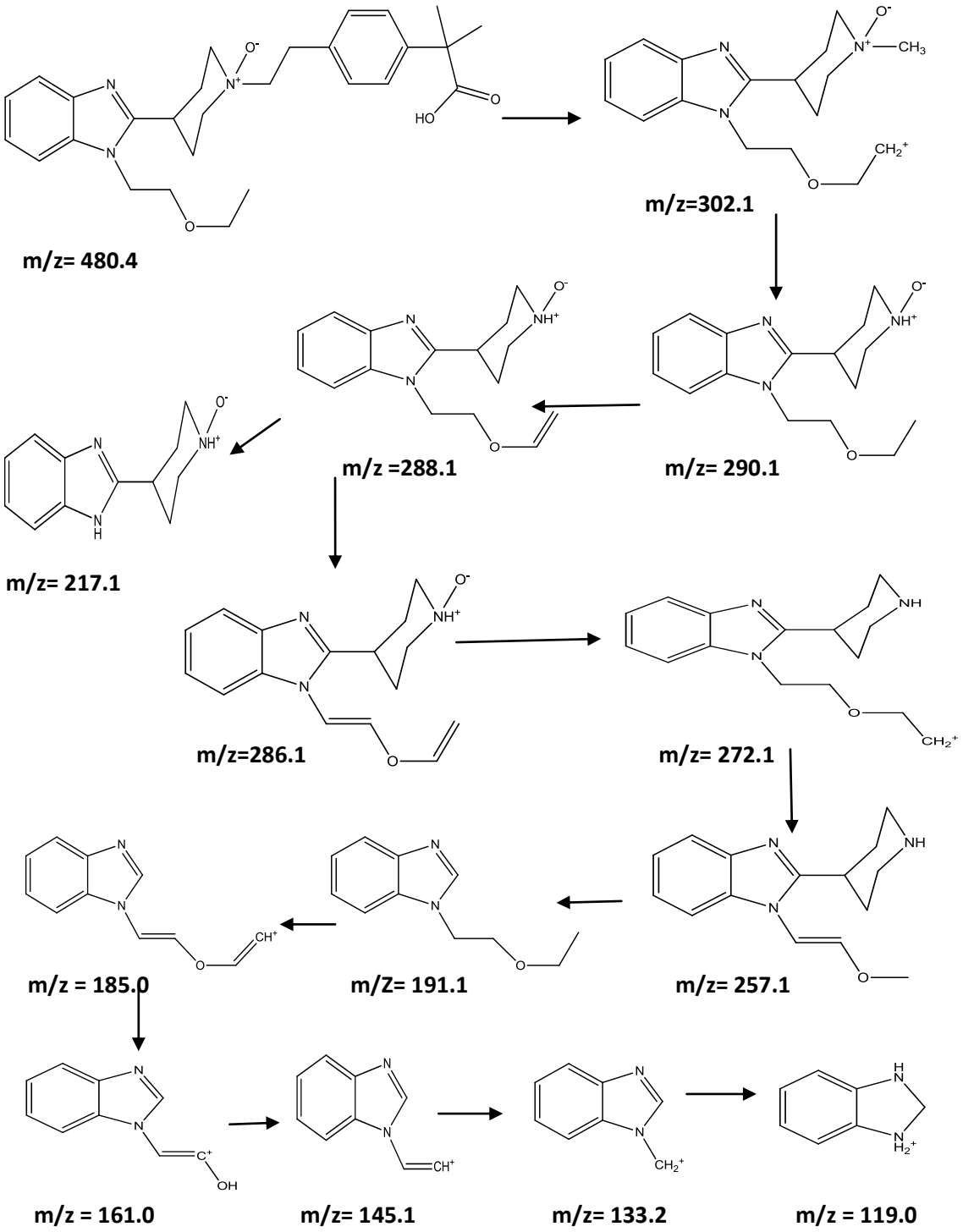

FIGURE 6.9 Likely fragmentation pattern of oxidation degradation product of Bilastine

TABLE 6.3 LC-MS/MS data of Bilastine and its degradation products and their major fragments

Substance	Experimental mass	Molecular formula	Major fragments
Bilastine	464.3	$C_{28}H_{37}N_3O_3^+$	286.1($C_{17}H_{24}ON_3^+$),272.1(($C_{16}H_{22}ON_3^+$), 257.1($C_{15}H_{19}ON_3^+$), 191.1($C_{11}H_{14}ON_2^+$), 185.1($C_{11}H_9ON_2^+$), 145.1($C_9H_7N_2^+$), 133.0($C_8H_7N_2^+$), 121.1($C_7H_7N_2^+$)
Acid degradation product of Bilastine	478.3	$C_{29}H_{39}N_3O_3^+$	286.1($C_{17}H_{24}ON_3^+$), 257.1($C_{15}H_{19}ON_3^+$), 191.1($C_{11}H_{14}ON_2^+$), 185.1($C_{11}H_9ON_2^+$), 145.1($C_9H_7N_2^+$), 133.0($C_8H_7N_2^+$)
Oxidation degradation product of Bilastine	480.4	$C_{28}H_{37}N_3O_4^+$	302.1($C_{17}H_{24}O_2N_3^+$), 290.1($C_{16}H_{24}O_2N_3^+$), 288.1($C_{17}H_{22}O_2N_3^+$), 286.1($C_{17}H_{20}ON_3^+$), 272.1(($C_{16}H_{22}ON_3^+$), 257.1($C_{15}H_{19}ON_3^+$), 217.1($C_{12}H_{14}ON_3^+$) 191.1($C_{11}H_{14}ON_2^+$), 185.1($C_{11}H_9ON_2^+$), 145.1($C_9H_7N_2^+$), 133.0($C_8H_7N_2^+$), 121.1($C_7H_7N_2^+$)

6.3 References

1. Jelena T, Igor P, Ana S, Anja T, Biljana J, 2016, Application Of Analytical Quality By Design Concept for Bilastine and its Degradation Impurities Determination by Hydrophilic Interaction Liquid Chromatographic Method, *Journal of Pharmaceutical and Biomedical Analysis*, 125, pp 385-93, ISSN No. 0731-7085.
2. 2016, Bilastine detection method, CN104730194B

CHAPTER 7

HPLC Method for Ebastine

7.1 Materials and Methods

7.1.1 Reagents and Materials

TABLE 7.1 List of reagents and materials used in HPLC method for Ebastine

Sr. No.	Reagent/material	Grade
1	Water	HPLC
2	Hydrochloric acid	Analytical
3	Sodium hydroxide	Analytical
4	Hydrogen Peroxide	Analytical
5	Methanol	HPLC
6	Acetonitrile	HPLC
7	Triethylamine	Analytical
8	Triflouroacetic acid	Analytical
9	Sodium dihydrogen phosphate	Analytical
10	Disodium hydrogen phosphate	Analytical
11	Ortho phosphoric acid (OPA)	Analytical
12	Ebastine BP procured from Kivi Labs, Vadodara	Reference standard

7.1.2 Instruments and Apparatus

TABLE 7.2 List of instrument and apparatus used in HPLC method for Ebastine

Sr. No.	Instrument/Apparatus	Manufacturer
1	Analytical weighing balance	Shimadzu
2	FTIR instrument	Bruker
3	Glass wares	Borosil
4	pH meter	Elico
5	HPLC	Shimadzu LC 2010 CHT with LC solution software

7.1.3 Identification of API

- **Melting point determination**

Melting point was determined by open capillary method.

- **Infra Red Spectra:**

Ebastine- KBr pellet was prepared and an IR spectrum was produced by FT-IR instrument. IR spectrum of sample was compared to reference IR spectrum[1].

7.1.4 Determination of Physicochemical Properties

- **Solubility test:**

Solubility of Ebastine in distilled water, methanol and acetonitrile was checked by taking 10 mg of drug in 100 mL flask and adding the solvent till the drug dissolved.

- **Wavelength maxima:**

From PDA data, a wavelength maximum of drug was determined.

7.1.5 Preparation of solutions

- **Preparation of standard stock solution**

Ebastine standard stock solution was made by taking accurately 100 mg of Ebastine in 100mL volumetric flask, adding 50 mL of diluent and was sonicated for 10 min. and making up to 100 mL with acetonitrile. Standard stock solution (1 mg/mL) was diluted as relevant with the acetonitrile to obtain the working concentration range. It was found that stock solution was stable for minimum 5 days when placed at room temperature.

- **Test solution preparation**

Accurately weighed 20 tablets (Ebast 10, label claim 10 mg) were taken and powdered, and powder equivalent to 20 mg Ebastine was mixed with acetonitrile (10 mL) and for 20 min. it was sonicated. Then it was filtered through Whatman no. 41 filter paper and the residue was thoroughly washed with acetonitrile. The filtrate and washings were combined in a 20 mL volumetric flask and dilution up to volume was made with acetonitrile. One mL of this solution was transferred to a 10 mL volumetric flask and made the volume with acetonitrile (100 µg/mL).

7.1.6 Selection of chromatographic condition

To develop stability indicating HPLC method for determination of Ebastine, Ebastine is highly basic drug as its pKa is 16.45 so highly non polar C_{18} column was selected and highly polar mobile phase were tried and system suitability test was performed for method optimization.

TABLE 7.3 selection of chromatographic condition

Sr. No.	Stationary Phase	Mobile Phase	Problem
1	Xterra C_{18} (150 x 4.6 mm, 5µm)	ACN: 0.1% OPA 60:40 v/v, 1.2 mL/min.	Tailing factor >2 Theoretical plates<1000
2	Xterra C_{18} (150 x 4.6 mm, 5µm)	Methanol: water 90:10 v/v, 1.0 mL/min.	Too broad peak Not good shape
3	Xterra C_{18} (150 x 4.6 mm, 5µm)	ACN: 0.1% OPA 70:30 v/v, 1.0 mL/min.	Theoretical plates<1000
4	Xterra C_{18} (150 x 4.6 mm, 5µm)	ACN: 0.1% OPA 75:25 v/v, 1.0 mL/min.	Theoretical plates<1000 Splitting of peak
5	Phenomenex C_{18} (250 x 4.6 mm, 5µm)	ACN: 0.1% OPA with 0.05% TEA 60:40 v/v, 1.0 mL/min	Too broad peak Not good shape
6	Phenomenex C_{18} (250 x 4.6 mm, 5µm)	ACN: 0.1%OPA with 0.05% TEA 70:30 v/v, 1.0 mL/min.	Fronting in peak and broad peak
7	Phenomenex C_{18} (250 x 4.6 mm, 5µm)	ACN:0.05% TFA with TEA (pH 3.0) 70:30 V/V, flow rate – 1 mL/min.	Resolution – 0.203 Peak purity – 0.973166
8	Phenomenex C_{18} (250 x 4.6 mm, 5µm)	ACN: 0.02% TFA 70:30 v/v, 1.0 mL/min.	Tailing factor – 1.540 Resolution – 0.653
9	Phenomenex C_{18} (250 x 4.6 mm, 5µm)	ACN: phosphate buffer pH 6.8 60:40 v/v, 1.0 mL/min.	Base line was not stable
10	Phenomenex C_{18} (250 x 4.6 mm, 5µm)	ACN: 0.5% OPA 68:32 v/v, 1.0 mL/min.	Tailing factor-1.305 Theoretical plates- 3335 Peak purity- 0.99997

7.1.7 Validation of the method

The developed method was validated for system suitability, specificity, linearity, precision, trueness, limit of detection, limit of quantification and robustness as per ICH guideline to ensure its appropriateness for the predetermined purpose.

- **System suitability test**

To ascertain suitability of selected HPLC testing system for the predetermined application, the system suitability test was checked. Seven replicate analyses of 100 µg/mL Ebastine solution were chromatographed as per optimized condition and theoretical plates and tailing factor were evaluated.

- **Linearity**

Accurately measured volumes of the Ebastine standard solution (1000 µg/mL) were successively transferred to a series of 10 mL volumetric flasks. They were diluted to the volume with acetonitrile to obtain final concentrations of 5-120 µg/mL and mixed properly. 20 µL aliquots of each solution were injected three times and analysis was executed by optimized method. The regression equation was derived from the plot of average area of Ebastine peak against the concentration of Ebastine.

- **Precision**

- **Repeatability**

To assess repeatability of the method, chromatographic analysis of six injections of 100 µg/mL Ebastine solutions was performed. The average, standard deviation and % RSD for retention time and area of Ebastine peak were estimated.

- **Intraday precision:**

Three concentrations (20, 40 and 60 µg/mL) were chromatographed for three times on same day by same analyst and area of Ebastine peak was reported to check the intraday precision. The %RSD of area of Ebastine peak was estimated.

- **Interday precision:**

Three concentrations (20, 40 and 60 µg/mL) were chromatographed for three different days by same analyst and area of Ebastine peak was reported to check the interday precision. The %RSD of area of Ebastine peak was calculated.

- **Accuracy**

20 tablets (brand name - Ebast 10) were crushed and powder equivalent to 20 mg Ebastine was transferred in 3 separate 100 mL volumetric flasks. 10 mg, 20 mg and 30 mg Ebastine standard was added to volumetric flasks containing tablet powder and 50 mL acetonitrile was added to each flask and they were sonicated for 40 min. with occasional shaking. The solutions were filtered through Whatman no. 41 filter paper and the residue was washed thoroughly with acetonitrile. The filtrate and washings were combined in respective 100 mL volumetric flask and dilution to the volume done with acetonitrile. Further they were diluted to have 40 µg/mL of sample in each flask. The experiment was executed triplicate. % recovery was calculated for each level.

- **Specificity**

The forced degradation studies were accomplished to determine whether the developed HPLC method was stability indicating and could uniquely determine the Ebastine though impurities and degradation products are present. Ebastine standard solution was stressed in acid, base, neutral hydrolytic, oxidative, thermal and photolytic conditions to achieve partial decomposition of the Ebastine.

- Acid induced degradation

2 mL of standard solution of Ebastine (1 mg/mL) was transferred to 20 mL volumetric flask and 2 mL of 0.1 N HCl was added to it. For 45 min. the mixture was refluxed in a thermostatic water bath at 80°C. Then it was cooled and dilution to 20 mL was done with acetonitrile. The resulted solution was chromatographed as per optimized conditions.

- Base induced degradation

2 mL of Ebastine (1 mg/mL) in methanol was transferred to 20 mL volumetric flask and 2 mL of 1.0 N NaOH was added to it. For 3 hr the mixture was heated in a thermostatic water bath at 80°C. Then it was cooled and dilution up to 20 mL was done with acetonitrile. The resulted solution was chromatographed as per optimized conditions.

- Wet heat degradation

2 mL of standard solution of Ebastine (1 mg/mL) was transferred to 20 mL volumetric flask followed by 2 mL distilled water. For 3 hr it was refluxed in a thermostatic water bath at 70°C. Then it was cooled and dilution up to 20 mL was done with acetonitrile. Then chromatography of the resulted solution was performed as per optimized conditions.

- Oxidative degradation

2 mL of the Ebastine standard solution (1 mg/mL) was transferred to 20 mL volumetric flask followed by 2 mL 1% H_2O_2. For 20 min. the mixture was refluxed in a thermostatic water bath at 70°C. Then it was cooled and dilution up to 20 mL was done with acetonitrile. Then chromatography of the resulted solution was performed as per optimized conditions.

- Thermal degradation

50 mg Ebastine powder in petridish was kept at 70°C in convection oven and exposed for 8 hr and further processed as per sample preparation. Then the resulted solution was chromatographed as per optimized conditions.

- Photolytic degradation

Ebastine drug (50 mg) was transferred to two petridish. One petridish was kept in UV chamber (365 nm) for 24 hr at room temperature and further processed as per sample preparation. Second petridish was kept in direct sunlight for 24 hr on wooden plank erected in terrace and further processed as per sample preparation. Then the resulted solutions were chromatographed as per optimized conditions.

- **Robustness**

The experimental conditions of the method were deliberately changed from optimized condition to determine the robustness. The mobile phase flow rate (1.0±0.1 mL/min), temperature of column oven (40±1°C) and acetonitrile composition (68±2 %) were varied. In each case, and %RSD for area of Ebastine peak and retention time were calculated. Comparison of the retention time and area of Ebastine peak were done with that obtained under the optimized method, also.

- **Limit of Detection and Limit of Quantitation**

Standard deviation of y intercept of calibration curve of linear lower concentrations (N) and slope (S) of the calibration curve were calculated and using their values LOD and LOQ were estimated as per ICH guideline.

7.1.8 Application of the developed method

Ebastine tablets (Ebast 10, label claim 10 mg) was exposed to 40°C 75% for 1 month in stability chamber. To determine the Ebastine content of a tablet after accelerated stress procedure of the test solution preparation was followed. And then the resulted solution was chromatographed as per optimized condition to check the effect of accelerated testing of drug product.

7.2 Results and Discussion

7.2.1 Identification of API

Melting point of the Ebastine was observed in the range of 81-82°C which is almost similar to the reported value (80-82 °C) for Ebastine.[2]

An IR spectrum of Ebastine API is given in Fig. 7.1 and a reference spectrum[2] is given in Fig. 7.2.

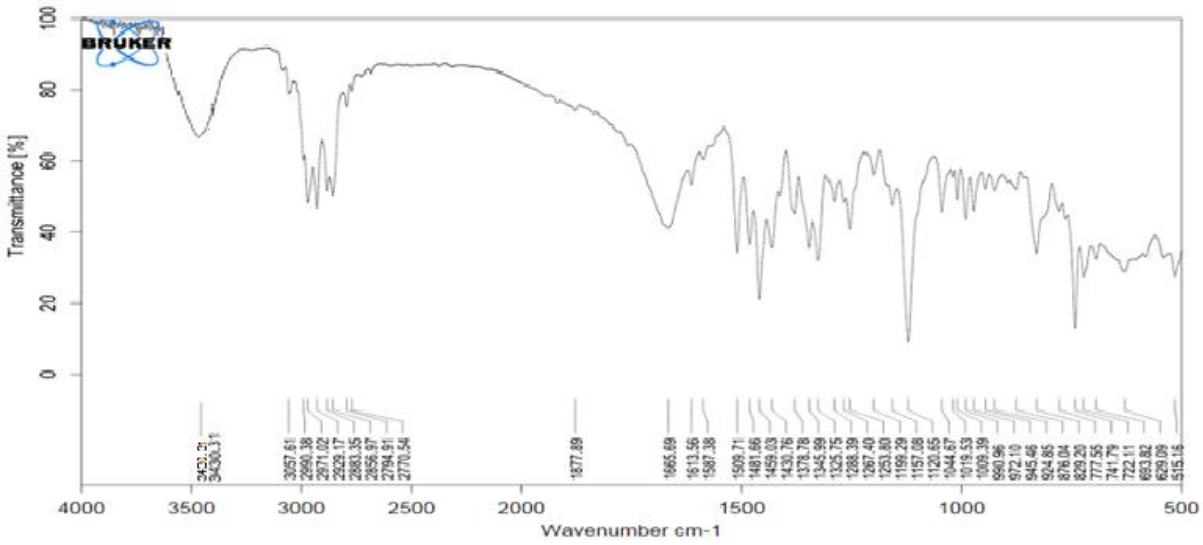

FIGURE 7.1 IR Spectra of Ebastine API

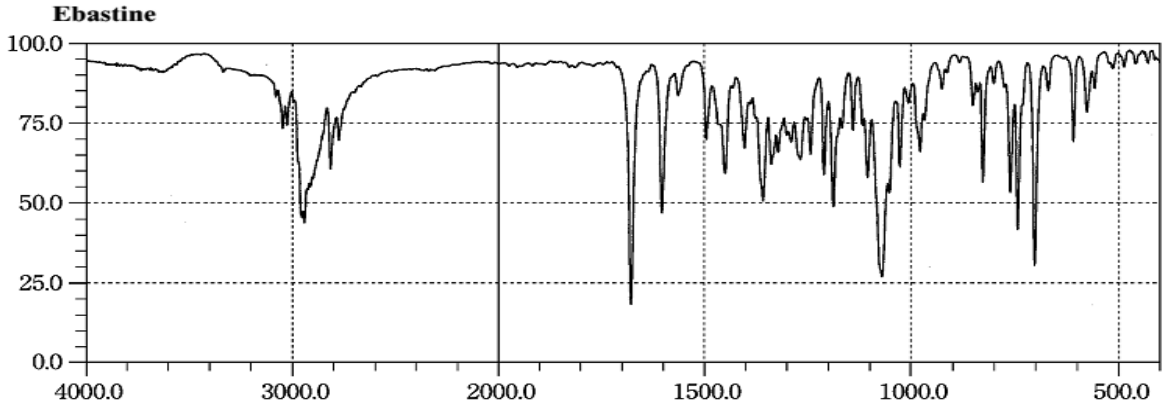

FIGURE 7.2 IR Spectra of Ebastine Reference

As sample spectrum completely matches with the reference spectrum and practical value of melting point also matches with theoretical value, it confirms the purity of Ebastine sample.

7.2.2 Determination of physicochemical properties

In Table 7.4, the results of solubility test are presented.

TABLE 7.4 Solubility results for Ebastine

Solvent	mL of solvent required to dissolve 10 mg Ebastine	Solubility
Methanol	0.3 mL (30 mL for 1 gm)	Soluble
Water	> 100 mL (>10000 mL for 1 gm)	Insoluble
Acetonitrile	0.9 mL (90 mL for 1 gm)	Sparingly soluble

From PDA data, 254 nm was found λ_{max} of Ebastine and it was selected as wavelength for study.

7.2.3 Development of method

HPLC method using Phenomenex C_{18} (250 mm x 4.6 mm, 5µm particle size) column and isocratic solvent system comprising acetonitrile: 0.5% OPA (68:32 v/v) with 1.0 mL/min. flow rate was found to provide sharp and well defined peak with very good symmetry(1.32), theoretical plates(>3000) and low retention time (4.253 min.). Hence it was selected throughout study of Ebastine by HPLC.

- **Optimized chromatographic condition:**

Column: Phenomenex C_{18} (250 mm x 4.6 mm, 5µm particle size)

Mobile phase: Acetonitrile: 0.5% OPA (68:32 v/v)

Flow rate: 1.0 mL/min

Volume of injection: 20 µL

Temperature of column: 40˚C

Run Time: 10 min

Diluent: Acetonitrile

Detector: PDA

Retention Time of Ebastine: 4.253 min.

RP-HPLC Chromatogram of Ebastine produced by optimized method is displayed in Fig. 7.3.

Purity plot of Ebastine peak is displayed in Fig.7.4.

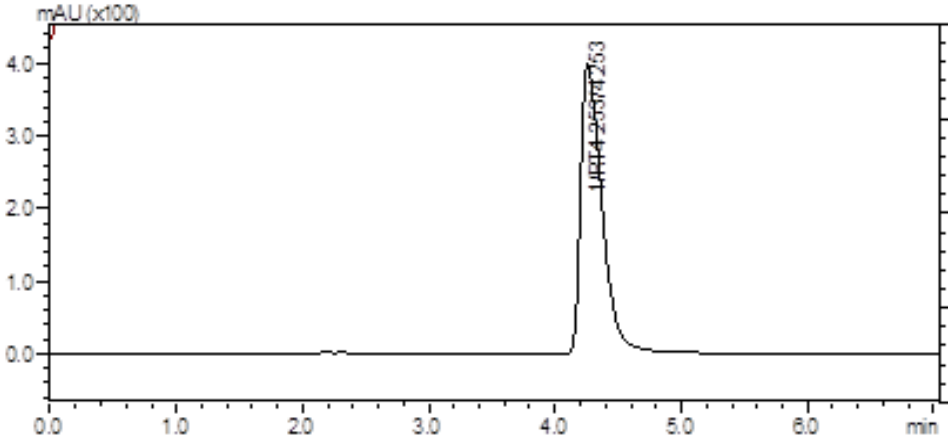

FIGURE 7.3 RP-HPLC Chromatogram of Ebastine

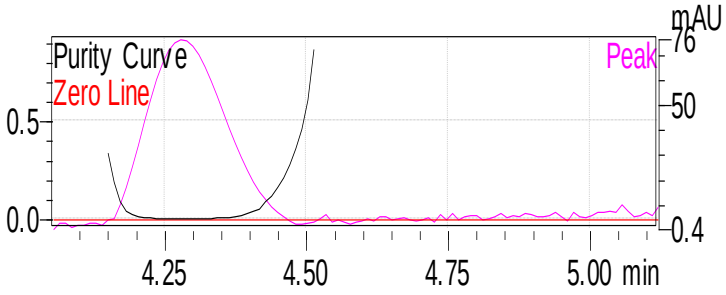

FIGURE 7.4 Purity plot of Ebastine peak

7.2.4 Method validation

To demonstrate appropriateness of method for the predetermined purpose, validation of the developed method was done as per ICH guideline.

- **System suitability test**

Table 7.5 presents the results of system suitability. As results of system suitability test are satisfactory it was ensured that HPLC testing system was adequate for routine analysis of Ebastine.

TABLE 7.5 system suitability data for Ebastine

Sr. No.	Conc.	Theoretical plates	Tailing factor
1	100 µg/mL	2980.2	1.213
2		3000.0	1.198
3		2888.7	1.211
4		2908.6	1.134
5		3138.6	1.257
6		3105.7	1.169
7		3235.6	1.172
Acceptance criteria		>2000	<2.0

- **Linearity**

Area of Ebastine peak and concentration were submitted to linear regression analysis to obtain the regresssion equation and correlation coefficient. Table 7.6 presents the data of linearity. The calibration graph was linear over the concentration range 5-120 µg/mL of Ebastine (r^2 ± SD = 0.9996 ± 0.0001). The calibration results revealed a good linear relationship of peak area of Ebastine over the concentration range of 25-150 µg/mL. Overlaid linearity chromatogram of Ebastine is displayed in Fig.7.5. Calibration curve for Ebastine is displayed in Fig. 7.6.

TABLE 7.6 Linearity data for Ebastine

Sr. No.	Stock solution taken in mL	Diluted to volume in mL	Concentration in µg/mL	Mean Area (n=3)
1	0.05	10	5	245970.0
2	0.1	10	10	500934.3
3	0.2	10	20	961812.7
4	0.3	10	30	1489953.0
5	0.4	10	40	1917200.0
6	0.5	10	50	2360659.0
7	0.6	10	60	2830005.0
8	0.7	10	70	3245293.0
9	0.8	10	80	3752450.0
10	0.9	10	90	4276039.0
11	1.0	10	100	4807239.0
12	1.2	10	120	5731412.0

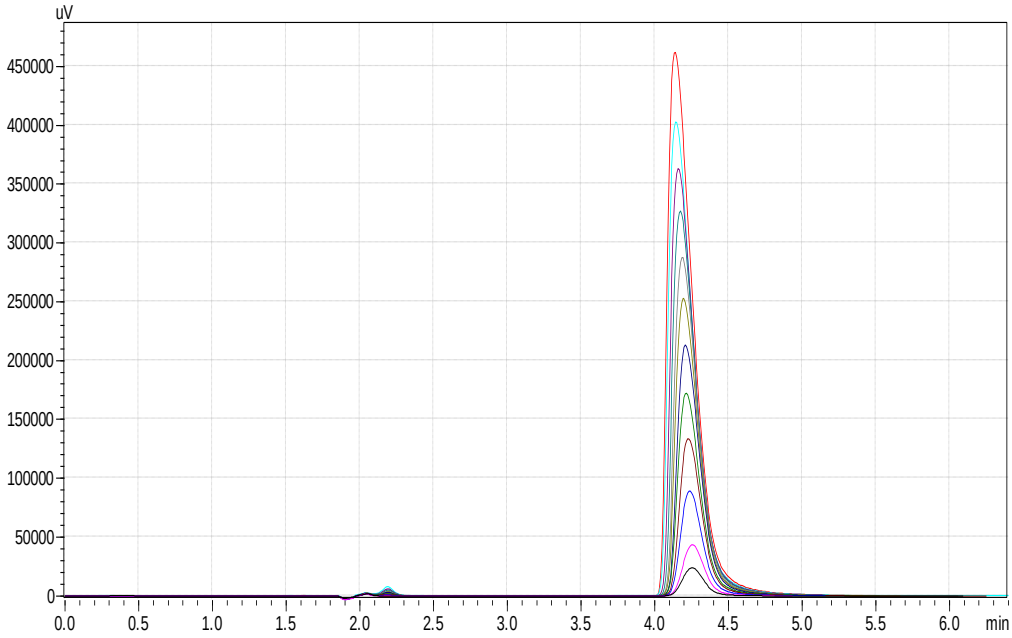

FIGURE 7.5 Overlaid linearity chromatogram of Ebastine

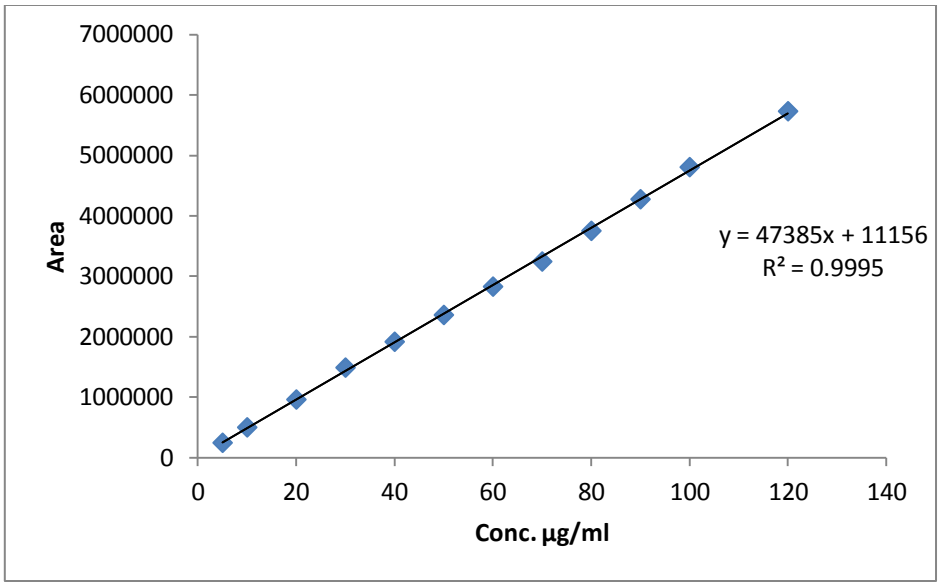

FIGURE 7.6 Calibration curve of Ebastine for RP-HPLC

- **Precision**

- **Repeatability**

Table 7.7 presents results of repeatability. % RSD of retention time and area of Ebastine peak determination was found less than 2% that proves the repeatability of the developed HPLC method.

TABLE 7.7 Repeatability data of RP-HPLC for Ebastine

Sr. No.	Retention time (Min.)	Peak area
1	4.261	4766840
2	4.274	4775358
3	4.277	4792576
4	4.330	4743848
5	4.376	4805719
6	4.292	4914856
7	4.287	4951082
Mean	4.299571	4821468
Std. dev.	0.04007	79308.58
%RSD	0.932	1.645

- **Intraday and interday precision**

Table 7.8 shows the intraday precision results. % RSD of peak area of Ebastine was found less than 1% for intraday precision. Table 7.9 shows the results of interday precision. % RSD of peak area of Ebastine was found less than 2% for interday precision. The values of %RSD were less than 2% which proved the high precision of the proposed method.

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TABLE 7.8 Intraday precision data of RP-HPLC for Ebastine

Conc. µg/mL	I	II	III	Avg.	Std. dev.	%RSD
20	917015	935146	926299	926153.3	9066.378	**0.9789**
40	1842731	1874533	1850938	1856067.0	16509.83	**0.8895**
60	2766821	2790915	2799899	2785878.0	17104.52	**0.6140**

TABLE 7.9 Interday precision data of RP-HPLC for Ebastine

Conc. µg/mL	I	II	III	Avg.	Std. dev.	%RSD
20	906365	924793	926299	919152.3	11099.73	**1.2076**
40	1808952	1847824	1850938	1835905	23393.57	**1.2742**
60	2698885	2776758	2799899	2758514	52920.6	**1.9184**

- **Accuracy**

TABLE 7.10 Accuracy data of RP-HPLC for Ebastine

Level of recovery	Conc. (added std. sol.) µg/mL	Conc. (sample sol.) µg/mL	Recovered conc.	% recovery	Mean % recovery ± S.D.
50%	20.00	40.00	20.39	101.95	100.83±1.35
			19.864	99.32	
			20.245	101.23	
100%	40.00	40.00	40.704	101.76	101.35±0.65
			40.238	100.60	
			40.676	101.69	
150%	60.00	40.00	60.891	101.49	101.28±0.57
			60.378	100.63	
			61.031	101.72	

When the method was used for analysis of Ebastine from previously analysed tablet powder after spiking of 50, 100 and 150% Ebastine standard, the recovery was found 99-100%. Table 7.10 presents the results of accuracy determination.

➢ **Specificity**

- **Acid induced degradation study**

As shown in Fig. 7.7, chromatogram of acid stressed Ebastine sample yielding the degradation product at 2.317 min. and Ebastine at 4.362 min. with good resolution. Purity curve of Ebastine peak after acid degradation is displayed in Fig. 7.8. Peak purity of the Ebastine peak is greater than 0.999. This study proves that the developed method determines the Ebastine specifically in presence of acid induced degradation product.

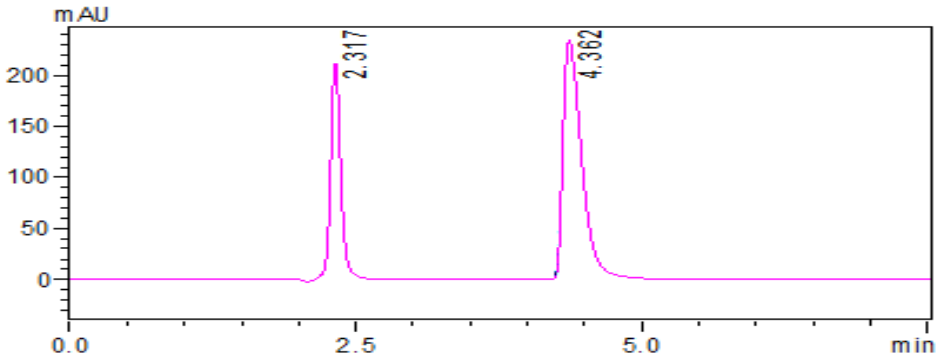

FIGURE 7.7 Chromatogram of Ebastine after degradation with 2 N HCl

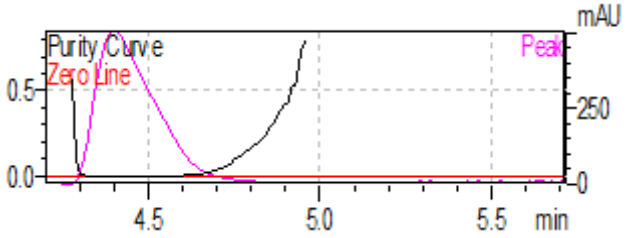

FIGURE 7.8 Purity curve of Ebastine peak after acid degradation

- **Base induced degradation study**

Peak area of Ebastine was not changed significantly and any additional peak was not observed when chromatographed after refluxing in 1N NaOH at 80°C for 3 hr. Peak purity of the Ebastine after base induced degradation is greater than 0.999, which shows the purity of Ebastine peak. Fig. 7.9 presents chromatogram of Ebastine after degradation with 1N NaOH at 80° C for 3 hr.

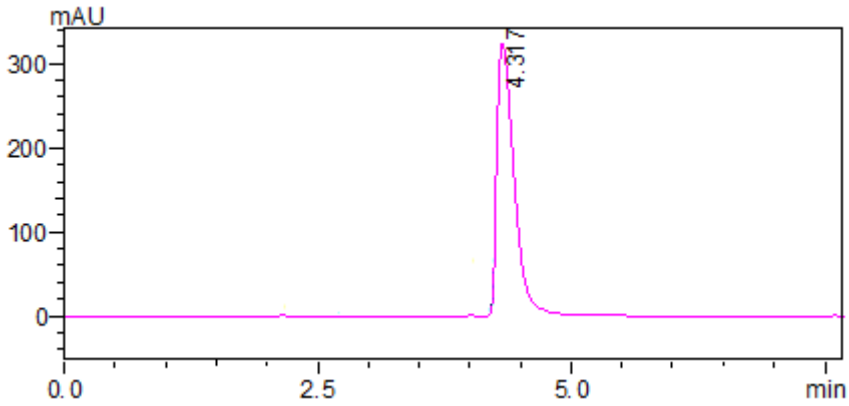

FIGURE 7.9 Chromatogram of Ebastine after degradation with 1 N NaOH

- **Wet heat degradation**

Peak area of Ebastine was not changed significantly and any additional peak was not observed when chromatographed after refluxing in distilled water at 70°C for 3 hr. Peak purity of Ebastine peak after wet heat degradation is greater than 0.999, which shows the purity of Ebastine peak. Chromatogram of Ebastine after degradation with water at 70° C for 3 hr is displayed in Fig. 7.10.

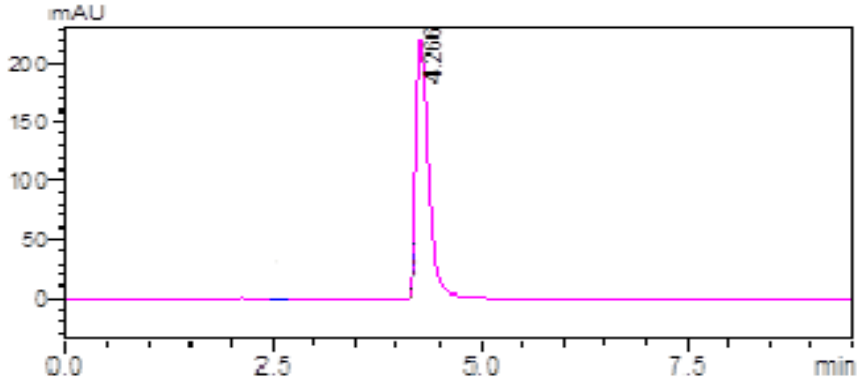

FIGURE 7.10 Chromatogram of Ebastine after degradation with water at 70° C for 3 hr

- **Oxidative degradation study**

Under oxidative condition, Ebastine degraded yielding the degradation product at 3.998 minute with resolution greater than 1.5 (Rs=1.56). Peak purity of Ebastine peak is greater than 0.999 which indicates the peak purity of Ebastine after oxidative degradation. Chromatogram of oxidative degradation study is given in Fig. 7.11 and purity curve of Ebastine peak after oxidative degradation is displayed in Fig. 7.12. Hydrogen peroxide retention time is 2.327 min. which was confirmed from chromatogram of 1% hydrogen peroxide.

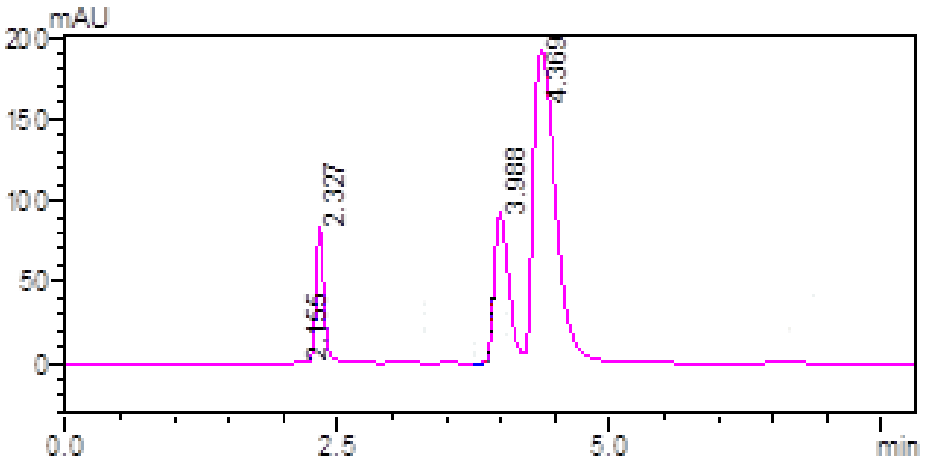

FIGURE 7.11 Chromatogram of Ebastine after degradation with 10% hydrogen peroxide at 80° C for 30 min.

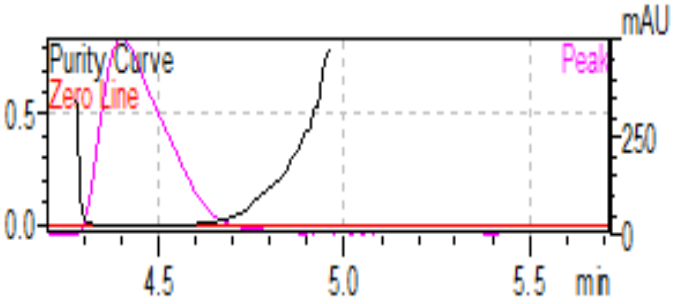

FIGURE 7.12 Peak purity curve of Ebastine after oxidative degradation

- **Thermal degradation**

Peak area of Ebastine was not changed significantly and any additional peak was not observed when chromatographed after exposing at 70°C for 8 hr. Peak purity of Ebastine peak is greater than 0.999. Chromatogram of Ebastine after thermal degradation is displayed in Fig. 7.13.

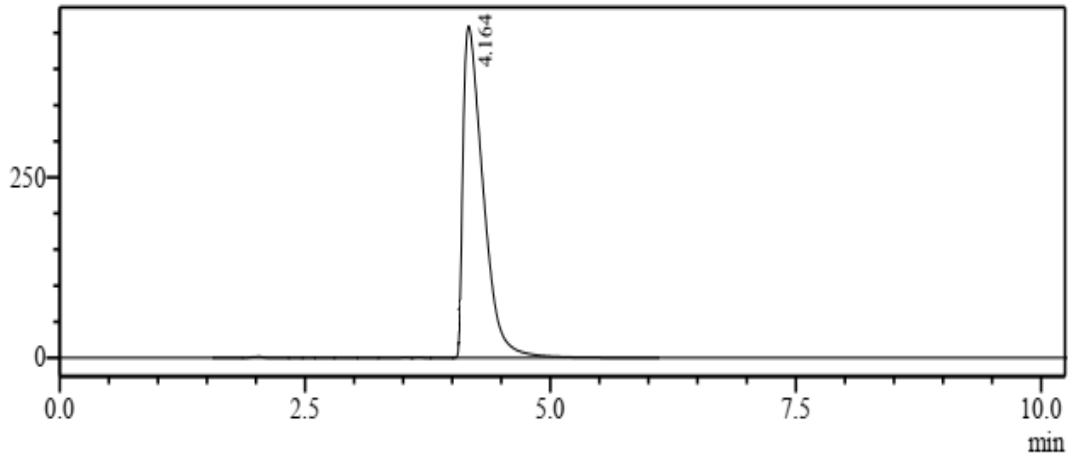

FIGURE 7.13 Chromatogram of Ebastine after thermal degradation

- **Photolytic degradation**

Peak area of Ebastine was not changed significantly and any additional peak was not observed when chromatographed after exposing at 365 nm for 24 hr. Peak purity of Ebastine peak is greater than 0.999. Chromatogram of Ebastine after photolytic degradationin under controlled condition is displayed in Fig. 7.14. Colour of the drug was changed to slight yellow and peak area of Ebastine was decreased about 5.7% and additional peaks were observed when chromatographed after exposing to direct sunlight for 24 hr. Peak purity of Ebastine peak is greater than 0.999. Fig. 7.15 shows chromatogram of Ebastine after exposure to direct sunlight.

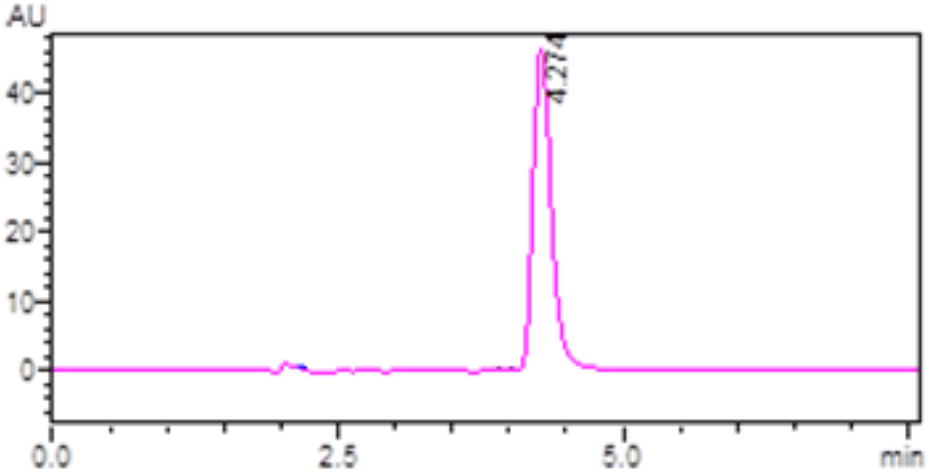

FIGURE 7.14 Chromatogram of Ebastine after photolytic degradation.

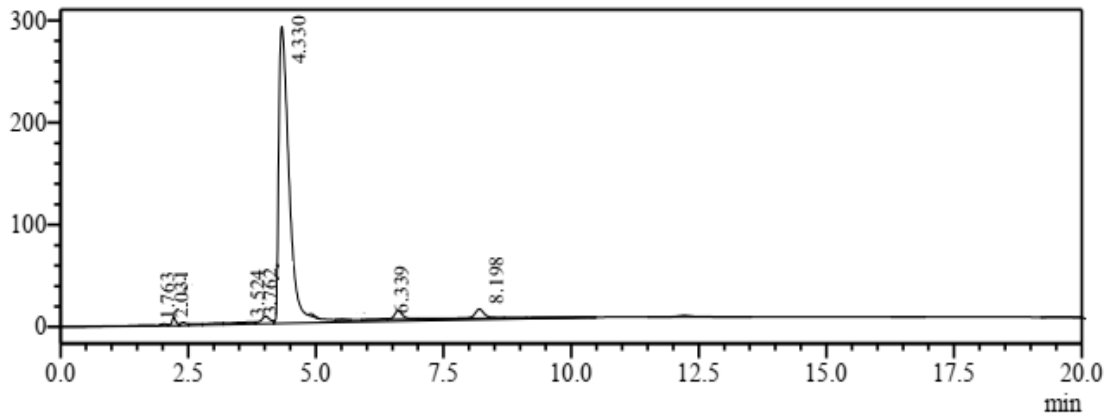

FIGURE 7.15 Chromatogram of Ebastine after exposure to direct sunlight.

The results of HPLC method for stress samples, including separation of the degradation products produced from acid degradation of Ebastine and degradation of Ebastine in oxidizing condition and quantification of Ebastine though degradation products are present shows the proposed analytical method is stability-indicating.

- **Robustness**

It was found that method was robust regarding any minor deviation in the column temperature (40 ± 1°c) as confirmed by the constant value of the retention time and the area of Ebastine peak. Small deviation in mobile phase flow rate (1.0 ± 0.1 mL/min.) resulted in a significant deviation in the retention time and area of the Ebastine peak and; in case of ratio of acetonitrile, small change resulted in considerable deviation in the retention of the Ebastine and symmetry of the peak. Table 7.11 shows the results of the robustness.

TABLE 7.11 Robustness data of RP-HPLC for Ebastine.

Parameter	Value	Retention time	% RSD	Area	% RSD
Flow rate (mL/min.)	0.9	4.895	11.093	5525211	10.983
	1.0	4.288		4696922	
	1.1	3.936		4512356	
Temperature (column)	39°C	4.296	0.223	4649354	0.139
	40°C	4.288		4645183	
	41°C	4.277		4636724	
Mobile phase composition (ACN:OPA)	70:30 v/v	4.05	8.526	4765591	1.287
	68:32 v/v	4.288		4645183	
	66:34 v/v	4.781		4695003	

- **Limit of Detection and Limit of Quantitation**

LOD and LOQ were estimated as per ICH guideline using mathematical formula. Obtained value for LOD was 0.3009µg/mL and obtained value for LOQ was 0.9119µg/mL. Small value of LOD and LOQ expressed that the developed method can be used for detection and quantification of Ebastine in QC samples.

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

= 3.3*(4320.801/47385)

= 0.3009 µg/mL

LOQ = 10 *(standard deviation of y-intercept/slope)

= 10* (4320.801/47385)

= 0.9119 µg/mL

7.2.5 Application of method

The proposed validated isocratic HPLC method was successfully applied to analyze Ebastine quantitatively in tablet dosage form after exposure to 40°C 75% for 1 month in stability chamber. The assay of drug product was 99.37% while the assay of drug product after accelerated stress was around 98.79%. In chromatograms of drug sample from nasal spray preparation, no interference was observed from excipients.

7.3 References

1. https://www.pmda.go.jp/files/000203134.pdf, Infrared Reference Spectra, JP XVI, 1844
2. https://www.chemicalbook.com/ChemicalProductProperty_US_CB8271800.aspx

CHAPTER 8

Degradation Kinetic Study of Ebastine

8.1 Materials and Methods

8.1.1 Reagents and Materials

TABLE 8.1 List of reagents and materials used in HPLC method for Ebastine

Sr. No.	Reagent/material	Grade
1	Water	HPLC
2	Hydrochloric acid (HCl)	Analytical
3	Hydrogen Peroxide	Analytical
4	Acetonitrile	HPLC
5	Ortho phosphoric acid (OPA)	Analytical
6	Ebastine BP procured from Kivi Labs, Vadodara	Reference standard

8.1.2 Instruments and Apparatus

TABLE 8.2 List of instrument and apparatus used in HPLC method for Ebastine

Sr. No.	Instrument/Apparatus	Manufacturer
1	Analytical weighing balance	Shimadzu
2	Glass wares	Borosil
3	HPLC	Shimadzu LC 2010 CHT with LC solution software

8.1.3 Preparation of solutions

- **Preparation of standard solution (1 mg/mL)**

Ebastine standard stock solution was made by taking accurately 100 mg of Ebastine in 100mL volumetric flask, adding 50 mL of diluent and was sonicated for 10 min. and making up to 100 mL with acetonitrile.

8.1.4 HPLC method

LC-2010 C_{HT} by Shimadzu furnished with quaternary solvent manager, auto sampler, and PDA detector controlled by LC solution software was used for quantitative analysis of Ebastine. Phenomenex C_{18} (250 x 4.6 mm, 5µm) column using solvent system comprising of Acetonitrile: 0.5% ortho phosphoric acid (68:32 %v/v) with was 1.0 mL/min. flow rate was employed for separation. The temperature of column was 40°C, and the volume of injection was 20 µL. 254 nm (λ_{max} of ebastine) was used for characterization and the

8.1.5 Degradation kinetic study of Ebastine in acid

To study order of reaction and degradation rate of Ebastine in acidic media, 2 mL of 1 mg/mL Ebastine standard solution was transferred to 20 mL volumetric flasks. To each volumetric flask 2 ml of 0.1 N HCl was added and the mixture was heated under reflux at 50, 60, 70, or 80 °C. Five volumetric flasks were placed at each temperature. After specific time interval volumetric flask was taken out from water bath, cooled it and dilution up to the volume was done with methanol. They were analyzed by the developed and validated HPLC method. The concentration of the undegraded Ebastine was estimated for each time period by putting the value of peak area in regression equation and the data were further treated to find order of reaction and degradation rate constant. Data got from order kinetics treatment were submitted to fitting to the Arrhenius equation and activation energy for the reaction was calculated. By processing the data further degradation rate, half time and t_{10} of the drug in 0.1 N HCl at 25 °C were obtained.

8.1.6 Degradation kinetic study in oxidative condition

To study order of reaction and rate of degradation of Ebastine in oxidative media, 2 mL of Ebastine standard stock solution (1 mg/mL) was taken in 20 mL volumetric flasks. To each volumetric flask 2 ml of 1% H_2O_2 was added and the mixture was refluxed at 50, 60, 70, or 80°C in thermostatic water bath. Five volumetric flasks were placed at each temperature. After specific time interval volumetric flask was taken out from water bath, cooled it and diluted to the volume with acetonitrile. These stressed samples were analyzed by the new validated HPLC method. The concentration of undegraded drug was estimated for each time period and

the data were further treated to find order of reaction and degradation rate constant. Data received from order kinetics treatment were submitted to fit the Arrhenius equation and activation energy was determined. By processing the data further degradation rate, half time and t_{10} of the drug in 2 N HCl at 25 °C were obtained.

8.2 Results and Discussion

8.2.1 Degradation kinetic study of Ebastine in acid

The concentration of the Ebastine in acidic condition was found to decrease with time. Overlay of chromatograms of Ebastine in acidic medium at different time interval at 70 °C is shown in Fig.8.1. The results of the percentage degradation of Ebastine at 50, 60, 70, or 80 °C at specific time interval are presented in Table 8.3 to Table 8.6, respectively.

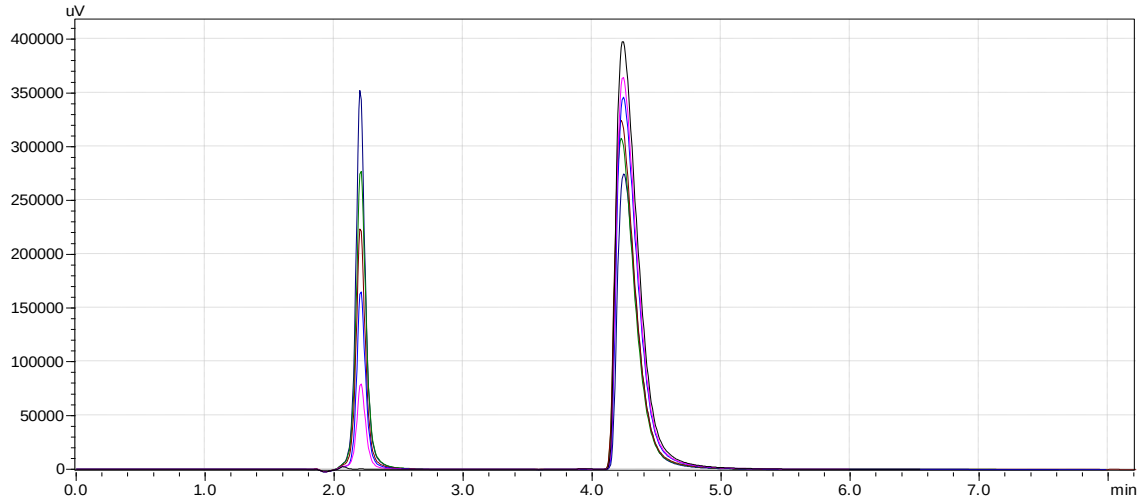

FIGURE 8.1 Overlay of chromatograms of Ebastine in acid at diiferent time at 70° C

TABLE 8.3 Degradation of Ebastine in 0.1 N HCl at 50°C

Time (min.)	Peak Area of Ebastine	%drug	% degradation
0	4478500	100	0
60	4229463	94.43928	5.560723
120	4027337	89.92602	10.07398
180	3876452	86.55693	13.44307
240	3776794	84.33167	15.66833

TABLE 8.4 Degradation of Ebastine in 0.1 N HCl at 60°C

Time (min.)	Peak Area of Ebastine	%drug	% degradation
0	4038021	100	0
30	3915770	96.9725	3.027498
60	3717460	92.06143	7.938567
90	3454877	85.55867	14.44133
120	3258544	80.69656	19.30344
180	3060485	75.79171	24.20829

TABLE 8.5 Degradation of Ebastine 0.1 N HCl at 70°C

Time (min.)	Peak Area of Ebastine	%drug	% degradation
0	4715636	100	0
30	4316017	91.52566	8.474339
60	3935644	83.45945	16.54055
90	3603297	76.41169	23.58831
120	3372084	71.50857	28.49143

TABLE 8.6 Degradation of Ebastine in 0.1 N HCl at 80°C

Time (min.)	Peak Area of Ebastine	%drug	% degradation
0	3855518	100	0
15	3552155	92.13172	7.868281
30	3094768	80.26854	19.73146
45	2789027	72.33858	27.66142
60	2312879	59.9888	40.0112

When drug concentration is plotted against time, if a straight line results then the reaction is zero order. If plot of log concentration of drug against time is linear then it is first order reaction. If plot of inverse concentration of drug against time is linear then it is second order. The regression co-efficient at 50, 60, 70, or 80 °C was calculated for zero order, first order and second order from graphical method and they are presented in Table 8.7. From regression co-efficient, acidic degradation of Ebastine was found most fit to the first order degradation. The effect of temperature (50, 60, 70 and 80° C) on the degradation of Ebastine in acidic condition is shown in Fig. 8.2. (Graph of log %undegraded drug vs time in min.)

TABLE 8.7 Order of reaction for Ebastine in 0.1 N HCl

Temperature	Zero order	First order	Second order
50	0.9744	0.9805	0.9855
60	0.9746	0.9886	0.9822
70	0.9904	0.9968	0.9988
80	0.9944	0.9821	0.9582

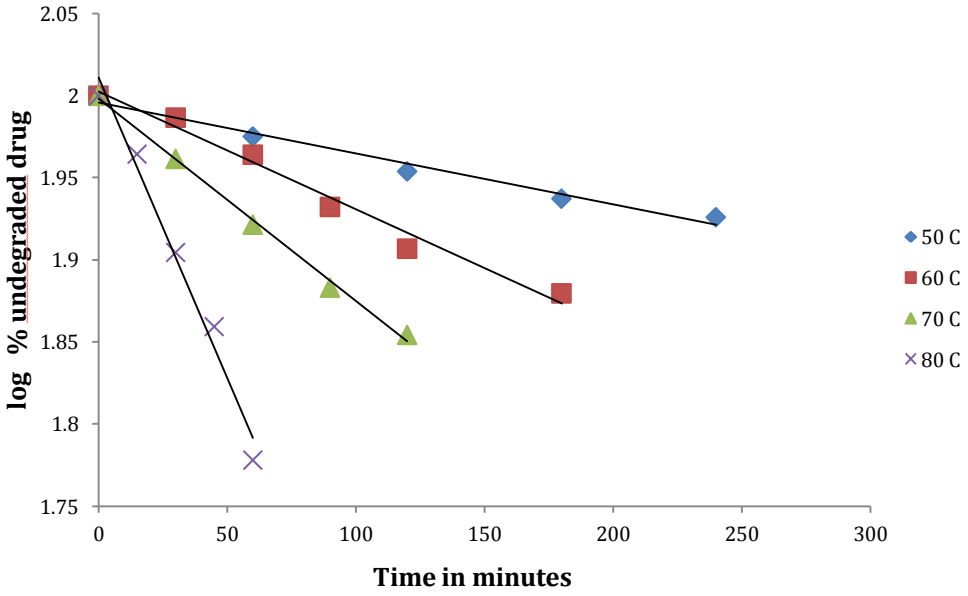

FIGURE 8.2 Temperature effect on degradation of Ebastine in 0.1 N HCl

The degradation under acidic condition follows first order kinetics. In accordance with equation (1) from the slopes of linear graphs of log (a/a-x) versus time t, the first order rate constants (K) were determined.

$Kt = 2.303 \log (a/a-x)$ ……..(1)

Here, "a" is the initial drug concentration and "a-x" is the undegraded Ebastine concentration.

$t_{1/2}$ and t_{10} for the first order degradation was determined in accordance with equation (2) and (3), respectively.

$t_{1/2} = 0.693/K$……(2)

$t_{10} = 0.104/K$……(3)

Results received from order kinetics were further submitted to fit the Arrhenius equation (equation 4)

log K = log A – Ea/2.303RT……(4)

Where K is the rate constant, A is the frequency factor, Ea is the activation energy (cal/ mol), R is the gas constant (1.987 cal /deg· mol), and T is absolute temperature. Fig. 8.3 presents Arrhenius plot, A plot of rate constant against 1000/T, gave straight line in the range of 50-80 °C for acidic degradation (Fig. 8.3). The energy of activation was calculated to be 18.272 Kcal/mol for reaction of Ebastine with acid. The degradation rate constant in 0.1 N HCl (acidic condition) at room temperature (K), was found when Arrhenius plot was extrapolated to 25 °C (where 1,000/T =3.356). K at room temperature was found 0.0000608 min^{-1}, and calculated $t_{1/2}$ and t_{10} were 11,403.67 min. (190.06 hr) and 1711.37 min.(28.52 hr), respectively. Degradation kinetic data for Ebastine in 0.1 N HCl is presented in Table 8.8

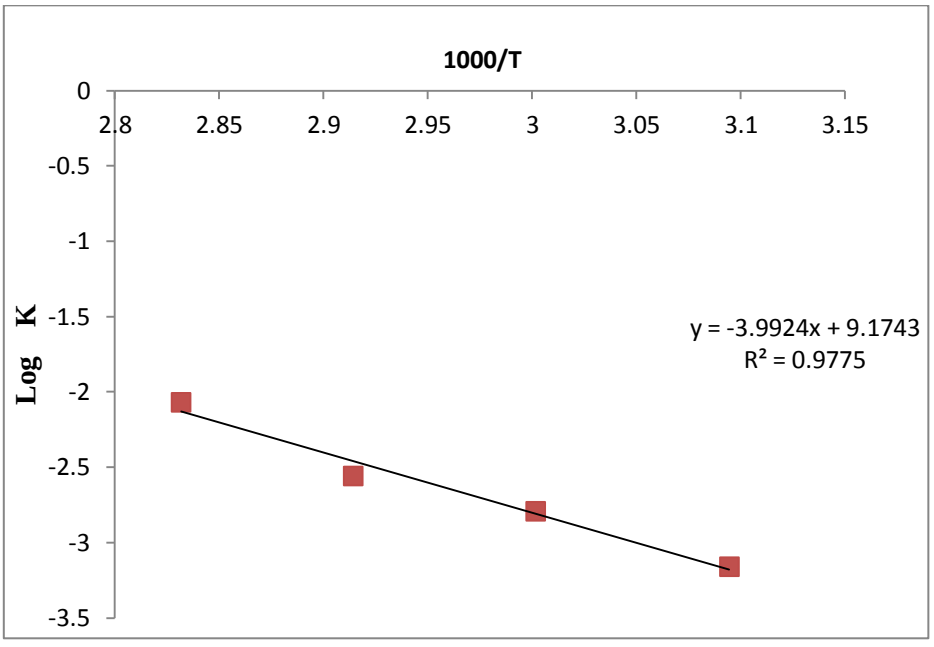

FIGURE 8.3 Arrhenius plot for Ebastine degradation in 0.1 N HCl

TABLE 8.8 Degradation kinetic data for Ebastine in the presence of 0.1 N HCl

Temperature (°C)	Degradation rate constant K (min^{-1})	Half time $t_{1/2}$ (min.)	t_{10} (min.)
50 °C (323.15 K)	0.00069	1003.04	150.53
60 °C (333.15 K)	0.00161	429.87	64.51
70 °C (343.15 K)	0.00276	250.75	37.63
80 °C (353.15 K)	0.00852	81.33	12.21
25 °C (calculated)	0.0000608	11,403.67	1711.37

8.2.1 Degradation kinetic study of Ebastine in oxidative condition

Concentration of the Ebastine in oxidative condition was found to decrease with time. Overlay of chromatograms of Ebastine in 1% H_2O_2 at different time interval at 70° C is shown in Fig.8.4. The results of the percentage degradation of Ebastine at 50, 60, 70, or 80 °C at specific time interval are presented in Table 8.9 to Table 8.12 respectively. Regression co-efficient at 50, 60, 70, or 80 °C was calculated for zero order, first order and second order from graphical method and they are presented in Table 8.13. From regression co-efficient, oxidative degradation of Ebastine was found most fit to the zero order degradation.

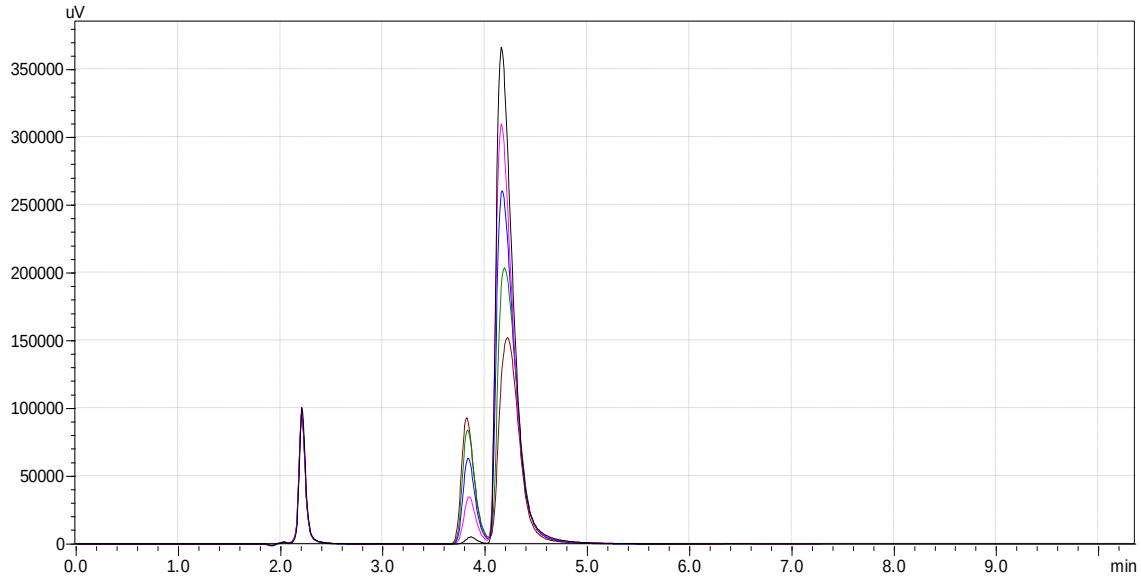

FIGURE 8.4 Overlay of chromatograms of Ebastine in 1% H_2O_2 at diiferent time at 70 °C

TABLE 8.9 Degradation of Ebastine in 1% H_2O_2 at 50°C

Time (min.)	Peak Area of Ebastine	%drug	% degradation
0	4118146	100	0
45	3567652	86.63248	13.36752
90	2918373	70.86619	29.13381
135	2290086	55.60964	44.39036
180	1723831	41.85939	58.14061

TABLE 8.10 Degradation of Ebastine in 1% H_2O_2 at 60°C

Time (min.)	Peak Area of Ebastine	%drug	% degradation
0	4449824	100	0
30	4126003	92.72284	7.277164
62	3630117	81.57889	18.42111
90	3240335	72.8194	27.1806
120	2780236	62.47968	37.52032
179	1884061	42.34012	57.65988

TABLE 8.11 Degradation of Ebastine in 1% H_2O_2 at 70°C

Time (min.)	Peak Area of Ebastine	%drug	% degradation
0	4161110	100.00	0
20	3668443	88.1602	11.8398
40	3225675	77.51958	22.48042
60	2683097	64.48032	35.51968
80	2031682	48.82548	51.17452

TABLE 8.12 Degradation of Ebastine in 1% H_2O_2 at 80°C

Time (min.)	Peak Area of Ebastine	%drug	% degradation
0	3921361	100	0
10	3489512	88.98727	11.01273
20	3212619	81.92612	18.07388
30	2772316	70.6978	29.3022
40	2230954	56.89234	43.10766

TABLE 8.13Order of reaction for Ebastine in 1% H_2O_2

Temperature (°C)	Regression co-efficient		
	Zero order	First order	Second order
50	0.9993	0.9846	0.9414
60	0.9980	0.9140	0.8300
70	0.9945	0.9680	0.9212
80	0.9888	0.9672	0.9323

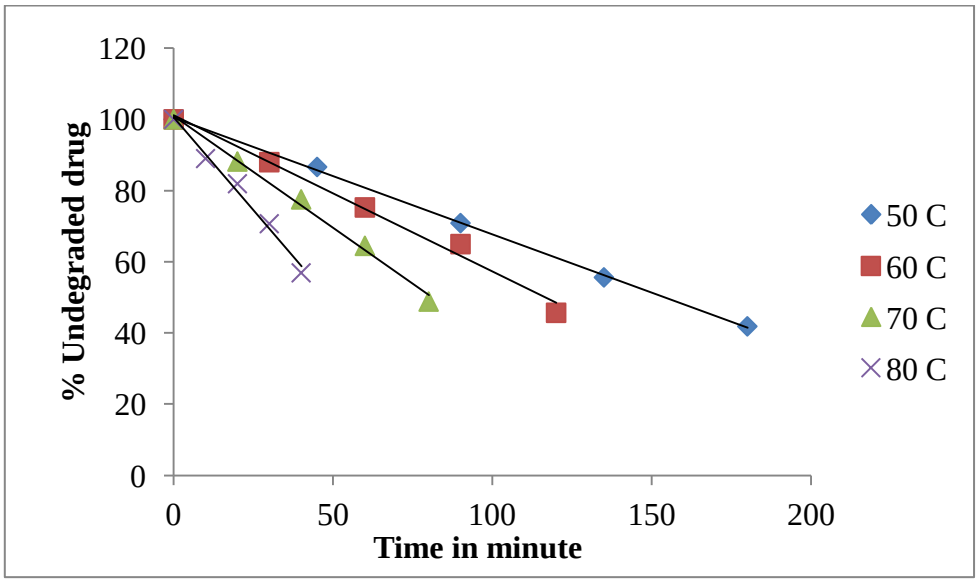

FIGURE 8.5 Temperature effect on degradation of Ebastine in 1% H_2O_2

The effect of temperature (50, 60, 70 and 80° C) on the degradation of Ebastine in oxidizing condition is shown in Fig. 8.5. (Graph of %undegraded drug vs time in min.)

The degradation of Ebastine in oxidizing condition follows zero order kinetics. Degradation rate constant (K) for the zero order is equal to slope of plot of undegraded drug versus time.

$K= (a-x)/t$…….. (5)

$t_{1/2}$ and t_{10} for the zero order degradation was determined as following.

$t_{1/2} = a/2K$…….(6)

t_{10} = a/10K…….. (7)

Here, "a" is the initial drug concentration and "a-x" is the undegraded Ebastine concentration.

Results received from order kinetics were further submitted to fitting to the Arrhenius equation (equation 4)

Arrhenius plot, a plot of K against 1000/T, gave straight line in the range of 50-80 °C for degradation of Ebastine in H_2O_2 (Fig. 8.6).

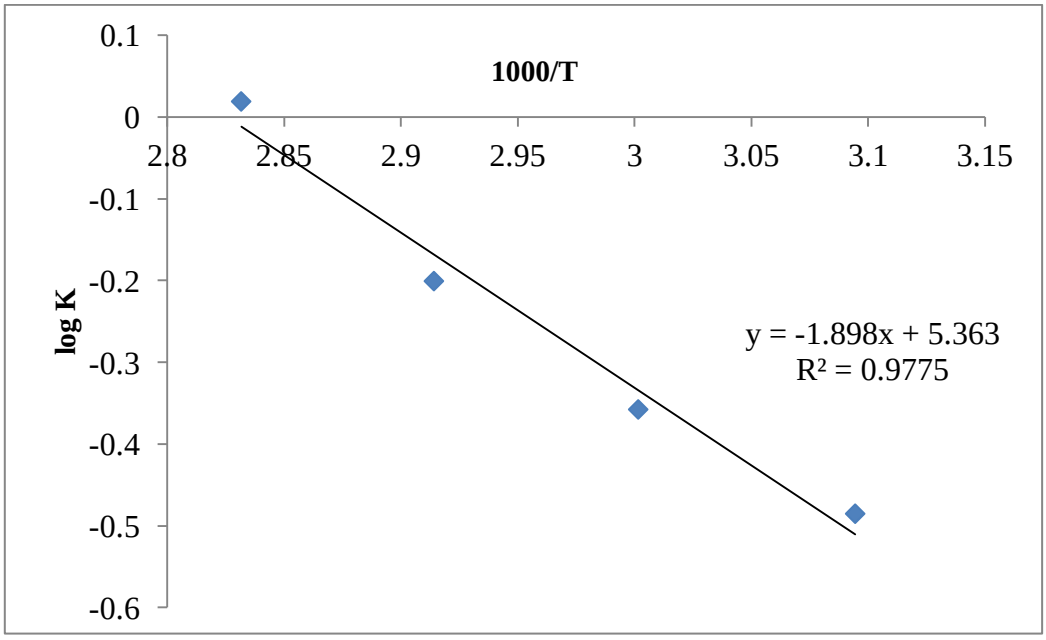

FIGURE 8.6 Arrhenius plot for Ebastine degradation in 1% H_2O_2

The energy of activation was calculated to be 8.685 Kcal/mol for reaction of Ebastine and H_2O_2. The degradation rate constant in 1% H_2O_2 (oxidizing condition) at room temperature (K), was found when Arrhenius plot was extrapolated to 25°C (where 1,000/T =3.354). K at room temperature was found 0.0993 min^{-1}, and calculated $t_{1/2}$ and t_{10} were 503.32 min. (8.39 hr) and 100.66 min.(1.68 hr), respectively. Table 8.14 presents degradation kinetic data for Ebastine in 1% H_2O_2.

TABLE 8.14 Degradation kinetic data for Ebastine in the presence of 1% H_2O_2

Temperature (°C)	Degradation rate constant K (min^{-1})	Half time $t_{1/2}$ (min.)	t_{10} (min.)
50°C (323.15 K)	0.327	152.765	30.553
60°C (333.15 K)	0.439	113.895	22.779
70°C (343.15 K)	0.630	79.352	15.870
80°C (353.15 K)	1.045	47.847	9.569
25° C (calculated)	0.0993	503.32	100.66

CHAPTER 9

Identification of Degradation Products of Ebastine by LC-MS

9.1 Materials and Methods

9.1.1 Reagents and Materials

TABLE 9.1 List of reagents and materials used in LC-MS method for Ebastine

Sr. No.	Reagent/material	Grade
1	Water	HPLC
2	Hydrochloric acid (HCl)	Analytical
3	Hydrogen Peroxide	Analytical
4	Acetonitrile	HPLC
5	Formic acid	Analytical
6	Ebastine BP procured from Kivi Labs, Vadodara	Reference standard

9.1.2 Instruments and Apparatus

TABLE 9.2 List of instruments and apparatus used in LC-MS method for Ebastine

Sr. No.	Instrument/Apparatus	Manufacturer
1	Analytical weighing balance	Shimadzu
2	Glass wares	Borosil
3	LC-800	Make by GL science with Analyst software
4	MS/ MS	AB sciex QTRAP 4500

9.1.3 Preparation of solutions

- **Preparation of standard stock solution**

Ebastine standard stock solution was prepared by taking accurately 100 mg of Ebastine in 100mL volumetric flask, adding 50 mL of diluent and was sonicated for 10 min. and making up to 100 mL with acetonitrile. The resulted solution was further diluted to have 10 µg/mL of Ebastine.

- **Acid induced degradation solution of Ebastine**

2 mL of standard solution of Ebastine (1 mg/mL) was taken in 20 mL volumetric flask and 2 mL of 0.1 N HCl was added to it. For 45 min. the mixture was refluxed in a thermostatic water bath at 80°C. Then it was cooled and dilution to 20 mL was done with acetonitrile. 1 mL of the resulted solution was taken further diluted to 10 mL with acetonitrile.

- **Oxidized degradation solution of Ebastine**

2 mL of the Ebastine standard solution (1 mg/mL) was taken in 20 mL volumetric flask followed by 2 mL 1% H_2O_2. For 20 min. the mixture was refluxed in a thermostatic water bath at 70°C. Then it was cooled and dilution up to 20 mL was done with acetonitrile. 1 mL of the resulted solution was further diluted to the volume with acetonitrile.

9.1.4 LC-MS method

Inertsil C_{18} column (150 mm length x 2.1 mm i. d., 3 µm particle size) and the mobile phase comprised of 0.5% formic acid and ACN (50:50 v/v) pumped at rate of 0.2 mL/min. was employed for chromatographic separation of Ebastine and its degradation products. The injection volume of drug was 10 µL and temperature of column was 40 °C. The eluted components were detected using UV detector. HPLC system is coupled with MS/MS, also. Fragmentation profile of Ebastine and its degradation products was established by performing mass spectral studies on Ebastine and its degradation products, respectively. The products were ionized by positive mode of electron spray ionization (ESI) for their mass data. The data was processed and monitored using Analyst software. The parameters MS were adequately adjusted that high intensity of molecular ions peaks and daughter ions peaks of degradation products were obtained.

9.2 Results and Discussion

9.2.1 Chromatographic analysis

For determination of Ebastine in presence of degradation products a novel HPLC method was developed and validated as described in chapter 7. From the study it was found that Ebastine produced one degradation product from acidic degradation and one degradation product from oxidizing degradation. This method was transferred to LC-MS with some modification to elucidate the structures of degradation products of Ebastine. 0.5% ortho-phosphoric acid was replaced by 0.5% formic acid. Inertsil C_{18} column (150 mm length x 2.1 mm i. d., 3 µm particle size) and the mobile phase comprised of 0.5% formic acid : ACN (50:50 v/v) pumped at rate of 0.2 mL/min. was employed for chromatographic separation.

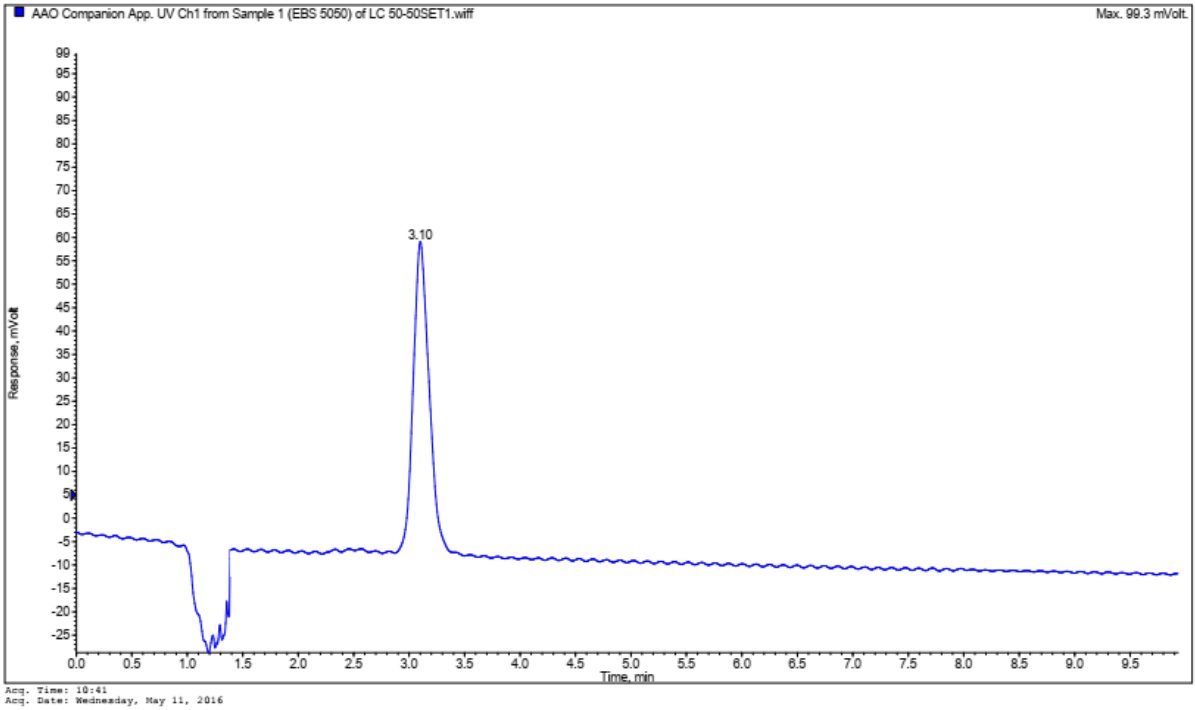

FIGURE 9.1 HPLC chromatogram of Ebastine standard

Chromatograms of Ebastine standard, acid stressed sample and hydrogen peroxide stressed sample are presented in Fig. 9.1 to Fig. 9.3, respectively. Retention time of Ebastine was found around 3.10 min. Degradation product of acidic condition and Ebastine were separated well. Retention time for acid degradation product was found at 1.45 min. Degradation product of oxidized condition and Ebastine were also separated well.

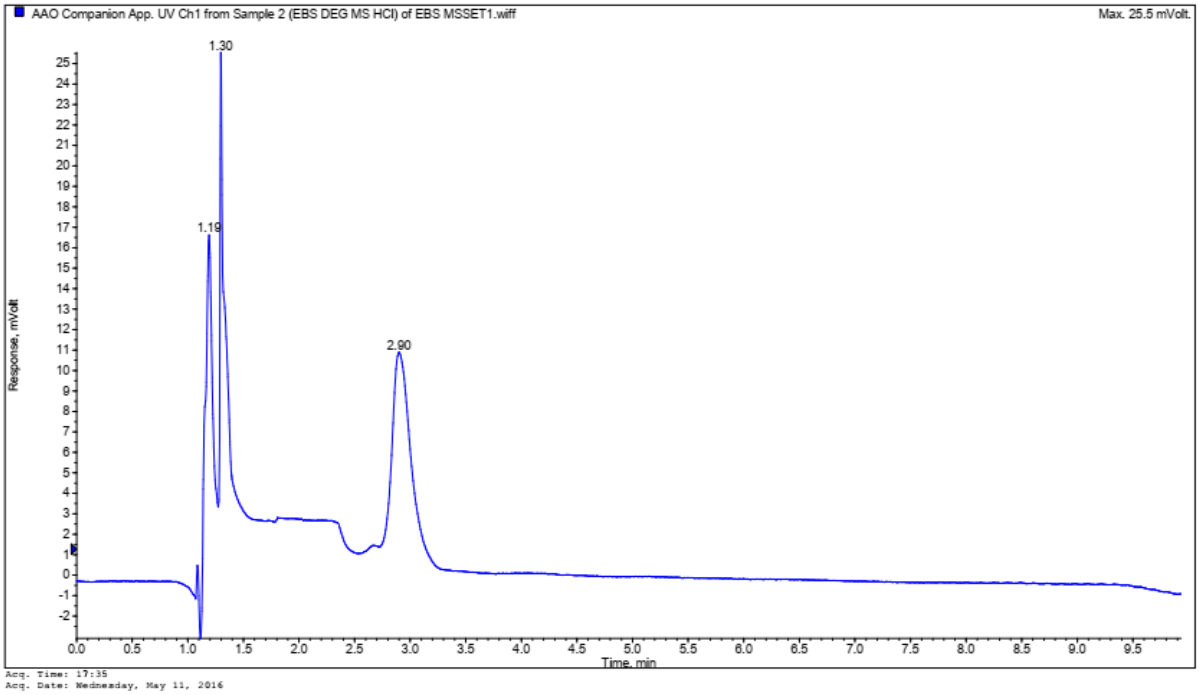

FIGURE 9.2 HPLC chromatogram of Acid stressed Ebastine sample

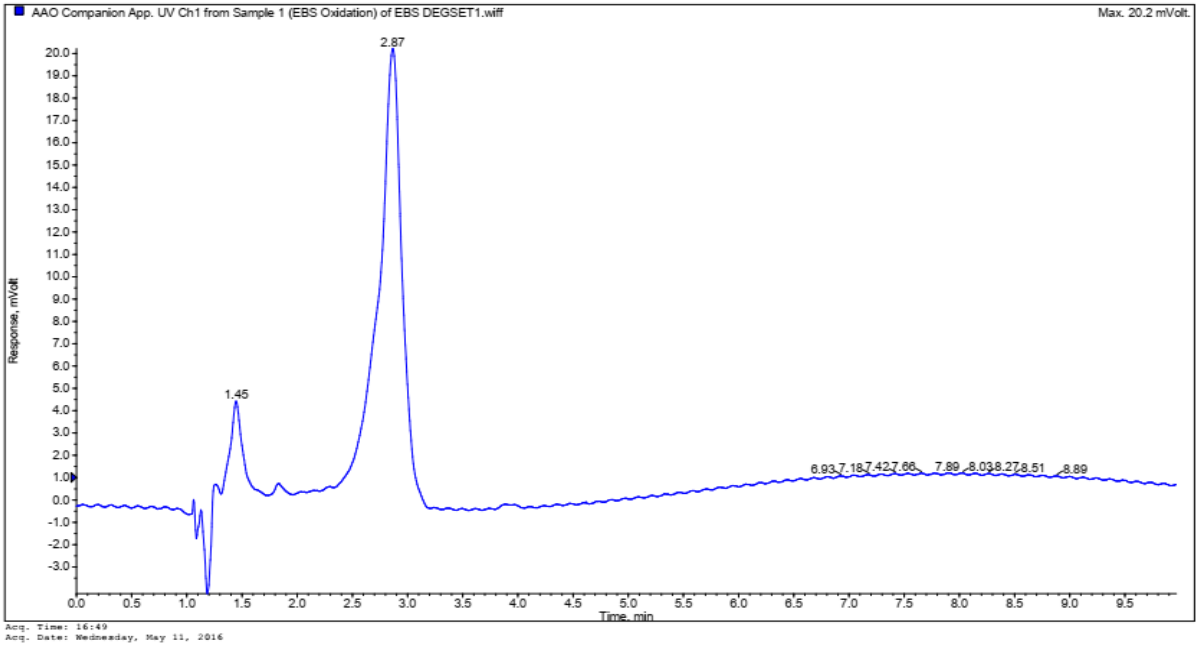

FIGURE 9.3 HPLC chromatogram of Hydrogen peroxide stressed Ebastine sample

9.2.2 LC-MS study of Ebastine

The optimization of the instrument conditions started from the tuning of detector and electron spray ionization in a positive mode. 10 µg/mL of Ebastine solution in acetonitrile was submitted to MS system in positive mode of ESI in the mass range of 50-500 Da. The following instrument settings were applied: de-clustering potential of 150.0 eV, entrance potential of 5.0 eV, cell exit potential of 6.0 eV and collision energy of 50 eV. The $[M + H]^+$ value observed in electron spray ionization positive mode was 470.2 and matched with molecular weight of Ebastine (469.65 gm/mole). A mass spectrum of product ion of Ebastine produced by LC-MS/MS is presented in Fig. 9.4. Fragmentation pattern of Ebastine is illustrated in Fig. 9.5 from the interpretation of fragments by the best suitable molecular formulae to the experimental value of m/z. Only two fragments at 203.1Da and 167.1Da were produced from the molecular ion of Ebastine.

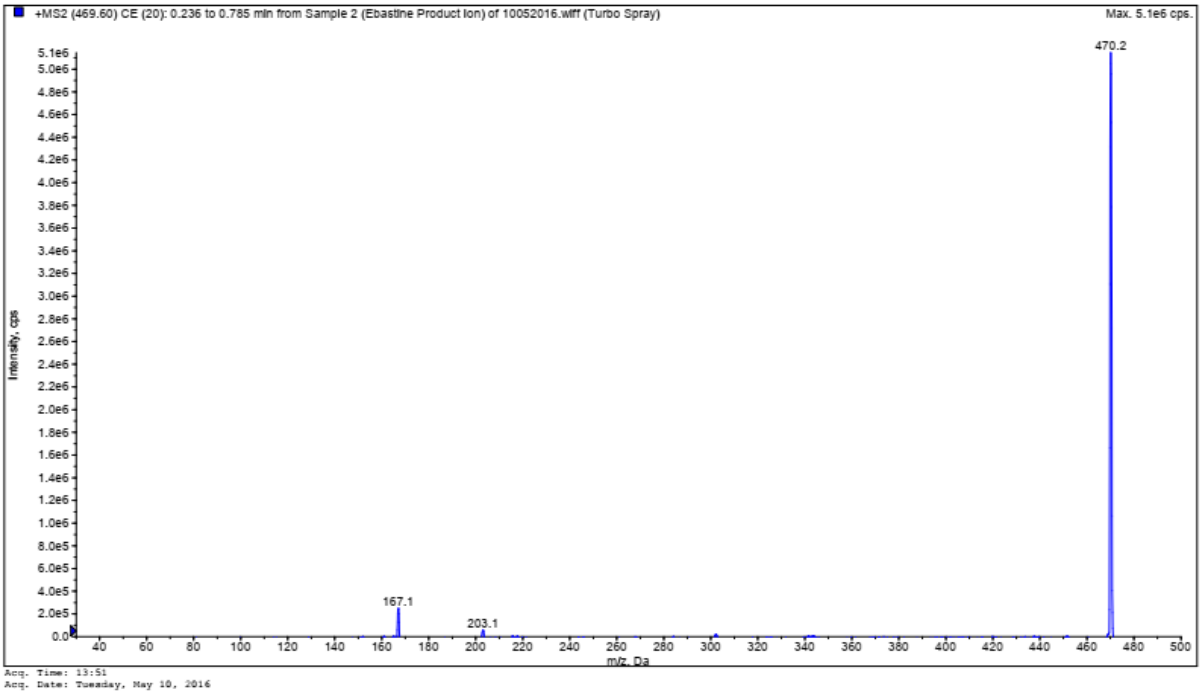

FIGURE 9.4 mass spectra of Ebastine (product ion).

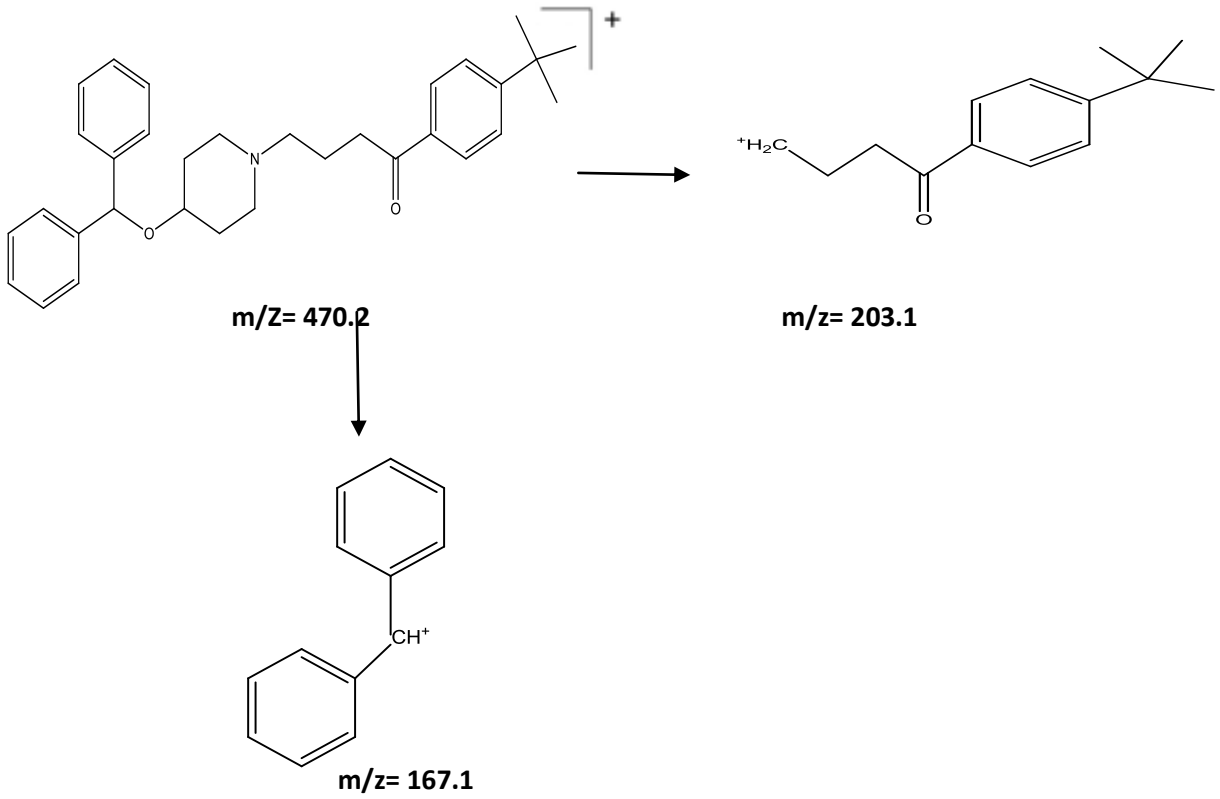

FIGURE 9.5 Probable fragmentation pattern of Ebastine

9.2.3 Characterization of degradation product of acid stressed sample

Fig. 9.6 presents a mass spectrum of degradation impurity of acid stressed sample. The results of LC–MS/MS analysis showed that the degradation product formed after acidic hydrolysis had molecular ion peak at 305.2 Da. Likely Fragmentation pattern of degradation product of acid stressed Ebastine is illustrated in Fig. 9.7. Mass spectra and likely fragmentation pattern of degradation product of acid stressed Ebastine sample confirms that reaction of Ebastine with hydrochloric acid does the hydrolysis of ether linkage and produces 1-(4-tert-butylphenyl)-4-[4-(hydroxy) piperidin-1-yl]butan-1-one. Molecular ion peak at 305.2 matched with the molecular weight of 1-(4-tert-butylphenyl)-4-[4-(hydroxy) piperidin-1-yl] butan-1-one (mol. wt. - 303.4 gm/mole). This acid degradation product is also reported as impurity of Ebastine in literature.[1]

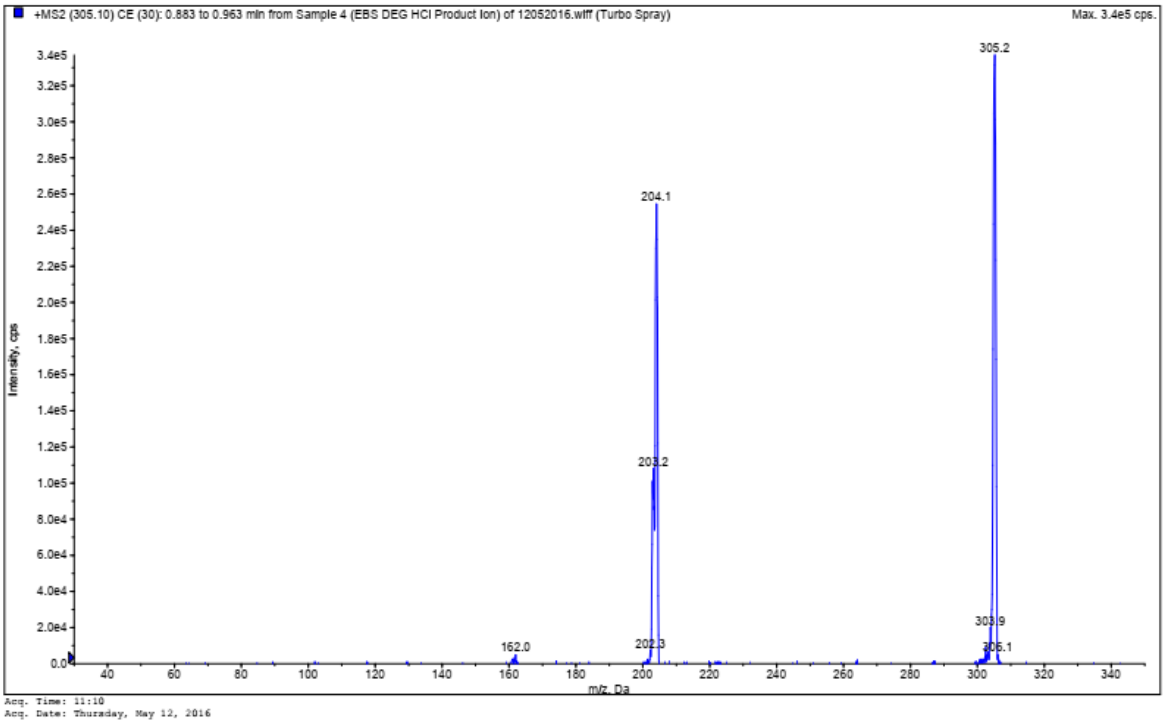

FIGURE 9.6 mass spectra of Acid stressed Ebastine (product ion)

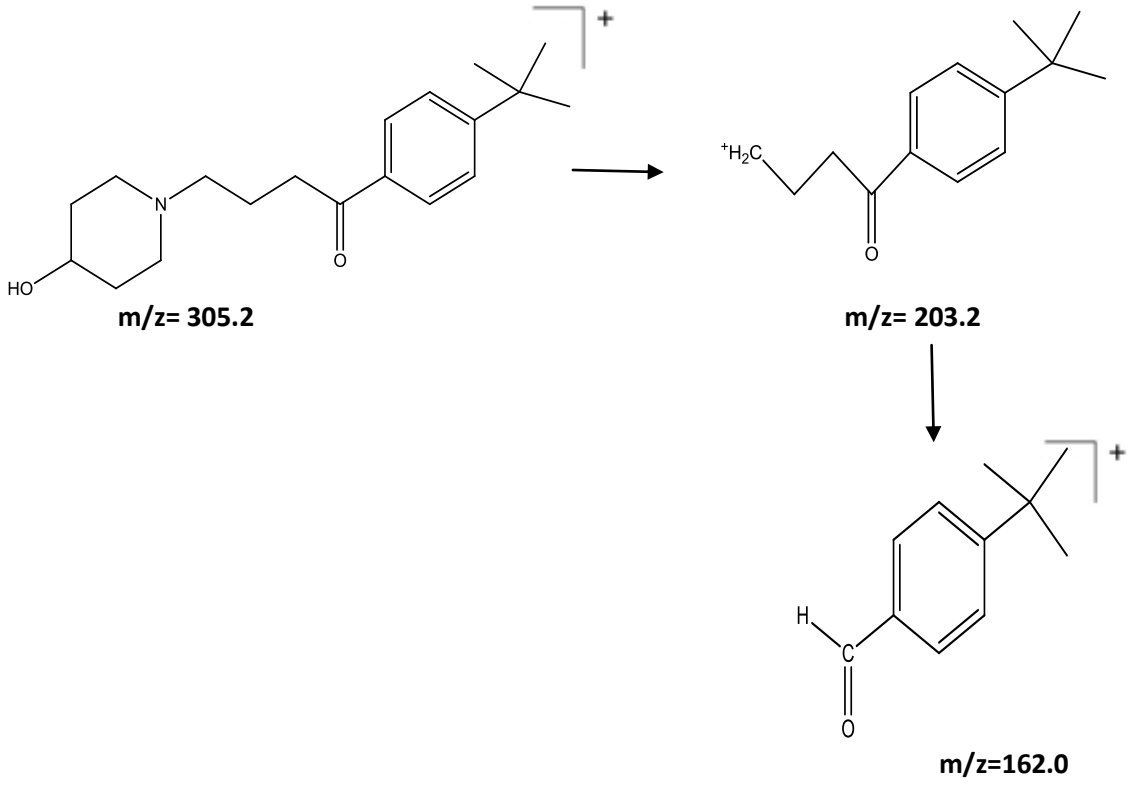

FIGURE 9.7 Fragmentation pattern of acid degradation product of Ebastine

9.2.4 Characterization of degradation product of oxidation stressed sample

The LC-MS/MS spectrum of degradation product after oxidation of Ebastine by hydrogen peroxide showed molecular ion peak at 486.4 Da ($[M+H]^+$) formed in positive ionization mode. Molecular ion at 486.1 Da shows the addition of m/z approx. 16 to the m/z of molecular ion of Ebastine which resembles to ion of oxygen. Molecular ion peak at 486.1 Da represents the formation of N-oxide of Ebastine (1-(4-tert-butylphenyl)-4-[4-(diphenyl methoxy) N-oxy piperidin-1-yl] butan-1-one) on reaction of Ebastine with hydrogen peroxide. N-oxide of Ebastine is reported as impurity of Ebastine in the literature also[1]. A mass spectrum of degradation product of hydrogen peroxide stressed Ebastine sample is presented in Fig. 9.8. Fragmentation pattern of degradation product of hydrogen peroxide stressed Ebastine is illustrated in Fig. 9.9. Mass spectra and fragmentation pattern of degradation product of hydrogen peroxide stressed Ebastine sample confirms that Ebastine reacts with oxidizing agent, hydrogen peroxide and produces (1-(4-tert-butylphenyl)-4-[4-(diphenyl methoxy)N-oxy piperidin-1-yl]butan-1-one). MS/MS data of Ebastine and degradation products of Ebastine and their major fragments along with molecular formula is presented in Table 9.3.

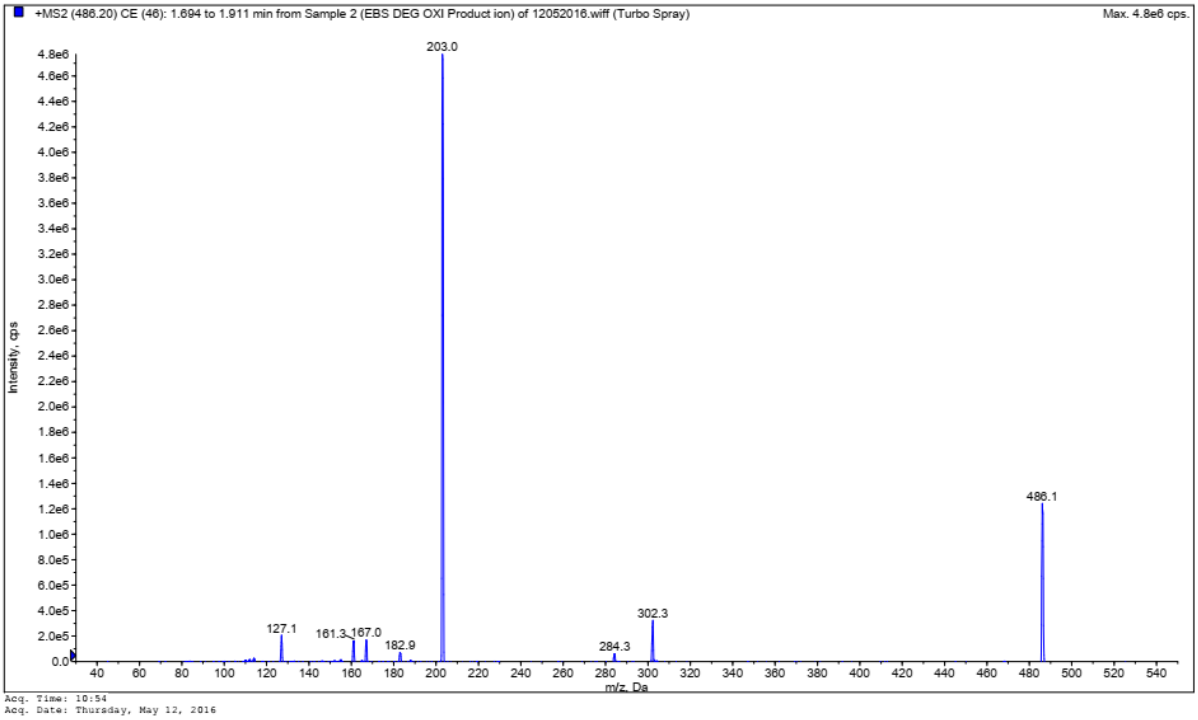

FIGURE 9.8 LC-MS/MS spectra of Hydrogen peroxide stressed Ebastine sample

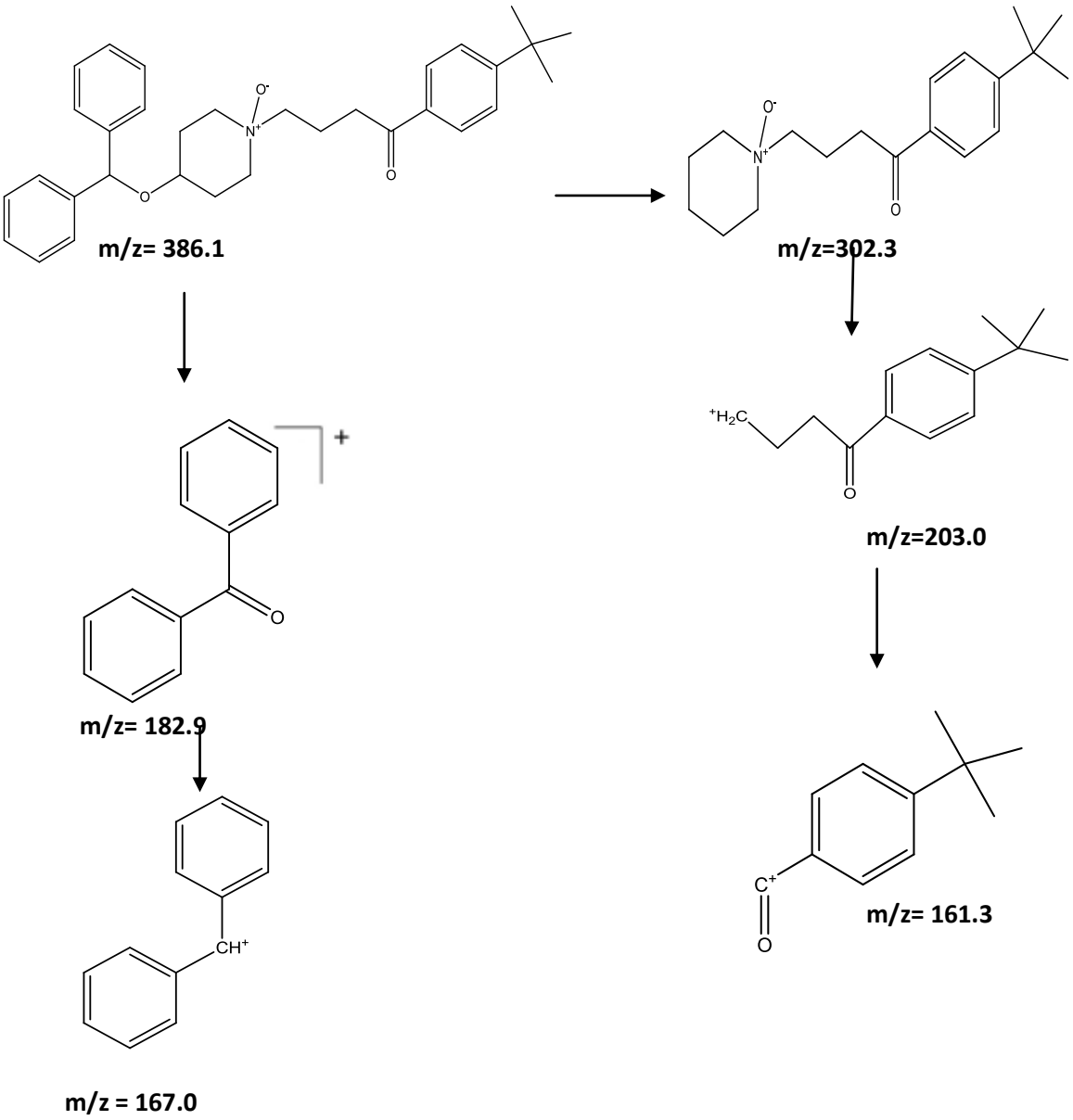

FIGURE 9.9 Fragmentation pattern of oxidation degradation product of Ebastine

TABLE 9.3 LC-MS/MS data of Ebastine and its degradation products and their major fragments

Substance	Experimental mass	Molecular formula	Major fragments (m/z)
Ebastine	470.2	$C_{32}H_{39}NO_2^+$	203.1($C_{14}H_{19}O^+$), 167.1($C_{13}H_{11}^+$)
Acid degradation product of Ebastine	305.2	$C_{19}H_{29}NO_2^+$	203.2($C_{14}H_{19}O^+$), 162.0($C_{11}H_{14}O^+$)
Oxidation degradation product of Ebastine	486.1	$C_{32}H_{39}NO_3^+$	302.3, 203.0($C_{14}H_{19}O^+$), 182.9($C_{13}H_{10}O^+$), 167.0($C_{13}H_{11}^+$), 161.3($C_{11}H_{13}O^+$)

9.3 References

1. Schmidt AH, Molnár I, 2013, Using an innovative Quality-by-Design approach for development of a Stability indicating UHPLC method for Ebastine in the API and pharmaceutical formulations, *Journal of Pharmaceutical and Biomedical Analysis*, 78, pp 65-74, ISSN No. 0731-7085

CHAPTER 10

HPLC Method for Azelastine Hydrochloride

10.1 Materials and Methods

10.1.1 Reagents and Materials

TABLE 10.1 List of reagents and materials used in HPLC method for Azelastine HCl

Sr. No.	Reagent/material	Grade
1	Water	HPLC
2	Hydrochloric acid	Analytical
3	Sodium hydroxide	Analytical
4	Hydrogen Peroxide	Analytical
5	Methanol	HPLC
6	Acetonitrile	HPLC
7	Potassium dihydrogen phosphate	Analytical
8	Ortho phosphoric acid (OPA)	Analytical
9	Triethylamine	Analytical
10	Azelastine Hydrochloride procured from Cadila Health Care Limited, Moraiya	Reference standard

10.1.2 Instruments and Apparatus

TABLE 10.2 List of instruments and apparatus used in HPLC method for Azelastine HCl

Sr. No.	Instrument/Apparatus	Manufacturer
1	Analytical weighing balance	Sartorius
2	FTIR instrument	Bruker
3	Glass wares	Borosil
4	pH meter	Elico
5	HPLC	Agilent 1260 with Ez Chrome software

10.1.3 Identification of API

- **Melting point determination**

Melting point was determined by open capillary method.

- **Infra Red Spectra:**

Azelastine Hydrochloride- KBr pellet was prepared and an IR spectrum was produced by FT-IR instrument. IR spectrum of sample was compared to reference IR spectrum[1].

10.1.4 Determination of Physicochemical Properties

- **Solubility test:**

Solubility of Azelastine hydrochloride in distilled water, methanol and acetonitrile was checked by taking 10 mg of Azelastine hydrochloride in 100 mL flask and adding the solvent till the drug dissolved.

- **Wavelength maxima:**

From PDA data, a wavelength maximum of drug was determined.

10.1.5 Preparation of solutions

- **Preparation of standard stock solution**

Azelastine hydrochloride standard stock solution was prepared by taking accurately 100 mg of Azelastine hydrochloride in 100mL volumetric flask, adding 50 mL methanol and was sonicated for 10 min. and making up 100 mL with methanol. Standard stock solution (1000 µg/mL) was diluted as relevant with methanol to obtain the working concentration range. Stock solution was stable for minimum 5 days when kept at ambient temperature.

- **Buffer preparation (pH 3.0)**

About 6.805g of potassium dihydrogen phosphate was dissolved in 600 mL of HPLC water and then final volume was made up to 1000 mL with HPLC water. Buffer pH was adjusted to 3.0 with OPA.

- **Test solution preparation**

To determine the Azelastine Hydrochloride content of a nasal spray (Arzep, label claim 1%w/v, 10 mL), solution of the container was taken out in 100 mL volumetric flask and 10 mL acetonitrile was added and sonicated for 20 min, and then made up to 100 mL with acetonitrile which made 1 mg/mL of Azelastine hydrochloride. 1 mL of this solution was transferred in 10 mL volumetric flask and dilution up to 10 mL made with acetonitrile to have 100µg/mL.

10.1.6 Selection of chromatographic condition

Stationary phase C_{18} and C_8 column were tried (Table 10.3) with different mobile phase on the basis of physico-chemical properties of the drug to develop stability indicating RPLC method for determination of Azelastine Hydrochloride, and system suitability test was performed for method optimization.

TABLE 10.3 selection of chromatographic condition

Sr. No.	Stationary Phase	Mobile Phase	Problem
1	Hypersil C_{18} (250 x 4.6 mm, 5µm)	ACN: 0.02 M KH_2PO_4 buffer pH 3.0, 60:40 v/v, 1.0 mL/min.	Tailing factor >2, Peak shape is not good
2	Phenomenex C_{18} (250 x 4.6 mm, 5µm)	ACN: 0.02 M KH_2PO_4 buffer pH 3.0, 60:40 v/v, 1.0 mL/min.	Splitting of peak
3	Waters CN column (250 x 4.6 mm, 5µm)	ACN: 0.05 M KH_2PO_4 buffer pH 6.7, 50:50 v/v, 1.0 mL/min.	Broad peak, System suitability test is not ok
4	Waters CN column (250 x 4.6 mm, 5µm)	ACN: 0.05 M KH_2PO_4 buffer pH 6.7, 55:45 v/v, 1.0 mL/min.	System suitability test is not ok
5	Waters CN column (250 x 4.6 mm, 5µm)	ACN: 0.05 M KH_2PO_4 buffer pH 3.0, 45:55 v/v, 1.0 mL/min.	Tailing factor = 1.622
6	Waters CN column (250 x 4.6 mm, 5µm)	ACN: 0.05 M KH_2PO_4 buffer pH 3.0 , 30:70 v/v, 1.0 mL/min.	Tailing factor = 1.7
7	Waters CN column (250 x 4.6 mm, 5µm)	ACN: 0.05 M KH_2PO_4 buffer pH 3.0 , 50:50 v/v, 1.0 mL/min.	Tailing factor = 1.3

10.1.7 Validation of the method

The developed method was validated for system suitability, specificity, linearity, precision, trueness, limit of detection, limit of quantification and robustness as per ICH guideline to ensure its suitability for the predetermined purpose.

- **System suitability test**

To ensure suitability of selected HPLC testing system for the expected application, the system suitability test was checked. Six replicate analyses of 100 µg/mL of Azelastine hydrochloride solution were chromatographed as per optimized method and theoretical plates and tailing factor were evaluated.

- **Linearity**

Accurately measured volumes of the Azelastine Hydrochloride standard solution (1000 µg/mL) were successively transferred into a series of 10 mL volumetric flasks to get final concentrations of 5-120 µg/mL. They were diluted with acetonitrile and mixed properly. 20 µL aliquots of each solution were chromatographed three times. The regression equation was derived by plotting the average area of Azelastine hydrochloride peak was plotted against the concentration of Azelastine hydrochloride.

- **Precision**

- **Repeatability**

Repeatability of the method was assessed by analysis of seven injections of 100 µg/mL Azelastine Hydrochloride solution. The average, standard deviation and % RSD for retention time and area of Azelastine hydrochloride peak were calculated.

- **Intraday precision:**

Three concentrations (40, 60 and 80 µg/mL) were chromatographed for three times on same day by same analyst and area of Azelastine hydrochloride peak was reported to check the intraday precision. The %RSD of Azelastine hydrochloride peak area was calculated.

- **Interday precision:**

Three concentrations (20, 40 and 60 µg/mL) were chromatographed for three different days by same analyst and area of Azelastine hydrochloride peak was reported to check the interday precision. The %RSD of peak area was calculated.

➢ **Accuracy**

0.8 mL Azelastine nasal solution (Arzep, label claim 1%w/v, 10 mL) equivalent to 0.8 mg of Azelastine hydrochloride was transferred in 3 different 20 mL volumetric flasks. 0.4 mL, 0.8 mL and 1.2 mL of 1.0 mg/mL of Azelastine hydrochloride solution was added to volumetric flasks containing nasal solution and dilution to volume was done with acetonitrile to have 40 µg/mL of sample in each flask. The resulted solutions were chromatographed triplicate as per optimized condition. % recovery was calculated for each level.

➢ **Specificity – forced degradation study**

The forced degradation studies were accomplished to determine whether the analytical method was stability indicating and could unambiguously evaluate the Azelastine Hydrochloride though impurities and degradation products are present in sample. Azelastine Hydrochloride standard solution was stressed in acid, base, neutral hydrolytic, oxidative, thermal and photolytic conditions to result in partial decomposition of Azelastine hydrochloride.

- Acid induced degradation

2 mL of standard solution of Azelastine Hydrochloride (1 mg/mL) was transferred in 20 mL volumetric flask and 2 mL of 5.0 N HCl was added to it. For 5 hr the mixture was refluxed in a thermostatic water bath at 80°C. Then dilution up to 20 mL was done with acetonitrile. The resulted solution was chromatographed as per optimized conditions.

- Base induced degradation

2 mL of Azelastine Hydrochloride (1 mg/mL) solution was transferred to 20 mL volumetric flask and 2 mL of 5.0 N NaOH was added to it. For 5 hr the mixture was refluxed in a thermostatic water bath at 80°C. Then dilution up to 20 mL was done with acetonitrile. Then the resulted solution was chromatographed as per optimized conditions.

- Wet heat degradation

2 mL of Azelastine Hydrochloride (1 mg/mL) was transferred to 20 mL volumetric flask followed by 2 mL distilled water. For 3 hr the mixture was refluxed in a thermostatic water bath at 70°C. Then dilution up to 20 mL was done with acetonitrile. Chromatography of the resulted solution was performed as per optimized conditions.

- Oxidative degradation

2 mL of Azelastine Hydrochloride (1 mg/mL) was transferred to 20 mL volumetric flask followed by 2 mL 30% H_2O_2. For 4 hr the mixture was refluxed in a thermostatic water bath at 80°C. Then dilution up to 20 mL was done with acetonitrile. Then chromatography of the resulted solution was performed as per optimized conditions.

- Thermal degradation

50 mg Azelastine Hydrochloride powder in petridish was kept at 70°C in convection oven and exposed for 8 hr and further processed as per sample preparation. Then the resulted solution was chromatographed as per optimized conditions.

- Photolytic degradation

Azelastine Hydrochloride drug (50 mg) was taken in petridish. One petridish was kept in UV chamber (365 nm) for 24 hr at room temperature and further processed as per sample preparation. Then the resulted solutions were chromatographed as per optimized conditions.

➢ **Robustness**

The experimental conditions of the method were deliberately changed from optimized condition to determine the robustness. The flow rate of the mobile phase (1.0±0.1mL/min), mobile phase pH (3.0±0.2), mobile phase composition (±2%), and column temperature (45±1°C) were varied. In each case, %RSD for area of Azelastine hydrochloride peak and retention time was calculated. Comparison of the retention time and area of Azelastine hydrochloride peak were done with that obtained under the optimized method, also.

- **Limit of Detection and Limit of Quantitation**

Standard deviation of y intercept of calibration curve of linear lower concentrations (N) and slope (S) of the calibration curve were calculated and using their values LOD and LOQ were estimated as per ICH guideline.

10.1.8 Application of the developed method

Azelastine Hydrochloride nasal solution (Arzep, label claim 1%w/v, 10 mL) was exposed to 40°C 75% for 1 month in stability chamber. To determine the Azelastine Hydrochloride content of a nasal spray after accelerated stress, procedure of the test solution preparation was followed. And then the resulted solution was chromatographed as per optimized condition to check the effect of accelerated testing of drug product.

10.2 Results and Discussion

10.2.1 Identification of API

Melting point of the Azelastine hydrochloride was observed in the range of 226-228°C which is almost similar to the reported value (224-228 °C) for Azelastine hydrochloride.[2]

An IR spectrum of Azelastine hydrochloride API is displayed in Fig. 10.1 and a reference spectrum[2] is displayed in Fig. 10.2.

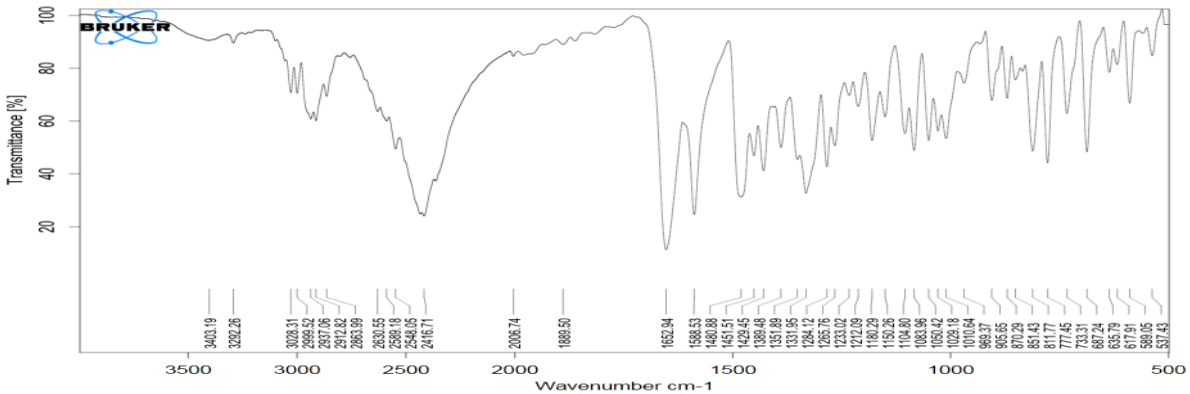

FIGURE 10.1 IR Spectrum of Azelastine hydrochloride API

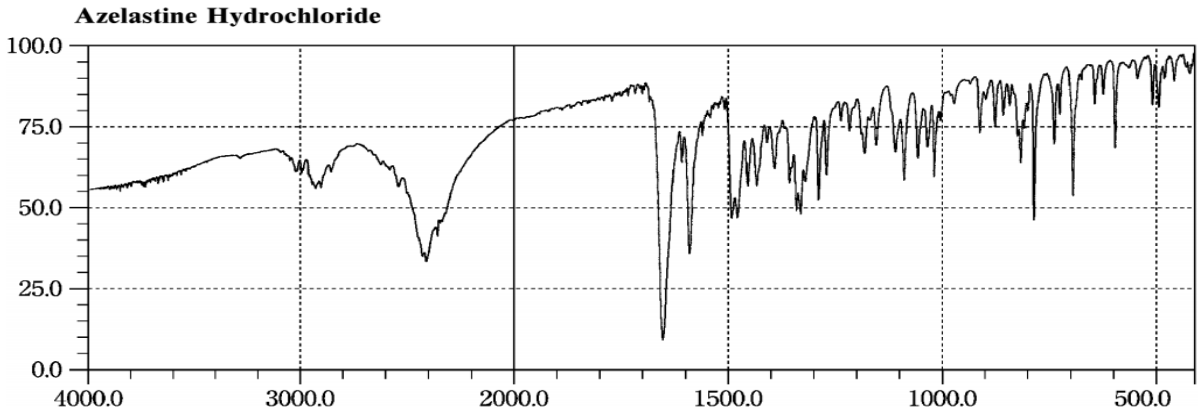

FIGURE 10.2 IR Reference Spectra of Azelastine hydrochloride

As Sample spectrum completely matches with the reference spectrum and practical value of melting point also matches with theoretical value, it confirms the purity of Azelastine hydrochloride sample.

10.2.2 Determination of physicochemical properties

Table 10.4 presents the results of solubility test.

TABLE 10.4 Solubility results for Azelastine hydrochloride

Solvent	mL of solvent required to dissolve 10 mg Azelastine hydrochloride	Solubility
Methanol	0.5 mL (50 mL for 1 gm)	Sparingly soluble
Water	1.0 mL (100 mL for 1 gm)	Sparingly soluble
Acetonitrile	2.0 mL (200 mL for 1 gm)	Slightly soluble

From PDA data, 215 nm is λ_{max} of Azelastine Hydrochloride and drug shows good absorbance at 290 nm also. So to avoid diluent interferences 290 nm was selected as wavelength for study.

10.2.3 Development of method

HPLC method using waters Spherisorb CN (250 mm x 4.6 mm i.d., 5µm particle size) column and isocratic solvent system comprising acetonitrile: 0.05 M Potassium Dihydrogen phosphate buffer of pH 3.0, 50:50 v/v pumped with 1.0 mL/min. flow rate was observed to

provide sharp and well defined peak with very good symmetry (1.3), theoretical plates(>3000) and low retention time (4.533 min.). Hence it was selected throughout study of Azelastine hydrochloride by HPLC.

- **Optimized chromatographic condition:**

  Column: Waters Spherisorb CN (250 mm x 4.6 mm i.d., 5µm particle size) column

  Mobile phase: 50 volumes of ACN: 50 volumes of 0.05 M Potassium Dihydrogen phosphate buffer pH 3.0.

  Flow rate: 1.0 ml/min

  Volume of injection: 20 µL

  Temperature of column: 45˚C

  Run Time: 10 min

  Diluent: Acetonitrile

  Detector: PDA

  Retention Time of Azelastine Hdrochloride: 4.533 min.

RP-HPLC Chromatogram of Azelastine hydrochloride produced by optimized method is displayed in Fig. 10.3. Peak purity of Azelastine hydrochloride is 1.00.

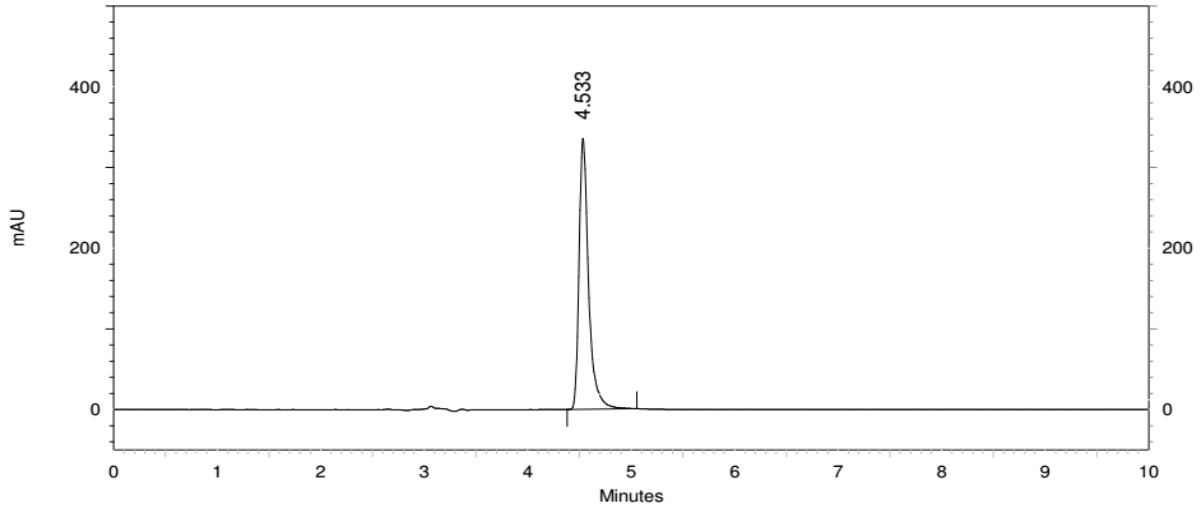

FIGURE 10.3 RP-HPLC Chromatogram of Azelastine hydrochloride

10.2.4 Method Validation

To demonstrate appropriateness of method for the predetermined purpose, validation of the developed method was done as per ICH guideline.

- **System suitability test**

Table 10.5 represents the results of system suitability test. As results of system suitability test are satisfactory it was ensured that HPLC testing system was adequate for routine analysis of Azelastine hydrochloride.

TABLE 10.5 system suitability data for Azelastine hydrochloride

Sr. No.	Conc.	Theoretical plates	Tailing factor
1	100 µg/mL	13049	1.50
2		12926	1.57
3		13041	1.49
4		13218	1.50
5		13295	1.50
6		13469	1.48
7		13718	1.54
Acceptance criteria		>2000	<2.0

- **Linearity**

Area of Azelastine HCl peak and concentration of Azelastine HCl were submitted to linear regression analysis to obtain the regression equation and correlation coefficient. The calibration results revealed a good good linear relationship of peak area of Azelastine HCl over the concentration range of 5-120 µg/mL with correlation coefficient more than 0.999. Table 10.6 represents the results of linearity. And overlaid linearity chromatogram of Azelastine hydrochloride is displayed in Fig.10.4. Calibration curve for Azelastine hydrochloride is displayed in Fig. 10.5.

TABLE 10.6 Linearity data for Azelastine hydrochloride

Sr. No.	Stock solution taken in mL	Diluted to volume in mL	Concentration in µg/mL	Average Area (n=3)
1	0.05	10	5	244548
2	0.1	10	10	465162
3	0.2	10	20	912898
4	0.3	10	30	1366001
5	0.4	10	40	1847118
6	0.5	10	50	2339408
7	0.6	10	60	2761394
8	0.7	10	70	3177241
9	0.8	10	80	3659064
10	0.9	10	90	4089412
11	1.0	10	100	4559944
12	1.1	10	110	4981191
13	1.2	10	120	5546493

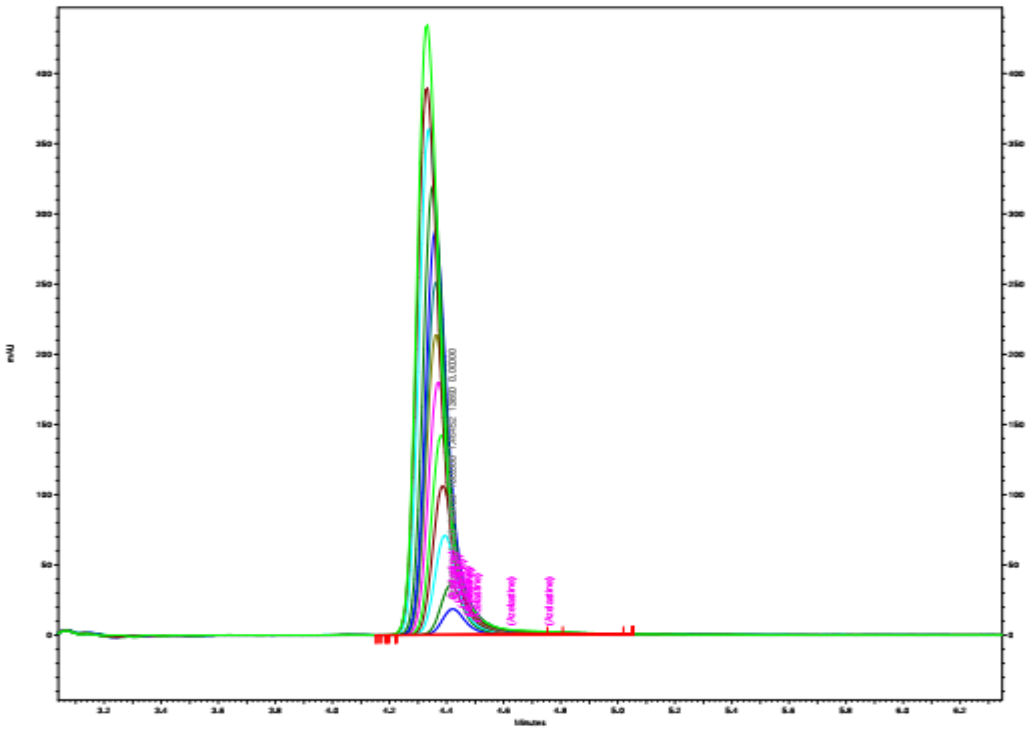

FIGURE 10.4 Overlaid linearity chromatogram of Azelastine hydrochloride

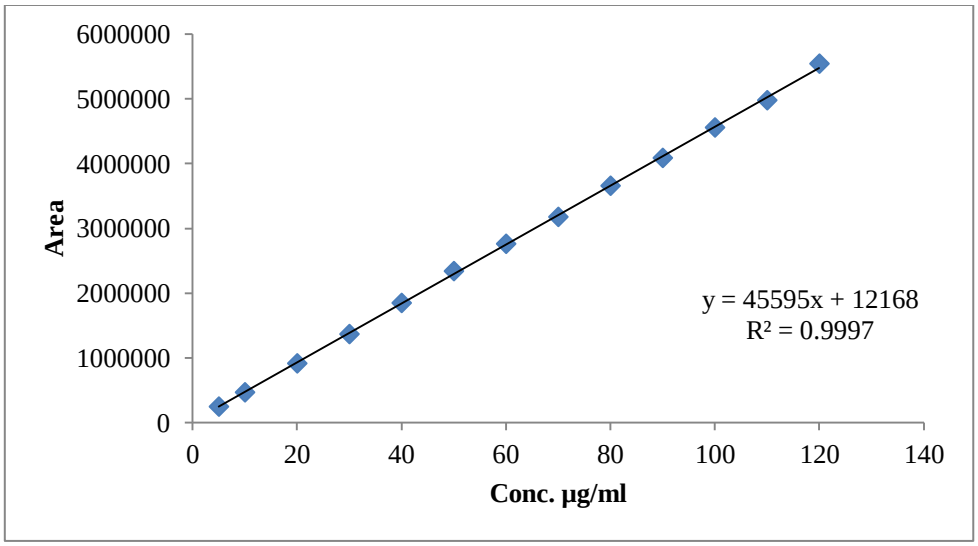

FIGURE 10.5 Calibration curve of Azelastine hydrochloride for RP-HPLC

➢ **Precision**

- **Repeatability**

Table 10.7 presents the results of repeatability. The % RSD of retention time and area of drug was found less than 2% that proves that method is repeatable.

TABLE 10.7 Repeatability data of RP-HPLC for Azelastine hydrochloride

Sr. No.	Retention time (Min.)	Peak area
1	4.56	4320481
2	4.533	4383485
3	4.51	4416390
4	4.492	4444033
5	4.475	4459652
6	4.459	4480677
7	4.447	4496922
Mean	4.497	4428806
Std. dev.	0.0405	61177.59
%RSD	0.907	1.381

- **Intraday and interday variation**

Table 10.8 shows the intraday precision results. % RSD of peak area of Azelastine hydrochloride was found less than 1% for intraday precision. Table 10.9 shows the results of

interday precision. % RSD of peak area of Azelastine hydrochloride was found less than 2% for interday precision. %RSD less than 2% proved the high precision of the proposed method.

TABLE 10.8 Intraday precision data of RP-HPLC for Azelastine hydrochloride

Conc. µg/mL	I	II	III	Avg.	Std. dev.	%RSD
40	1801808	1807705	1814355	1807956	6277.25	0.347
60	2672560	2694569	2681276	2682802	11083.54	0.413
80	3594238	3610145	3584819	3596401	12800.76	0.356

TABLE 10.9 Interday precision data of RP-HPLC for Azelastine hydrochloride

Conc. µg/mL	I	II	III	Avg.	Std. dev.	%RSD
40	1801808	1815436	1820248	1812497	9564.79	0.528
60	2672560	2697321	2731324	2700402	29502.88	1.093
80	3594238	3628672	3650467	3624459	28350.26	0.782

- **Accuracy**

When the method was used for analysis of Azelastine hydrochloride from a previously analysed test solution after spiking of 50, 100 and 150% Azelastine hydrochloride standard, the recovery was found 99-102%. Table 10.10 presents the results for accuracy.

TABLE 10.10 Accuracy results for Azelastine hydrochloride by RP-HPLC

Level of recovery	Conc. (added std. sol.) µg/mL	Conc. (sample sol.) µg/mL	Recovered conc.	% recovery	Mean % recovery ± S.D.
50%	20.00	40.00	20.02	100.10	99.54±1.29
			20.09	100.47	
			19.61	98.07	
100%	40.00	40.00	40.71	101.77	101.14±0.70
			40.15	100.38	
			40.51	101.28	
150%	60.00	40.00	60.07	100.12	99.46±0.72
			59.21	98.69	
			59.74	99.57	

➢ **Specificity**

- **Acid induced degradation study**

Peak area of Azelastine hydrochloride was not changed significantly and any additional peak was not observed when chromatographed after refluxing in 5N HCl at 80°C for 5 hr. Chromatogram of Azelastine hydrochloride after degradation is displayed in Fig. 10.6.

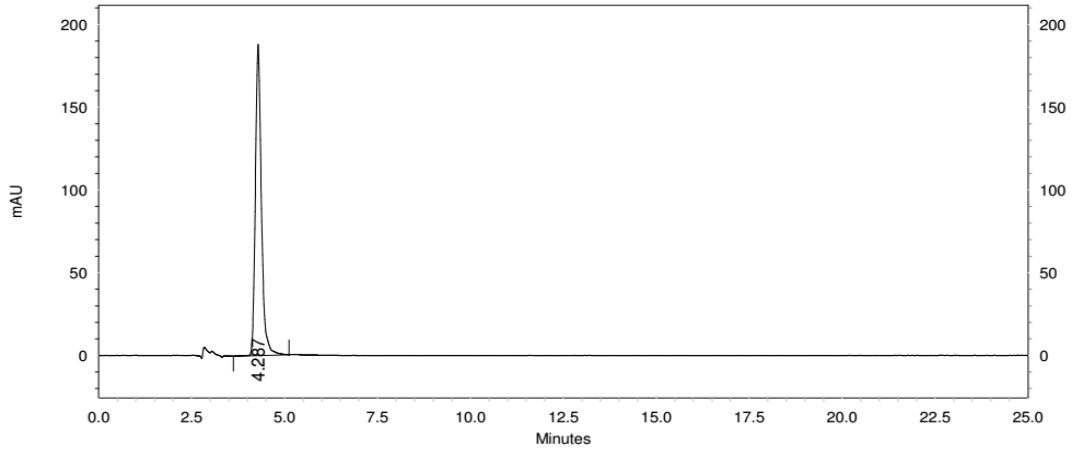

FIGURE 10.6 Chromatogram of Azelastine hydrochloride after acid degradation.

Peak purity of the Azelastine hydrochloride after acid induced degradation is 1.000, which shows studied drug was stable in acid induced stress condition.

- **Base induced degradation study**

Peak area of Azelastine hydrochloride was not changed significantly and any additional peak was not observed when chromatographed after refluxing in 5 N sodium hydroxide at 80°C for 5 hr. Peak purity of the Azelastine hydrochloride after base induced degradation is 1.000, which shows the purity of Azelastine hydrochloride peak. Chromatogram of Azelastine hydrochloride after degradation with 5 N NaOH at 80° C for 5 hr is displayed in Fig. 10.7.

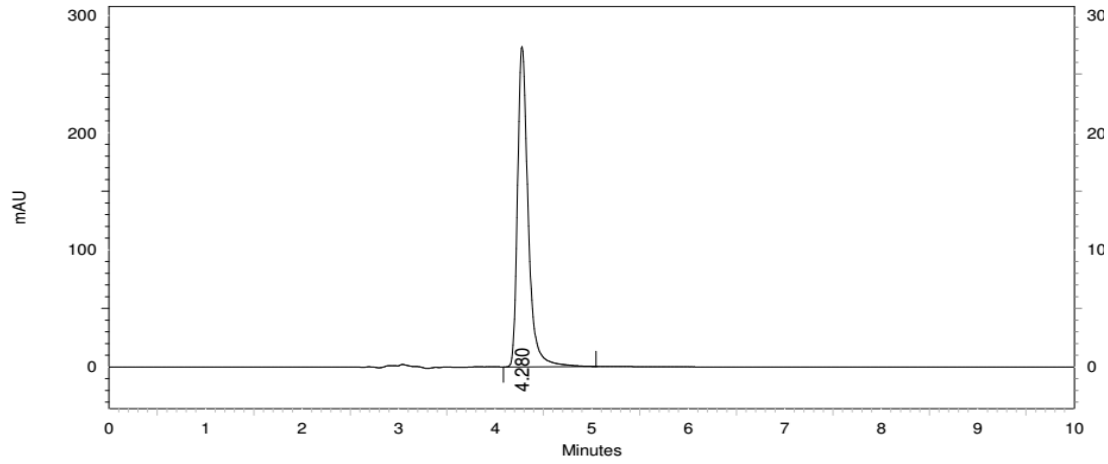

FIGURE 10.7 Chromatogram of Azelastine hydrochloride after base degradation.

- **Wet heat degradation**

Peak area of Azelastine hydrochloride was not changed significantly and any additional peak was not observed when chromatographed after refluxing in distilled water at 80°C for 5 hr. Peak purity of Azelastine hydrochloride peak after wet heat degradation is 1.000. Chromatogram of Azelastine hydrochloride after degradation with water at 80° C for 5 hr is displayed in Fig. 10.8.

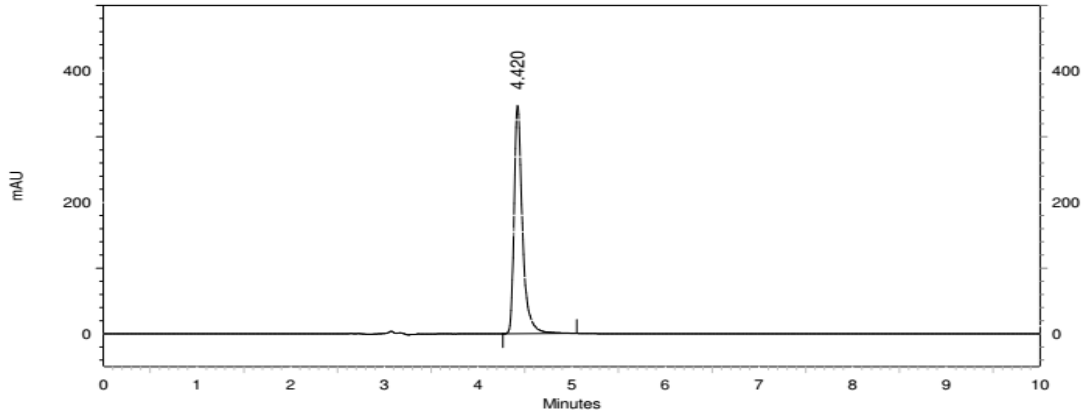

FIGURE 10.8 Chromatogram of Azelastine HCl after degradation with water

- **Oxidative degradation study**

Peak area of Azelastine hydrochloride was not changed significantly and any additional peak was not observed when chromatographed after refluxing in 30% H_2O_2 at 80°C for 5 hr. Peak purity of Azelastine hydrochloride peak after oxidative degradation is 1.000. Chromatogram of Azelastine hydrochloride after degradation with 30% H_2O_2 at 80° C for 5 hr is displayed in

Fig. 10.9. Hydrogen peroxide retention time is 2.9 min. which was confirmed from chromatogram of 30% hydrogen peroxide.

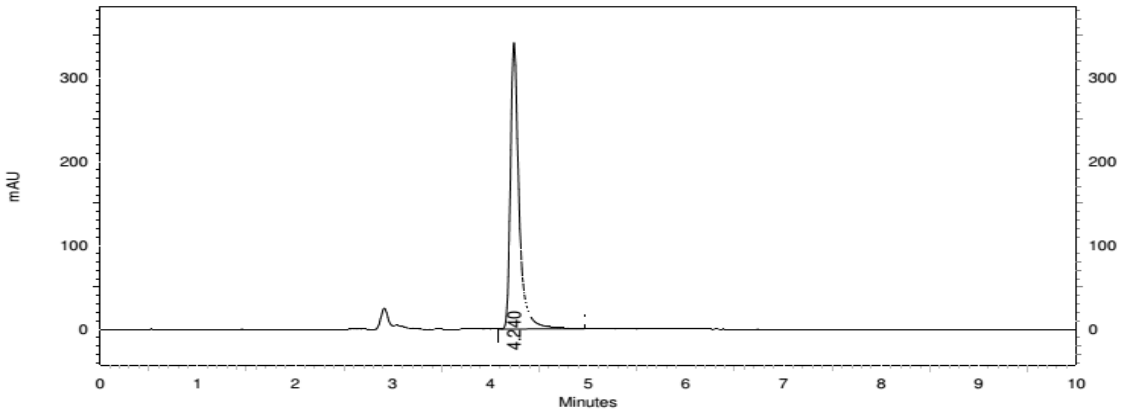

FIGURE 10.9 Chromatogram of Azelastine HCl after degradation with H_2O_2.

- **Thermal degradation**

Peak area of Azelastine hydrochloride was not changed significantly and any additional peak was not observed when chromatographed after exposing at 70°C for 8 hr. Peak purity of Azelastine hydrochloride peak is 1.000. Chromatogram of Azelastine hydrochloride after thermal degradation is displayed in Fig. 10.10.

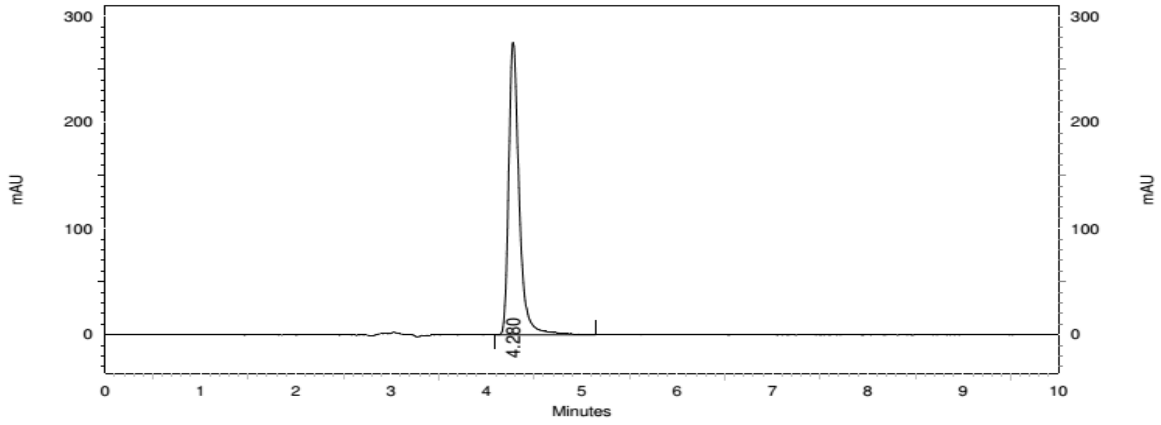

FIGURE 10.10 Chromatogram of Azelastine hydrochloride after thermal degradation

- **Photolytic degradation**

Peak area of Azelastine hydrochloride was not changed significantly and any additional peak was not observed when chromatographed after exposing at 365 nm for 24 hr. Peak purity of Azelastine hydrochloride peak is 1.000. Chromatogram of Azelastine hydrochloride after photolytic degradationin under con trolled condition is displayed in Fig. 10.11.

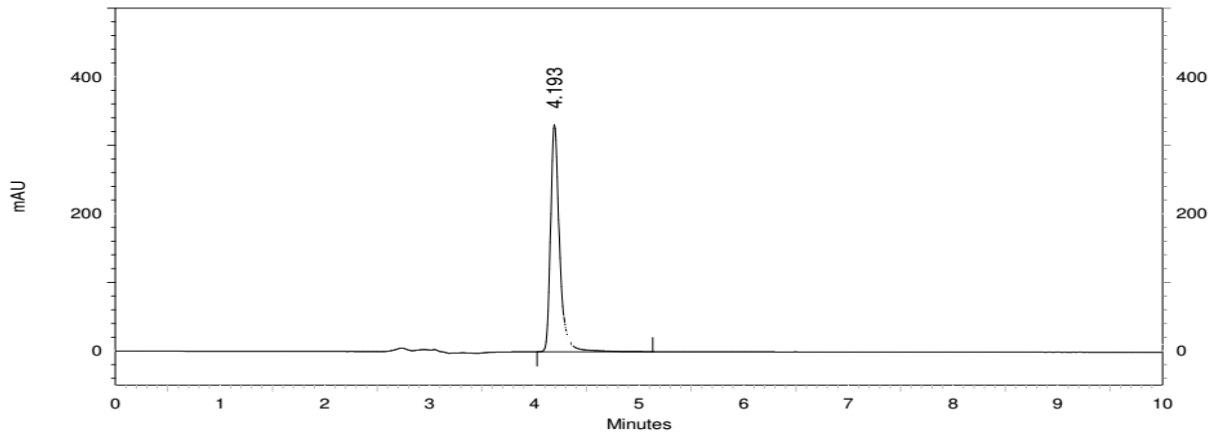

FIGURE 10.11 Chromatogram of Azelastine hydrochloride after photolytic degradation.

- **Robustness**

It was found that method was robust regarding any minor deviation in column temperature (45±1°C), mobile phase pH (3.0±0.2), and mobile phase composition (±2%) as confirmed by the constant value of retention time and the peak area of Azelastine hydrochloride. Small deviation in mobile phase flow rate (1.0 ± 0.1 mL/min.) resulted in significant deviation in the retention time and area of the Azelastine hydrochloride peak. Table 10.11 shows the results of the robustness.

TABLE 10.11 Robustness results of RP-HPLC for Azelastine hydrochloride.

Parameter	Value	Retention time	% RSD	Area	% RSD
Flow rate (mL/min.)	0.9	4.7835	10.1015	4915124	7.587
	1.0	4.5465		4588800	
	1.1	3.92		4221270	
Temperature (column)	44°C	4.313	0.2879	4646260	0.1460
	45°C	4.3095		4644688	
	46°C	4.29		4637742	
pH	2.8	4.127	0.1056	4759309	0.5586
	3	4.128		4716047	
	3.2	4.12		4764238	
Composition (ACN:OPA v/v)	48:52	4.273	1.6170	4695003	0.7669
	50:50	4.27		4716047	
	52:48	4.153		4765591	

- **LOD and LOQ**

LOD and LOQ were determined as per ICH guideline using mathematical formula. Obtained value for LOD was 0.8056µg/mL and obtained value for LOQ was 2.4410µg/mL. Small value of LOD and LOQ expressed that the developed method can be used for detection and quantification of Azelastine HCl in QC samples.

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

= 3.3 * (11129.94/45594.67)

= 0.8056 µg/mL

LOQ = 10 *(standard deviation of y-intercept/slope)

= 10* (11129.94/45594.67)

= 2.4410 µg/mL

10.2.5 Application of the method

The proposed liquid chromatographic method was used to analyze Azelastine hydrochloride in Arzep nasal spray solution after exposure to 40°C 75% for 1 month in stability chamber. The assay of drug product was 99.78% while the assay of drug product after accelerated stress was around 98.51%. In chromatograms of drug sample from nasal spray preparation, no interference was observed from excipients.

10.3 References

1. https://www.pmda.go.jp/files/000203134.pdf, Infrared Reference Spectra, JP XVI, 1788
2. https://www.drugbank.ca/drugs/DB00972

CHAPTER 11

Summary and Conclusion

11.1 Summary

11.1.1 Development of method

Table 11.1 shows the chromatographic conditions of the developed methods for selected antihistaminic drugs.

TABLE 11.1 Chromatographic conditions of the developed methods

Parameter	Bilastine	Ebastine	Azelastine hydrochloride
Stationary Phase	Discovery C_8 (250 x 4.6 mm, 5µm) column	Kromasil C_{18} (250 x 4.6 mm, 5µm) column	Waters Spherisorb CN (250 x 4.6 mm, 5µm) column
Mobile phase	Methanol and 0.1% Ortho-phosphoric acid in the ratio of 55:45 v/v	Acetonitrile and 0.5% ortho-phosphoric acid in ratio of 68:32 v/v	Acetonitrile and 0.05 M KH_2PO_4 buffer pH 3.0 in ratio of 50:50 v/v
Flow rate	1.0 mL/min.	1.0 mL/min.	1.0 mL/min.
volume of injection	10 µL	20 µL	20 µL
Temperature of column	35˚C	40˚C	45˚C
Diluent	Methanol	Acetonitrile	Acetonitrile
Detector	PDA	PDA	PDA
Retention time	3.833 min.	4.27 min.	4.38 min.

11.1.2 Validation of method

Table 11.2 presents summary of validation results for the developed HPLC method for selected drugs.

TABLE 11.2 Validation results of developed methods

Validation parameter	Ebastine	Azelastine	Bilastine
Linearity	5-120 µg/mL	5-120 µg/mL	25-150 µg/mL
Correlation co-efficient (r^2±SD)	0.9995	0.9997	0.9996
Precision (%RSD)			
Repeatability	1.645 (n=7)	1.381 (n=7)	0.253 (n=6)
Intraday precision (n=3)	0.979	0.413	0.941
Interday precision(n=3)	1.918	1.092	1.107
Accuracy (% recovery)	101.15±0.28	100.05±0.95	99.55±0.26
LOQ	0.91 µg/mL	2.44 µg/mL	0.57 µg/mL
LOD	0.30 µg/mL	0.81 µg/mL	0.19 µg/mL
Robustness	Robust in column temperature	Robust in temperature of column, composition of mobile phase and pH of mobile phase	Robust in column temperature

11.1.3 Forced degradation study

Chromatograms of Bilastine degraded with 2 N HCl yielding the degradation product at 4.824 min. with resolution of 3.6. Bilastine degraded with 10% hydrogen peroxide yielding the degradation product at 4.612 min. with resolution of 2.3. In all degradation sample purity threshold value was found greater than purity angle for Bilastine peak which indicates that purity of the drug peak. No significant change in area of Bilastine peak and no additional peak were observed when chromatographed after application of base, neural hydrolytic, thermal and photolytic stress condition.

Chromatograms of Ebastine degraded with 0.1 M HCl yielding the degradation product at 2.317 minute with good resolution and Ebastine having peak purity of 0.999. Ebastine degraded with 1% hydrogen peroxide yielding the degradation product at 3.943 minute with resolution of 1.56 and Ebastine having peak purity of 0.999. Chromatograms of stressed samples of Ebastine after refluxing with 1 N NaOH for 5 hr at 80° C, with distilled water for 5 hr at 80° C and in dry heat conditions furnished no additional peaks. No additional peak and no significant change in initial concentration of Ebastine were observed for drug powder kept at 365nm (long wavelength U.V.) for 2 days.

Significant change in area of Azelastine hydrochloride peak and any additional peak was not observed when chromatographed after refluxing in 5M HCl at 80°C for 5hr, 5M NaOH at 80°C for 5hr, 30% H_2O_2 at 80°C for 5hr, for drug in oven at 70°C for 8 hr and drug kept at 365 nm for 1 day.

11.1.4 Degradation kinetic study

Degradation kinetic study of Ebastine in 0.1 N HCl and 1% Hydrogen Peroxide and degradation kinetic study of Bilastine in 2 N HCl were performed.

It was found that degradation of Ebastine in acidic condition followed first order kinetics and in oxidizing condition provided by hydrogen peroxide followed zero order kinetics. Half time of Ebastine at room temperature in acidic and oxidative condition was calculated to be 11,403.67 min. (190.06 hr) and 503.32 min. (8.39 hr), respectively. The activation energy was calculated 18.272 and 8.685 Kcal/mol for acidic and oxidative condition, respectively.

It was found that degradation of Bilastine in acidic condition followed first order kinetics. Half time of Bilastine at room temperature in acidic was calculated to be 1776.92 min. (29.62 hr). The activation energy was calculated 11.340 Kcal/mol for acidic condition.

11.1.5 Identification of degradation products

Mass spectra and likely fragmentation pattern of degradation product of acid stressed Bilastine sample confirms that Bilastine in presence of hydrochloric acid reacts with methanol and produced methyl ester of Bilastine. Mass spectra and fragmentation pattern of degradation product of hydrogen peroxide stressed Bilastine sample confirms that Bilastine reacts with oxidizing agent, hydrogen peroxide and produces N-oxide of bilastine.

Mass spectra and likely fragmentation pattern of degradation product of acid stressed Ebastine sample confirms that reaction of Ebastine with hydrochloric acid does the hydrolysis of ether linkage and produces 1-(4-tert-butylphenyl)-4-[4-(hydroxy) piperidin-1-yl]butan-1-one. Mass spectra and fragmentation pattern of degradation product of hydrogen peroxide stressed Ebastine sample confirms that Ebastine reacts with oxidizing agent, hydrogen peroxide and produces N-oxide of Ebastine.

11.2 Conclusion

Statistical analysis proves that the method is accurate, precise, specific and suitable for analysis of Bilastine in the pharmaceutical product without interference from excipients. 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 acidic hydrolysis and oxidation; further kinetic investigation was carried out to check rate of reaction in acidic medium. All validation parameter is permitting the application of suggested method in quality control laboratory for assessment of Bilastine. Described method is stability indicating one. Degradation products of Bilastine produced in acidic condition and oxidizing condition were also identified by LC-MS study.

Conclusion from the results states that the proposed method for estimation of Ebastine is precise, specific, accurate and stability-indicating. This method was validated as per ICH guideline. A stress degradation study as per ICH guideline was carried out to investigate the degradation behaviour of Ebastine; further kinetic investigation was preferred to check rate of degradation in acidic and oxidation condition. All validation parameter is permitting the application of suggested method in quality control laboratory for the quantitative estimation of Ebastine in tablet dosage form. Degradation products of Ebastine produced in acidic condition and oxidizing condition were characterized by LC-MS study.

It can be concluded from the results that the proposed RP-HPLC method for determination of Azelastine hydrochloride is simple, precise, specific, accurate and stability-indicating. This method is adequate for analysis of Azelastine hydrochloride in the raw material and the pharmaceutical product without interference from excipients. A stress degradation study was performed under ICH recommended condition to investigate the degradation behaviour of Azelastine hydrochloride; it shows that Azelastine hydrochloride is stable drug.

CHAPTER 12

Publications

1. Shital Patel, T Y Pasha, "Stability Indicating HPLC Method for Antihistamine Drug Ebastine along with Degradation Kinetic Study", International Journal of Current Advanced Research, Vol. 7(1), pp. 9372-9376, ISSN: O: 2319-6475, P: 2319-6505.
2. Shital Patel, TY Pasha, "Stability-Indicating High-Performance Liquid Chromatography Method for Determination of Antihistamine Drug Azelastine", Asian Journal of Pharmaceutical and Clinical Research, Vol. 11(8), pp. 248-251, ISSN: O: 2455-3891, P: 0974-2441.

- Arising article- Stability-Indicating Method and LC–MS-MS Characterization of Forced Degradation Products of Bilastine