ANALYSIS OF THE PHARMACEUTICALS BY CHROMATOGRAPHIC TECHNIQUES

A THESIS

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IN

THE FACULTY OF SCIENCE (CHEMISTRY)

BY

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UNDER THE GUIDANCE

OF

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CERTIFICATE

This is to certify that the work entitled "**Analysis of the Pharmaceuticals by Chromatographic Techniques"** is a piece of research work done by Mr. Prakash M. Davadra under my guidance and supervision for the degree of Doctor of Philosophy. The thesis has been prepared under my supervision for the submission at Saurashtra University, Rajkot, India.

To the best of my knowledge, the thesis contains original work. Any related work from the literature has been duly acknowledged.

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DECLARATION

I declare that the thesis entitled "**Analysis of the Pharmaceuticals by Chromatographic Techniques**" is my own work conducted under the supervision of Dr. Atul H. Bapodra, Chemistry Department, M. D. Science College, Porbandar. However, the work was conducted at the Dept. of Biopharmaceutics, Zydus Research Centre, Ahmedabad.

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Dedicated
To my
Parents

ACKNOWLEDGEMENTS

First and foremost I wish to pay my homage and respects to "My Parents" without whose blessing this task would not have been accomplished. I bow my head in utter humility and complete dedication from within my heart. My salutations to the Omnipresent, Omniscent and Almightly God ***(Lord Shreeram),*** *the glorious fountain and continuous source of inspirations!*

A journey is easier when you travel together. Interdependence is certainly more valuable than independence. This thesis is the result of three years of work whereby I have been accompanied and supported by many people. It is with great pleasure that I have now the opportunity to express my gratitude for all of them. (My sincere apologies to anyone inadvertently omitted).

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Doing a Ph.D. is one of the best decisions of my life and it is impossible to forget stimulating best wishes, whole hearted support, affection and constant encouragement of my dear friends ***Dr. Kachhadia, Dr. Pritam, Dr. Meera, Dr. Rupesh, Mr. Ram Vijay,*** *and* ***Mr. Renish.***

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While discussing literature review and references, due credit has been given to authors and their references have been appropriately acknowledged. However, if any shortcomings are observed in this regard, it should be treated as completely unintentional.

Prakash

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Synopsis

A comprehensive summary of the work to be incorporated in the thesis entitled "**Analysis of the Pharmaceuticals by Chromatographic Techniques**" has been presented in two parts as follows.

PART - [A]: APPLICATION OF THE CHROMATOGRAPHY FOR THE SEPARATION OF ISOMERS OF SOME PHARMACEUTICALS.

PART - [B]: APPLICATION OF THE CHROMATOGRAPHY FOR THE IMPURITY PROFILING OF ACTIVE PHARMACEUTICAL INGRADIENT.

Aim of Work

The objective of the work is to study the use of chromatographic techniques for the analysis of pharmaceuticals for different applications. Pharmaceutical drugs are very specific in their action therefore, it is very essential to have analytical methodology for the separation of isomers, impurities and degradants from the desired substance.

Experimental Approach

Development of Analytical Method

- Selection of mobile phase
- Selection of stationary phase
- Selection of column temperature and flow rate
- Selection of wave-length for detection
- Injection volume / Concentration
- Selection of diluent

Validation of Analytical Method

- System suitability/Specificity study
- Linearity study
- Precision & Intermediate precision study
- Accuracy/Recovery study

- Robustness study
- Solution stability study
- Limit of detection and limit of quantification

PART - [A]: APPLICATION OF THE CHROMATOGRAPHY FOR THE SEPARATION OF ISOMERS OF SOME PHARMACEUTICALS.

The research work undertaken in these studies mainly addresses liquid chromatographic separation of different types of isomers, such as, enantiomers, diastereomers and *E/Z* isomers. It covers method development and its validation as per ICH guidelines for pharmaceutical compounds.

Section-I : HPLC Separation of Enantiomers of Duloxetine Hydrochloride

Duloxetine (Fig. 1), [*(S)*-N-Methyl-3-(1-naphthalenyloxy)-3-(2-thienyl) propanamine] is an antidepressant drug. Duloxetine is a potent and balanced dual re-uptake inhibitor of serotonin (5-hydroxytryptamine, 5-HT) and norepinephrine(NE). Its pharmacological effects are mainly due to *(S)*-enantiomer while the *(R)*-enantiomer is considered to be inactive.

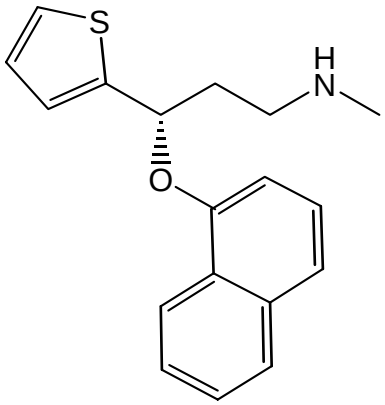


Fig. 1 : Chemical Structure of *S*(+)-Duloxetine

Developed Chromatographic Parameters

- Mobile phase: - Sodium acetate buffer (pH 3.8; 10 mM)-acetonitrile (93:07, v v)
- Column: - Chiral-AGP (150 mm 4.0 mm, 5 m)
- Flow rate: - 1 mL/min
- Column temperature: - 20° C
- Wavelength: - 220 nm
- Injection volume: - 5 µL
- Diluent: - Mobile phase

The developed method has been validated as per ICH guideline. Interestingly, distomer was eluted prior to eutomer in the developed method. The proposed method was found to be suitable, precise, linear, accurate and robust for the quantitative determination of *(R)*-enantiomer in bulk drug.

Section-II: HPLC Separation of Two Diastereomers of 2-[4-(Methylsulfonyl)Phenyl]-3-(3*(R)*-oxocyclopentyl)Propanoic Acid

The title compound, 2-[4-(methylsulfonyl)phenyl]-3-(3*(R)*-oxocyclopentyl)propanoic acid (Fig. 2) has novel skeleton and is an interesting molecule for generation of pharmacologically active compounds. This study examines the efficiency of crystallization induced resolution and monitors chiral enrichment/separation of two diastereomers.

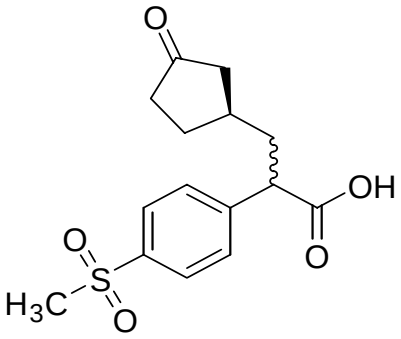


Fig. 2 : Chemical Structure of 2-[4-(methylsulfonyl)phenyl]-3-(3*(R)*-oxocyclopentyl)propanoic acid

Developed Chromatographic Parameters

- Mobile phase: - 0.05 % Triflouroacetic acid in water-Acetonitrile (85:15, v/v)
- Column: - J'sphere-ODS-H80 (150 mm 4.6 mm, 4 m, YMC make, Japan)
- Flow rate: - 1 mL/min
- Column temperature: - 30° C
- Wavelength: - 228 nm
- Injection volume: - 5 µL
- Diluent: - Mobile phase

The developed procedure has been evaluated over the system suitability, peak homogeneity, precision, limit of detection, limit of quantification, robustness and solution stability in order to ascertain the stability of the analytical method. It has been proved that proposed method is linear, precise, suitable and robust for its intended use.

Section-III: HPLC Separation of *E/Z* isomers of Acrivastine

Acrivastine is a second generation H1-receptor antagonist antihistamine and works by blocking H1 histamine receptors. It is chemically, (*E*)-3-{6-[(*E*)-1-(4-methylphenyl)-3-pyrrolidin-1-yl-prop-1-enyl]pyridin-2-yl}prop-2-enoic acid (Fig. 3). The current method , which may be further optimized is discussed below.

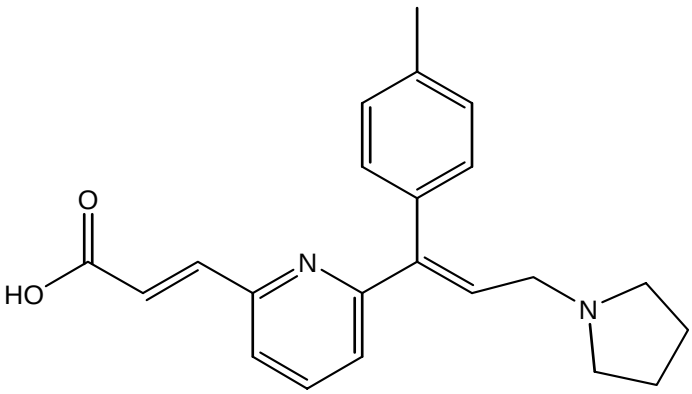


Fig. 3: Chemical Structure of Acrivastine

Chromatographic Parameters

- Mobile phase: - 0.05 % Triflouroacetic acid in water-Acetonitrile (72:28, v/v)
- Column: - Cosmosil packed column, 5C18-PAQ (250 x 4.6, 5µm)
- Flow rate: - 1.0 mL/min
- Wavelength: - 229 nm
- Injection volume: - 5 µL

The method has been validated as per ICH guideline. The proposed method was found to be rapid, precise, linear, accurate and robust for the quantitative determination of undesired Z-isomer in bulk drug.

PART - [B]: APPLICATION OF THE CHROMATOGRAPHY FOR THE IMPURITY PROFILING OF ACTIVE PHARMACEUTICAL INGRADIENT.

Impurity profiling constitutes a fascinating science in pharmaceutical industries. One encounters several adjectives eg. known, unknown, specified, unspecified, polymeric, organic volatile, metallic, inorganic, fibrous, polymorphic, etc. linked with the world of impurities. There are significant regulatory and IP consequences also linked with the world of impurities.

The research work undertaken in these studies mainly addresses use of chromatographic for the organic impurity profiling of API. Method development and validation for the determination of process related impurities is presented in section-I while formation, isolation and characterization of degradant impurities are discussed in section-II for drug substance.

Section-I : A UPLC Method For The Determination of Process-Related Impurities in Azathioprine

Azathioprine (Fig. 4) is the nitro imidazole derivatives of 6-mercaptopurine. It is an immunosuppressive drug co-administered with cyclosporine and corticoids to prevent rejection after transplantation.

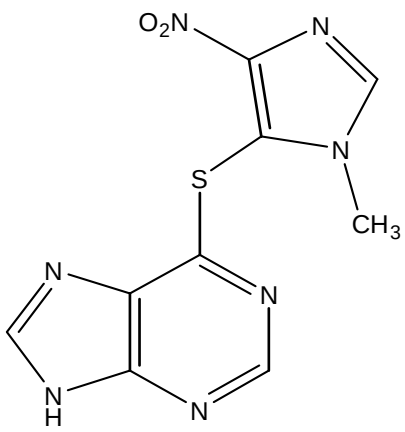


Fig. 4: Chemical Structure of Azathioprine

Chromatographic Parameters

- Mobile phase: - (A) 0.05 % Triflouroacetic acid in water : (B) Acetonitrile
- Gradient Program: Time/B (%) : 1/3, 3.5/60, 4/60, 4.1/3, 5/3
- Column: - Acquity UPLC BEH C18 (100 mm 2.1 mm, 1.7 m)
- Flow rate: - 0.35 mL/min
- Wavelength: - 220 nm
- Injection volume: - 1 µL
- Diluent: - 0.1 % Ammonia in methanol

The developed method has been validated for the process-related impurities. Validation parameters such as, specificity, precision, accuracy, linearity, limit of detection and limit of quantification have been studied. The proposed method was found to be useful for rapid monitoring of impurities during process development.

Section-II :Isolation and Characterization of Degradation products of Pioglitazone Hydrochloride

Pioglitazone hydrochloride (Fig. 5), is an oral anti-hyperglycemic agent which acts primarily by decreasing insulin resistance. It is used in the treatment of type-II diabetes.

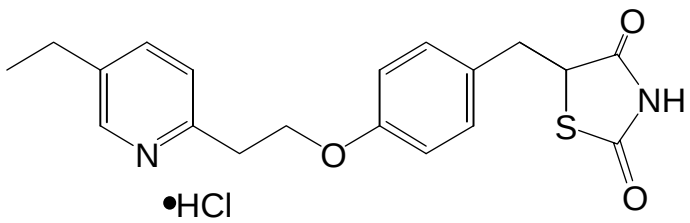


Fig. 5: Chemical Structure of Pioglitazone hydrochloride

Stress Testing

- Acid degradation
- Base degradation
- Peroxide degradation
- Thermal degradation
- Photolytic degradation

Stress testing has been monitored by HPLC. The significant degradation was observed under base treatment. The major degradation products were isolated by preparative HPLC and characterized by spectroscopic teqchniques such as, Mass, NMR (^{1}H and ^{13}C), IR and UV.

Introduction

1.0 Analysis of Pharmaceuticals

Pharmaceutical analysis simply means analysis of a pharmaceutical(s). Webster's dictionary defines a pharmaceutical as a medicinal drug. It is generally known that a pharmaceutical is a chemical entity of therapeutic interest. In an ideal world, the need for analysis of pharmaceuticals should be driven by the desire to assure the quality of a drug substance and product. However, in the real world the need for pharmaceutical analysis is driven largely by regulatory requirements. This stems from the fact that regulatory considerations loom large when a commercial product does not meet its purported quality. A dose examination of new drug development quality can not be tested into the drug; it has to be built into it. This means that a great deal of consideration has to go in from the very start, when NCE is developed to assure that it has the proposed structure, crystal form and stereo chemical structure. Additionally, it is critical that it meet high quality standards necessary for a product that is to be administrated to humans.

The pharmaceutical industry in under increased scrutiny from the government and public interest groups to contain costs and get consistently deliver to market safe, efficacious drugs that fulfill unmet medical needs. Analytical chemistry has played a major role in the changes facing the pharmaceutical industry today. Traditionally, viewed as a service organization, the analytical department has become a significant partner in the drug development process. Indeed, the demand for analytical data has become a critical path activity for the selection of candidate molecules for full development. The science and technology utilized today coupled with new regulation that are now binding, have made pharmaceutical analysis much more complicated compared to what it has as little as fifteen years ago.

The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the

pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs.

Also quality is important in every product or service but it is vital in medicines as it involves life. Quality control is a concept, which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stage of production. The decision to release or reject a product is based on one or more type of control action. With the growth of pharmaceutical industry during last several years, there has been rapid progress in the field of pharmaceutical analysis involving complex instrumentation.

The drugs is very specific in their action, other than drug itself, anything present in bulk is consider as a impurity of that drug. It may be undesired enantiomer, undesired diastereomer, undesired geometric isomer, related substances, degradant, residual solvents, inorganic substances and any contaminant. Therefore, to control quality of the drug and to support process research, we needs reliable analytical methods for the separation and determination of all this different kinds of impurities.

2.0 Chromatography

When discussing chromatography, the first question usually asked refers to the name and its meaning. In other words: *What is chromatography?*

Tswett in his basic papers published in 1906 [1, 2] gave a very pragmatic definition: he simply stated that the method in which different components of a mixture are separated on an adsorbent column in a flowing system is a "chromatographic method." The authors of the first books on chromatography (e.g., Zechmeister and Cholnoky [3] and Strain [4]) did not try to exactly define the technique either. The first detailed definition we could find was given by Zechmeister in his second book: :The term 'chromatography' designates those processes which allow the resolution of solute mixtures by selective fixation and liberation on a solid surface of a support , with the aid of a fluid streaming in a definite direction ."

As is true with technique in science, chromatography was not developed at a certain instant, on a single day of the calendar. In fact, "chromatography " actually exists in nature: the composition of fluids –liquids and gases-migrating underground through clay, soil , and other sediments can often be explained by a "chromatographic separation " during migration.

Chromatography is now more than 105 years old. It was originally developed as a technique for the separation of plant pigments, which, according to present day terminology, would be termed liquid-solid (adsorption) chromatography. For the first 20 years, may be a dozen or even fewer people utilize the new technique. The real upswing in the use of chromatography started in the 1930s and, in less than a decade, chromatography became an important laboratory technique. During this period new variants, such as thin-layer and ion-ex-change chromatography and electrophoresis also came into existence. The 1940s saw the invention of partition chromatography and the development of paper chromatography, another important variant of liquid chromatography, and, finally, gas chromatography, albeit first in the gas –solid form. The early 1950s brought about the development of gas-liquid partition chromatography, which by 1955, started an evolution hitherto unparalleled in laboratory techniques. Other important results originating in the 1950s are the utilization of ion-exchange chromatography for the analysis of amino acids, the development of the theory of chromatography as a differential migration process, and, by the end of the decade, gel filtration technique. Finally, by the middle of the 1960a the knowledge accumulated in the previous two decades in general chromatographic theory and in the practice of liquid and gas chromatography initiated the modernization of liquid chromatography, which, by the end of the 1960s the started another exponential development rapidly "catching up " gas chromatography.

Chromatography, in its various forms, is also a very versatile technique. It can be carried out in a column or on a plate, and the sample may equally be a solid, a liquid, or a gas. Present-day instruments used in chromatographic techniques are highly sophisticated, with automated controls and data handling.

3.0 High Performance Liquid Chromatography (HPLC)

HPLC was introduced to pharmaceutical analysis not long after its discovery in the late 1960s. By now it has developed into a generally applicable analytical method providing rapid and versatile separation possibilities that meet the increasing requirements for purity testing of bulk pharmaceuticals and pharmaceutical products. It is also suitable for the determination of drugs in biological and environmental samples. It provides a number of highly selective variants to resolve almost every type of separation problem: on the basis of this, HPLC and related techniques can be regarded as the most importance of HPLC can be characterized by the very high

number of books devoted to this subject. The most important ones published in the last decade [5-11] with emphasis of pharmaceutical and biomedical applications [12-16] are listed.

In the beginning, major attempts were first made to develop highly efficient systems leading to a dramatic improvement in column technology using micro-particulate packing and to an optimized design of column hardware resulting in extremely low extra-column peak dispersion through the column. In the later stage, development of selective stationary phases was attempted. The fundamentally important steps in creating highly selective phase systems include rigorous control of surface chemistry and adjustment of the final stationary phase properties by appropriate mobile phase selection, leading to specific solute-surface interactions and suppressing undesired interactions. Similarly, significant advances in the instrumentation to further improve separation selectivity (detection, raw data manipulation, etc.) and efficiency (injection techniques, extra-column peak broadening, etc.) have been made. The high level of HPLC instrumentation regarding speed, accuracy, precision and reliability meets the special requirements of purity tests in pharmaceutical analysis.

3.1 Instrumentation of HPLC

The basic components of HPLC are pumping system, sample introduction device, chromatographic column, detector and data handling device [17-21]. A schematic diagram of HPLC equipment is shown in Fig. 1 [22].

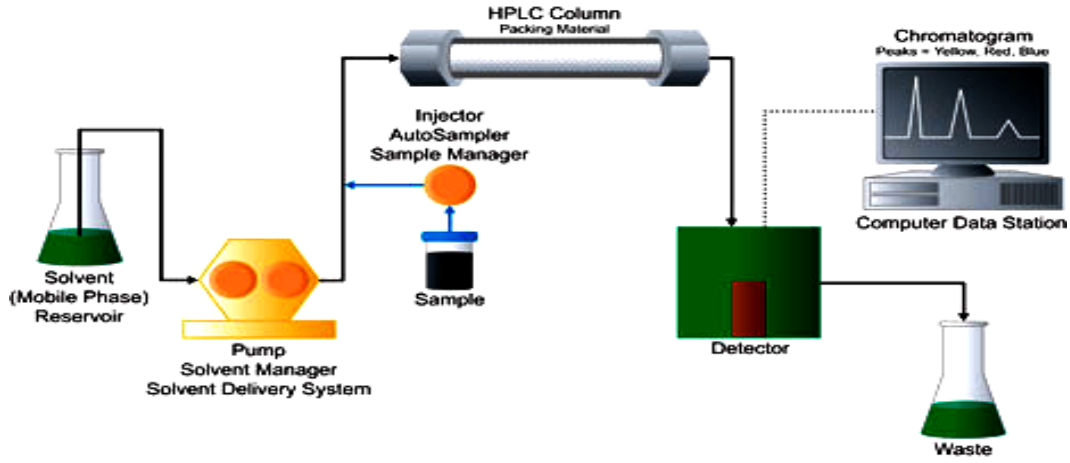


Fig. 1 : Schematic diagram of HPLC equipment

3.1.1 Pumping System

The HPLC pump is very important component of the system. It delivers the constant flow of the mobile phase. Its performance directly affects retention time, reproducibility and detector sensitivity. There are mainly, three types of pumps are used in HPLC to propel the liquid mobile phase through the system are as under

Displacement pump: It produces a flow that tends to independent of viscosity and backpressure and also output is pulse free. But it possesses limited capacity (250 ml).

Reciprocating pump: It has small internal volume (35 to 400 µl), their high output pressure (up to 10,000 psi) and their constant flow rates. But it produces a pulsed flow.

Pneumatic or constant pressure pump: They are pulse free, suffer from limited capacity as well as a dependence of flow rate on solvent viscosity and column backpressure. They are limited to pressure less than 2000 psi.

There are two type of pumping systems mode are used: isocratic and gradient.

Isocratic: In the system, the things are kept constant throughout the run. In the case of pumping of mobile phase, the mobile phase composition is kept constant throughout the run. The nominal flow rate accuracy required is ± 1 % of the set flow

Gradient: There is some change purposely incorporated during the particular sample run to achieve a better or/and faster separation. In case of pumping mobile phase, the composition of mobile phase is continuously varied during the particular run. The gradient accuracy of ± 1 % of the step gradient composition is typical.

3.1.2 Sample Introducing Device

It is not possible to use direct syringe injection on column like GC, as the inlet pressure in LC is too high. Insertion of the sample onto the pressurized column must be as a narrow plug so that the peak broadening attributable to this step is negligible. The injection system itself should have no dead (void) volume. There are three important ways of introducing the sample into injection port.

Loop injection: In which, a fixed amount of volume is introduced by making use of fixed volume loop injector.

Valve injection: In which, a variable volume is introduced by making use of an injection valve.

On column injection: In which, a variable volume is introduced by means of a syringe through a septum.

3.1.3 Chromatographic Column

Column is a heart of chromatography. The column is usually made up of heavy glass or stainless steel tubing to withstand high pressure. The columns are usually 10-30 cm long and 4-10 mm inside diameter containing stationary phase at particle diameter of 25 μm or less.

The packing used in modern HPLC column consists of small, rigid particles having a narrow particle size distribution. There are three main types of column packing in HPLC.

Porous, polymeric beds: Porous, polymeric beds based on styrene divinyl benzene co-polymers used doe ion exchange and size exclusion chromatography. For analytical purpose these have now been replaced by silica based, packing which are more efficient and more stable.

Porous layer beds: Consisting of a thin shell (1-3 μm) of silica or modified silica on an spherical inert core (e.g. Glass). After the development of totally porous micro particulate packings, these have not been used in HPLC.

Totally Porous silica particles (dia. < 10 μm): These packing have widely been used for analytical HPLC in recent years. Particles of diameter > 20 μm are usually dry packed. While particles of diameter < 20 μm are slurry packed in which particles are suspended on a suitable solvent and the slurry so obtained is driven into the column under pressure.

3.1.4 Detector

The function of the detector in HPLC is to monitor the mobile phase as it merges from the column. There are several detectors available in the market. However UV-Visible detector, photo diode array detector, fluorescence detector, conductometric and coulometric detector are more commonly used. The new ELSD detector is proving to be important detector, while the MS detector is outstanding. Detectors are usually of two types:

Bulk property detectors: It compares overall changes in a physical property of the mobile phase with and without an eluting solute e.g. refractive index, dielectric constant or density.

Solute property detectors: It responds to a physical property of the solute, which is not exhibited by the pure mobile phase e.g. UV absorbance, fluorescence or diffusion current.

4.0 Ultra Performance Liquid Chromatography (UPLC)

Increasing demand for greater pharmaceutical analysis throughput promoted the testing of the Ultra Performance Liquid Chromatography. This system claims to provide faster analysis through the use of a novel separation material of a very fine particle size (1.7 μm) and unique core chemistry.

The UPLC System is up to nine times faster, has up to twice the peak resolution, and is up to three times more sensitive than traditional HPLC. Combine UPLC System with mass spectrometry systems to satisfy regulatory detection limits and significantly increase the throughput of laboratory.

UPLC takes advantage of technological strides made in particle chemistry performance, system optimization, detector design and data processing and control. This new category of analytical separation science retains the practically and principles of HPLC while increasing the overall interlaced attributes of speed, sensitivity and resolution. The picture of UPLC is shown in Fig. 2.

The UPLC system has been holistically designed to match the performance needs of innovative column chemistries with robust hardware, easy-to-use software.

Small, pressure-tolerant particles

High-pressure fluidic modules

Minimized system volume

Negligible carryover

Reduced cycle times

Last response detectors

Integrated system software and diagnostics

Fig. 2: Waters ACQUITY UPLC system

The UPLC system's high-pressure fluidics optimizes flow rates to make the most of small particle technology. The UPLC system's sample-handling design is designed to ensure exceptionally low carryover and reduced cycle time. And when interfaced with the sample organizer, it increases unattended sample capacity by ten times. High-speed detectors, both optical and mass, contribute to increased sensitivity and help manage the heightened speed and resolution requirements of UPLC. The both MassLynx™ and Empower™ software support data management capabilities.

5.0 Method Development

A variety of patterns have been used to develop analytical separations. The discussion here is limited to conventional chromatographic approaches. These approaches are advanced and often based on intuitive judgment and the knowledge of the chromatographer. Where as individual approaches may exhibit considerable diversity, method development often follows the series of steps summarized below.

Before starting the method development, it is needed to review what is known about the sample. The aim of method development should also define at separation

stage. The important information about sample of interest, which may help in method development strategy, there are nature of sample, number of components present, chemical structure, molecular weight, pKa value, solubility and sample type (ionic or non-ioinic).

The choice of the mode of HPLC method should be made principally from the properties of the sample to be analyzed such as, molecular weight (MW), polarity and solubility.

After selecting mode of HPLC, the choice of the column (stationary phase) should be made after thorough consideration of mode of chromatography, column-to-column variability, and a number of other considerations. The silica particles may be acquired in a variety of sizes, shapes, and degrees of porosity. In addition, various functional groups or polymeric layers can easily be attached to the silica surface, extending the usefulness of these particles for applications to any specific HPLC method. Sufficient time is also allowed for column equilibration before starting the analysis.

The selection of mobile phase is very important parameter in HPLC method development as changing the mobile phase altars the selectivity. When selecting organic solvents for use in mobile phases, various physical and chemical properties of the solvent should be considered. Selected solvent should have low viscosity, compatible with the detection system, easily available in pure form, and low flammability and toxicity, if possible. UV cutoff values of solvents are also important consideration from the stand point of detection. The term polarity concerns the ability of a sample or solvent molecule to interact by combination of dispersion, dipole, hydrogen boding, and dielectric interactions. The combination of these four intermolecular attractive forces constitutes the solvent polarity.

Application of reagents such as buffers, ion-pairing reagents, or other modifiers (such as triethylamine) to the mobile phase is known to improve reproducibility, selectivity, or peak shape. Buffers are used primarily to regulate the pH and the acid-base equilibrium of the solute in the mobile phase. They may also be utilized to affect the retention times of ionizable compounds. The buffer capacity should be at maximum and should not vary in the pH range of 2 to 8 commonly used in HPLC. The buffers should be soluble, stable, inert to analytes and compatible to the detector.

By employing gradient elution mode, required % of the organic phase can be estimated rapidly. For aqueous sample mixtures, the best way to start is with gradient reversed phase chromatography. Gradient run can be started with 5 to 10% organic phase (acetonitile or methanol) in the mobile phase and can be increased up to 90% within 30 to 40 minutes. Separation can then be optimized by changing the initial mobile phase composition and the slope of the gradient according to the chromatogram obtained from the preliminary run.

For each samples, good analytical results should be obtained by careful selection of the wavelength used for detection. This choice requires known UV spectra of the sample. If analyte standard is available, its UV spectra can be measured prior to HPLC method development. Alternatively, PDA detector permits the acquisition of UV spectra for all sample components during method development. The wavelength selected for detection should provide acceptable response by the various components in the sample and no interference of baseline noise. Alternatively, relative response factor can be determined if two components do not have similar absorbance at single wavelength. In most cases HPLC method development is carried out with UV detector. Alterative detectors are required when sample have low or no UV absorbance.

Diluent for test preparation shall be selected at initial stage of development on the basis of solubility of the drug. However, optimization of the diluent is based on its extraction efficiency, effect on peak symmetry, peak interference in estimation and stability of analyte in diluent. Test concentration and injection volume shall be set according to suitability with extraction process and detector response. Wherever it is necessary to filter the test preparation, filter compatibility shall be check for selection of proper type of filter.

Unfortunately, theoretical predictions of mobile phase and stationary phase interactions with a given set of sample components are not always accurate, but they do help to narrow down the choices for method development. The separation scientist must usually perform a series of trial and error experiments with different mobile phase compositions until a satisfactory separation is achieved.

The parameters that are affected by the changes in chromatographic conditions are: resolution (R_s) capacity factor (k'), selectivity ( ), column efficiency (N) and peak asymmetry factor (A_s).

- **Resolution (Rs):**

The resolution of a column provides a quantitative measure of its ability to separate two analyses. Column resolution is defined as

$$Rs = \frac{2\,(t_2-t_1)}{W_1+W_2}$$

Where,

t_1 and t_2 are retention times of the first and second adjacent bands.

W_1 and W_2 are baseline at bandwidth of the peaks

Resolution for a given stationary phase can be improved by lengthening the column, thus increasing the number of plates. Resolution can be estimated in three ways.

Calculation based on above equation

Comparison with standard resolution curves

Calculations based on the valley between the two bands.

Resolution can be expressed in terms of three factors (k, a and N)

$$Rs = \frac{1}{4} \times \frac{(\alpha-1)}{\alpha} \times \frac{k}{1+k} \times N^{1/2}$$

Where,

k – Average retention factor for the two bands

N - Number of plates making up column

α – Selectivity factor

- **Capacity factor (k):**

It is the measure of the how well the sample molecule is retained by the column during and isocratic separation

$$k = \frac{t_R-t_0}{t_0}$$

Where,

t_R - band retention time

t_O - column dead volume

- **Selectivity ( ):**

It measures relative retention of two components. Selectivity is found of chromatographic surface (column), melting point and temperature

$$\alpha = \frac{k'_2}{k'_1} = \frac{V_2 - V_o}{V_1 - V_0}$$

Where,

V_1, V_2 – retention volume

V_0 –void volume

- **Column efficiency (N):**

It is called as number of theoretical plates. It indicates good column and system performance. It measure the band separation of a peak when band spread is smaller, the number of theoretical plate is higher.

Column performance can be defines in terms of values of N

$N = 16 (t_R/W)^2$ or 3500L (cm) dp (μm)

HETP = L/N

L-length of the column packing (cm)

Efficiency of column can be expressed by

$$HETP = H = A + B/\mu + C\mu$$

Where,

H = plate height in centimeters

μ = linear velocity of mobile phase in cm/sec

A = Multipath effect (Eddy diffusion)

B/μ = Longitudinal diffusion

Cμ = Mass transfer

- **Peak asymmetry**

Peak asymmetry and symmetry can result in inaccurate plate number, imprecise quantitation, decrease in detection limits and poor retention reproducibility It is calculated by

$$T = \frac{W_{0.05}}{2\,f}$$

Where,

$W_{0.05}$ = is the width of peak at 5 % peak height

f = distance at 5 % height from leading edge of peak to distance of peak maximum as measured at 5 % height.

The increased peak asymmetry value 1.5 the sign that the column should be changed.

6.0 Method Validation

Validation is an act of proving that any procedure, process, equipment, material, activity or system performs as expected under given set of conditions and also give the required accuracy, precision, sensitivity, ruggedness, etc.

When extended to an analytical procedure, the analytical method validation is the process of demonstrating that analytical procedures are suitable for their intended use. More specifically, analytical method validation is a matter of establishing documented evidence that provides a high degree of assurance that the specified method will consistently provide accurate test results that evaluate a product against its defined specification and quality attributes [23]. The U.S. Federal register states that, "validation data must be available to establish that the analytical procedures used in testing meet proper standards of accuracy and reliability [24]." All analytical procedures require some form of validation, regardless of whether the method is used for stability, in-process analysis, release, or acceptance testing.

Literature survey revealed that method validation has received considerable attention of industrial committees and regulatory agencies. The U.S. FDA cGMP [25] states for validation for the test methods employed by the firm. The U.S. FDA has also proposed industry guidance for Analytical Procedures and Methods Validation [26]. ISO/IEC 17025 includes a chapter on the validation of methods [27] with list validation parameters. The ICH [28] has developed a consensus text on the validation of analytical procedures. It has also developed guidance on detailed methodology [29]. The U.S. EPA prepared guidance for method's development and validation for the Resource Conservation and Recovery Act (RCRA) [30]. The AOAC, the EPA and other scientific organizations provide methods that are validated through multi-

laboratory studies. The USP has published specific guidelines for method validation for compound evaluation [31]. The WHO published validation guidelines under the title, 'Validation of analytical procedures used in the examination of pharmaceutical materials' in the 32nd report of the WHO expert committee on 'specifications for pharmaceutical preparations.'

There were papers published by representatives of the pharmaceutical and chemical industry on the validation of analytical methods. Hokanson [32, 33] applied the life cycle approach, developed for computerized systems, to the validation and revalidation of methods. Green [34] gave a practical guide for analytical method validation, with a description of a set of minimum requirements for a method. Wegscheider [35] has published procedures for method validation with a special focus on calibration, recovery experiments, method comparison and investigation of ruggedness. Seno et al.[36] have described how analytical methods are validated in a Japanese QC laboratory. The AOAC [37] has developed a Peer-Verified Methods validation program with detailed guidelines on exactly which parameters should be validated. Winslow and Meyer [38] recommend the definition and application of a master plan for validating analytical methods. J. Breaux and colleagues have published a study on analytical methods development and validation [39].

6.1 Advantages of Analytical Method Validation

- The biggest advantage of method validation is that it builds a degree of confidence, not only for the developer but also to the user.
- Although the validation exercise may appear costly and time consuming, it results inexpensive, eliminates frustrating repetitions and leads to better time management in the end.
- Minor changes in the conditions such as reagent supplier or grade, analytical setup are unavoidable due to obvious reasons but the method validation absorbs the shock of such conditions and pays for more than invested on the process.

6.2 Validation Terminology and Definitions

It is important to define the terms used in regulatory guidelines when discussing method validation. Following parameters are generally validated for the analytical method to prove its validity for intended use.

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. Method accuracy is the agreement between the difference in the measured analyte concentrations of fortified and unfortified samples, the fortification procedure is called spiking.

Precision:

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements from multiple sampling of the same homogeneous sample under prescribed conditions. Precision may be considered at three levels: Repeatability, intermediate precision, and reproducibility.

Repeatability:

Repeatability expresses the precision under the same operating conditions over a short interval of time.

Intermediate Precision:

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

Reproducibility:

Reproducibility expresses the precision between laboratories.

Specificity:

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present.

Detection Limit:

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value,

Quantitation Limit:

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

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 the analyte in the sample.

Range:

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

Robustness:

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

Ruggedness:

The degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions, such as different labs, different analysts, and different lots of reagents. Ruggedness is a measure of reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst. See intermediate precision.

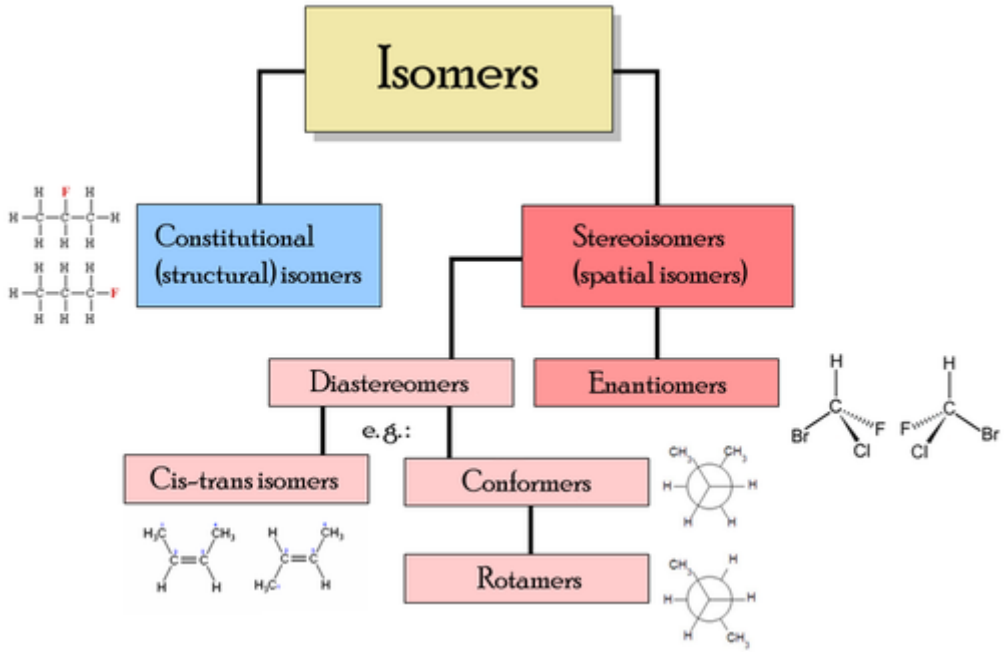
Sensitivity:

The sensitivity of an analytical method is equal to the slope of the calibration line in a linear system.

7.0 Isomerization

Isomerism was first noticed in 1827, when Friedrich Woehler prepared cyanic acid and noted that although its elemental composition was identical to fulminic acid (prepared by Justus von Liebig the previous year), its properties were quite different. This finding challenged the prevailing chemical understanding of the time, which held that chemical compounds could be different only when they had different elemental compositions. After additional discoveries of the same sort were made, such as Woehler's 1828 discovery that urea had the same atomic composition as the chemically distinct ammonium cyanate, Jöns Jakob Berzelius introduced the term *isomerism* to describe the phenomenon. In 1849, Louis Pasteur separated tiny crystals of tartaric acid into their two mirror-image forms. The individual molecules of each were the left and right optical stereoisomers, solutions of which rotate the plane of polarized light in opposite directions.

In chemistry, isomers are compounds with the same molecular formula but different structural formulas. The word is derived from the Greek ισομερης, isomerès; isos = "equal", méros = "part". There are many different classes of isomers, like stereoisomers, enantiomers, geometrical isomers, etc. (see chart below). Isomers do not necessarily share similar properties, unless they also have the same functional groups. There are two main forms of isomerism: structural isomerism and stereoisomerism (spatial isomerism).



In structural isomers, the atoms and functional groups are joined together in different ways. This group includes chain isomerism whereby hydrocarbon chains have variable amounts of branching; position isomerism which deals with the position of a functional group on a chain; and functional group isomerism in which one functional group is split up into different ones.

In skeletal isomers the main carbon chain is different between the two isomers. This type of isomerism is most identifiable in secondary and tertiary alcohol isomers.

Tautomers are structural isomers of the same chemical substance that spontaneously interconvert with each other, even when pure. They have different chemical properties, and consequently, distinct reactions characteristic to each form are observed. If the interconversion reaction is fast enough, tautomers cannot be isolated from each other. An example is when they differ by the position of a proton, such as in keto/enol tautomerism, where the proton is alternately on the carbon or oxygen.

In stereoisomers the bond structure is the same, but the geometrical positioning of atoms and functional groups in space differs. This class includes enantiomers where different isomers are non-superimposable mirror-images of each other, and diastereomers when they are not. Diastereomerism is again subdivided into "cis-trans isomers"", which have restricted rotation whithin the molecule (typically isomers containing a double bond) and "conformational isomers" (conformers), which can rotate about one or more single bonds whithin the molecule. Note that although conformers can be referred to as diastereomers, they are not stable diastereomers, since bonds in conformers can be rotated to make them mirror images.

An obsolete term for "cis-trans isomerism" is "geometric isomers". For compounds with more than two substituents E-Z notation is used in stead of cis and trans. If possible, *E* and *Z* (written in italic type) is also preferred in compounds with two substituents.

In octahedral coordination compounds fac- (with facial ligands) and mer- (with meridional ligands) isomers occur.

8.0 Chirality and Drug Development

Although the optical isomers of a recemic drug can exhibit different pharmacological activities in living systems [40-48], bioactive synthetic compounds, which comprise most of the chiral drugs, are administered as recemates [49]. Obviously, the more chiral centers present in a drug molecule, the more complex the situation. To ensure the optimum therapeutic effect, it would seem to be convenient to administer the eutomer. However, applying a single enantiomer to humans does not necessarily prevent side effects or tissue/organ damage, since, for example, the formation of harmful metabolites, as well as chiral inversion or racemization, can occur in vivo. To protect patients from unwanted and harmful enantiomers [40] and side effects, the possibility of a different action of the individual enantiomers with regard to pharmacology and toxicology had to be taken into account. The quality of the stereoisomer is served by chromatographic techniques, such as HPLC, GC, SFC, CEC and TLC.

Among the chromatographic techniques, HPLC remains the best modality owing to several advantages. High speed, sensitivity, and reproducible results make HPLC the method of choice in almost all laboratories. Moreover, HPLC can be used successfully at the preparative scale. There is no serious limitation of this technique. About 90% of the work in chiral resolution has been achieved by means of the HPLC mode of chromatography. Almost all the chiral selectors are available in the form of HPLC columns, owing to the wide range of applications of HPLC in chiral resolution [44-48]. A variety of mobile phases including normal, reversed, and new polar organic phases are used in HPLC. The composition of the mobile phases may be changed by the addition of various aqueous and non-aqueous solvents. A number of parameters are used in the optimization of the chiral resolution.

9.0 Impurities

During the manufacturing process, whether by chemical synthesis, extraction, cell culture/fermentation, recovery from natural sources, or any combination of these processes, impurities may arise. There is an ever increasing interest in impurities present in API's. Recently, not only purity profile but also impurity profile has become essential as per various regulatory requirements. Impurities are extraneous compounds that are not the drug substance (API), but arise during the synthesis, extraction, purification, or storage of the drug. Understanding the origin, control, and

measurement of impurities is critical to the production of high-quality drug substances.

In addition to guidances from the local authorities of many countries, a series of guidances developed in recent years by the Expert Working Group of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, commonly known as ICH, have been increasingly accepted by the pharmaceutical community.

Impurities present in excess of 0.1% should be identified and quantified by selective methods. It is essential to know the structure of these impurities in the bulk drug in order to alter the reaction condition and to reduce the quantity of impurity to an acceptable level. Isolation, identification and quantification of impurities help us in various ways, to obtain a pure drug substance with less toxicity and, safety in drug therapy. Quantitative determination of these impurities could be used as a method for the quality control and validation of drug substances. Regulatory authorities such as US FDA, CGMP, TGA (Therapeutic Good Administration, Australia), and MCA (Medical Control Agency, UK) insist on the impurity profiling of drugs.

9.1 Origin of impurities

Impurities generally fall into three main categories: process impurities, degradation impurities, and contaminant impurities. Additionally, enantiomers and polymorphs may be considered impurities under some circumstances.

Process impurities arise during the manufacture of the drug substance.

Degradation impurities arise during the storage of the drug substance.

Contaminant impurities are not drug related but are inadvertently introduced during processing or storage, and are not part of the synthesis, extraction, or fermentation process. Impurities that cause the greatest concern are those that are toxic, defined by the US Pharmacopeia (USP) as impurities that have significant undesirable biological activity [50].

9.2 Process Impurities

These include inorganic impurities, organic impurities and residual solvents. Organic impurities may be unwanted by-products of a chemical synthesis. They may arise by many different routes, for example, by the reaction of an intermediate with the solvent rather than the desired substrate, by cyclization in the wrong direction.

Organic impurities can also arise from impurities in the starting materials, for example, traces of propylamine in butylamine may lead to the propyl analog of the drug substance or they may be reagents used during the reaction. Unreacted starting materials and intermediates may also be present as impurities in the drug substances. In some cases, enzymes are used during a chemical synthesis. These materials may be present in the drug substance as impurities. Very frequently, batches of drug substance made by different synthetic routes will contain different impurities.

In a chemical synthesis, the unwanted compounds that are not removed during the synthetic or purification steps will become impurities. In a similar fashion, the extraction, purification, and later synthetic steps for natural, fermentation, or recombinant products may also give rise to such impurities. Biotechnological processes may give rise to impurities such as media components and host cell proteins. For animal and plant sources, the manufacturing process begins with the organ, fluid, or tissue of the animal or the whole plant or part of the plant. Understanding the source of the impurity will make it easier to devise a means of eliminating the impurity, thus resulting in a drug substance of improved quality.

Inorganic impurities include water, salts from buffers, reagents, ligands, catalysts, heavy metals, or other residual metals, and inorganic compounds used in processing, such as filter aids and charcoal. Inorganic impurities can also arise by leaching from equipment as a result of the unit manufacturing process.

Residual solvents are considered a subset of organic impurities. Solvents used to create a solution or suspension during the manufacturing process may not be completely eliminated in the course of manufacture. Solvents used later in the synthesis are more likely to be present in the drug substance, although solvents that have low volatility may persist from earlier steps.

9.3 Degradation Impurities

Normally, degradants are chemical breakdown compounds of the drug substance formed during storage. In rare cases, degradants are formed when the drug substance chemically interacts with other compounds or contaminants. In addition, degradants may also be formed by physical degradation, for example, aggregates of proteinaceous material, dimmers, trimers, and so forth, of synthetic compounds, polymorphs of synthetic compounds. Degradants may be chemically identical to process impurities. However, the levels of degradants will increase during storage,

while the levels of process impurities will remain constant. The rate of increase of degradants resulting from storage is dependant on the chemical nature of the drug substance. An understanding of the potential degradation pathways of the drug substance will lead to optimization of the storage conditions and will result in less impurities.

9.4 Contamination Impurities

Contamination impurities are unexpected adulterating compounds found in the drug substance. Current manufacturing technology has reduced many of the contaminant impurities observed in drugs prepared decades ago. For example, heavy metals like lead that leached from pipes or manufacturing/storage tanks gave rise to the commonly used limit test for heavy metals in the drug substance. Current pipes and tanks are primarily stainless steel or glass-lined to reduce this concern, although the type of material is ultimately dependent on the nature of the reactions, the nature of the drug substance, and the nature of the manufacturing unit operations. Other contaminants could be, but not likely agents sprayed to improve the environment in the manufacturing plant or accidental droppings like human hair or paint chips from walls. Disinfectants such as mono-, di-, or trichloroacetic acids or chloramines may be present.

9.5 Control of Drug Substance Impurities

In theory, all impurities should be eliminated. In practice, it is generally not economically feasible to totally eliminate all impurities. However, the levels of all impurities should be controlled to provide a consistent product. In most cases, only low levels of impurities should be allowed, but in rare cases, even quite high levels of impurities are tolerated.

Generally, the reaction conditions are adjusted to reduce the amounts of by-products produced during each step of the reaction. The reaction conditions are tightly controlled to prevent varying levels of impurities, or even new impurities, from arising. High-quality starting materials may also lead to lower amounts of impurities in the final product when starting material impurities are carried through to drug substance impurities. Similarly, the use of high-quality reagents may help avoid the generation of unwanted by-products. Other options to reduce these impurities are the introduction of additional intermediate or final purification steps.

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Part-A

Application of the Chromatography for the Separation of Isomers of Some Pharmaceuticals

Section-I

HPLC Separation of Enantiomers of Duloxetine Hydrochloride

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1. Introduction

The separation of enantiomers is an important area in the pharmaceutical industry. The reasons for the separation and quantitation of enantiomers in a racemic mixture are being addressed by the regulatory agencies of the USA, Japan and the European Economic Community [1-5]. The physiological environment within a living organism is chiral, therefore, chiral discrimination has been an issue in the development and use of pharmaceutical drugs. The enantiomers of chiral drugs often exhibit different pharmacological properties, and more and more chiral drugs are being developed as single isomers to avoid the use of larger doses or the risk of toxic effects [1, 6]. Determination of enantiomeric purity, or enantiomeric excess (ee), is of special importance in the control of the purity of chiral synthetic materials and chiral pharmaceuticals. Fast, simple and rugged stereoselective analytical methods to control the enantiomers are urgently needed. In the recent years, research has been intensified to understand the aspects of the molecular mechanism for stereoselective biological activities of the chiral molecules. The development of analytical methods for the assessment of enantiomeric purity is extremely challenging due to the fact that enantiomers possess virtually identical properties [7].

In the present study Duloxetine hydrochloride, an anti-depression drug has been selected. Depression is a chronic, debilitating mental disease affecting more than 10% of the general population [8–12]. Traditional tricyclic antidepressants (TCAs) increase the synaptic availability of norepinephrine (NE) [13, 14]. However, conventional tricyclic antidepressants have a relatively high affinity for a broad range of receptors, including adrenergic, muscarinic and histaminergic receptors [15]. This low selectivity causes a wide range of adverse side effects that contribute to low compliance among patients. The introduction of the selective serotonin reuptake inhibitors (SSRIs), which have a more favourable side effect profile, shifted the emphasis toward a potential crucial role of serotonergic dysfunction in affective disorders.

1.1 Drug Profile

1.1.1 Description

Duloxetine (Fig.1), [*(S)*-N-Methyl-3-(1-naphthalenyloxy)-3-(2-thienyl) propanamine] is an antidepressant drug. It is a potent and balanced dual re-uptake inhibitor of serotonin (5-hydroxytryptamine, 5-HT) and norepinephrine (NE). Its pharmacological effects are mainly due to *(S)*-enantiomer while the *(R)*-enantiomer is considered to be inactive. Its molecular formula is $C_{18}H_{19}NOS$ having molecular weight 333.88 g/mole.

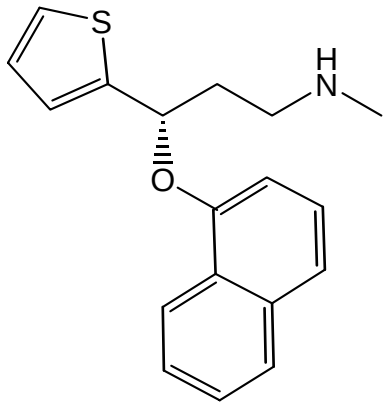


Fig. 1: Chemical Structure of *S*(+)-Duloxetine

1.1.2 Mechanism of Action

Duloxetine belongs to a family of drugs called serotonin-norepinephrine reuptake inhibitors (SNRIs). Other members of this family include venlafaxine (Effexor XR, Effexor) and sibutramine (Meridia, Reductil). Although the exact mechanism of duloxetine is not precisely known, its actions are believed to be related to its effect on the levels of the neurotransmitters serotonin and norepinephrine in the central nervous system. Duloxetine weakly inhibits dopamine reuptake and has no significant affinity for histaminergic, dopaminergic, cholinergic or adrenergic receptors [16].

Lab studies have shown that duloxetine is a strong inhibitor of serotonin and norepinephrine reuptake by brain cells. This action would theoretically raise levels of serotonin and norepinephrine in the spaces between brain cells (called "synapses").

Increased levels of serotonin and norepinephrine in the synapses is believed to alleviate the symptoms of depression and anxiety.

1.1.3 Clinical Use

Duloxetine is a prescription drug for treatment of anxiety, depression, fibromyalgia, and diabetic neuropathy [17].

Major Depressive Disorder (MDD): For MDD, duloxetine is taken at a total dose of 40 mg daily (given as 20 mg twice daily) to 60 mg maximum daily (either 60 mg once daily or 30 mg twice daily). Treatment of MDD remains a challenge, and a recent study has shown that many recently-approved antidepressants (bupropion, duloxetine, mirtazapine, and venlafaxine) offer comparable success rates [18].

Diabetic Peripheral Neuropathic Pain: For diabetic peripheral neuropathic pain, duloxetine is taken at a total dose of 60 mg daily given once a day. The drug does not appear to prevent nerve damage; rather, its effects seem to be based on its activity at serotonin and norepinephrine receptors in mediating pain. It tends to increase blood glucose to a small extent, but also tends to improve patients' reported quality of life [19].

Generalized Anxiety Disorder (GAD): For GAD, duloxetine is taken at a total dose of 60 mg once daily. Duloxetine offers improvements in GAD symptoms as well as improvements in reported well-being and quality of life[20].

1.1.4 Pharmacokinetic

The Applicant has appropriately described the non-clinical pharmacokinetics properties for duloxetine. A number of studies concerning the absorption, distribution, metabolism, and excretion of duloxetine in mice, rats, and dogs have been performed.

Rats might be suggested to be the most appropriate species for toxicology studies since major biotransformation pathways for duloxetine, in female rats as in man, were similar. However, mice and dogs are also valuable since exposure to specific human circulating metabolites could be seen in their plasma. Duloxetine was well absorbed in the mentioned species, and absorption was maximum at the duodenum. It did not show evidence of accumulation when duration of exposure increased. Duloxetine presented high protein binding across all species. Although it is not widely distributed it crosses the blood-brain barrier, undergoes placental transfer

and is excreted into milk. Duloxetine is extensively metabolised, primarily by the liver. The pharmacokinetics for duloxetine is summerized in Table 1.

Table 1: Summary of Pharmacokinetics for Duloxetine

Absorption	Well absorbed. After oral administration: t_{max} = 1.5 h in mice, rats, dogs and monkeys t_{max} =6 h in humans; with food t_{max} delayed to 10 hrs (variable)
Bioavailability	Rat approximately 21%. Dog approximately 5%. Human approximately 50% (32%-80%)
Dose proportionality	Linear kinetics was seen in mice and rats. In dogs, linear kinetics was observed at low daily doses (< 30 mg/kg). Non-linear at and above daily doses of 30 mg/kg in dog. Non-linear kinetics in humans
Terminal plasma half-life	Mouse: 2.1 h, radioactivity half life: 27 h Rat: 1.5 h, radioactivity half life: 122 hrs Dog: 4 h, radioactivity half life: 93 h Monkey: 3.5 h, radioactivity half life: 91 h Human: 12.1 h, radioactivity half life: 120 h
Distribution	High protein binding (93.6%-97.3% in all species). Not widely distributed but crosses the blood-brain barrier. Distribution over placenta and excreted into milk.
Metabolism	Rapid and extensive metabolism. Some differences related to the metabolic profile and exposure exists between animals species and humans.
Excretion	Mice, rats and dogs: main route via faeces (46%-77%). Monkey: main route via urine (58%) Human: main route via urine (72%) Biliary excretion in rats, dogs.

1.1.5 Dosage

Oral dose for adults:

Major Depressive Disorder: Initial: 40-60 mg/day; dose may be divided (ie, 20 or 30 mg twice daily) or given as a single daily dose of 60 mg; maintenance: 60 mg once daily; for doses >60 mg/day, titrate dose in increments of 30 mg/day over 1 week as tolerated to a maximum dose: 120 mg/day. Note: Doses >60 mg/day have not been demonstrated to be more effective.

1.1.6 Side Effects

The most common side effects observed with duloxetine use are:

Nausea, Vomiting, Constipation, Diarrhea, Dry mouth, Dyspepsia (indigestion), Loose stools, Fatigue, Asthenia (feelings of weakness), Fever, Nasopharyngitis (common cold symptoms), Decreased appetite, Anorexia (loss of appetite), Muscle cramp, Myalgia (muscle aches), Somnolence (sleepiness), Headache, Dizziness, Tremor, Insomnia, Frequent daytime urination (pollakiuria), Erectile dysfunction, Cough, Sore throat and Increased sweating.

1.1.7 Risks and Precautions

As with many other antidepressants, person taking duloxetine may develop suicidal thoughts, especially when the drug is first started or when the dose is changed [21].

1.1.8 Drug Interactions

Since the liver enzymes CYP1A2 and CYP2D6 are responsible for duloxetine metabolism, concurrent use of CYP1A2 inhibitors (such as fluvoxamine and quinolone antibiotics) and CYP2D6 inhibitors (such as paroxetine, fluoxetine, and quinidine) with duloxetine is to be avoided.

Duloxetine must not to be used with any MAO inhibitors (MAOIs) nor with any anti-migraine medications called triptans.

1.1.9 International Brand Names

Ariclaim (AT, BE, BG, CH, CZ, DE, DK, ES, FI, FR, GB, GR, HN, IE, IT, NL, NO, PT, RU, SE, TR)

Cymbalta (AR, AT, BE, BG, BR, CH, CL, CN, CO, CR, CZ, DE, DK, DO, ES, FI, FR, GB, GR, GT, HK, HN, ID, IE, IL, IT, KP, MX, MY, NI, NL, NO, PA, PE, PH, PT, RU, SE, SG, SV, TH, TR, TW)

Delok (IN)

Duxetin (UY)

Xeristar (AT, BE, BG, CH, CZ, DE, DK, ES, FI, FR, GB, GR, HN, IE, IT, NL, NO, PT, RU, SE, TR)

Yentreve (AT, BE, BG, CH, CZ, DE, DK, ES, FI, FR, GB, GR, HN, IE, IL, IT, MX, NL, NO, PT, RU, SE, TR, TW)

1.2 Chiral AGP Column

Chromtech Ltd. make Chiral-AGP column contains **human alpha-acid glycoprotein** as a stationary phase. The alpha-acid glycoprotein is a protein based chiral stationary phase (CSP). Proteins CSP's are defined as Type V CSP's where separations rely on a combination of hydrophobic and polar interactions.

Human alpha-acid glycoprotein (shown in Fig. 2) is a polypeptide with 181 amino acid residues and 40 sialic acid residues. It has an acidic character, its iso-electricpoint being 2.7. It is very stable protein having two disulphide bridges. It may be covalently bonded to silica to produce a reverse-phase column. A very wide range of molecules have been separated using AGP columns. The AGP column can be used for the resolution of an extremely broad range of chiral compound. Such as a amines (primary, secondary, tertiary and quaternary ammonium.), acids, esters, sulphoxides, amides, alcohols etc. The enantioselectivity and the retention can easily be regulated by the pH of the mobile phase, the buffers concentration and the nature and the concentration of the organic modifier.

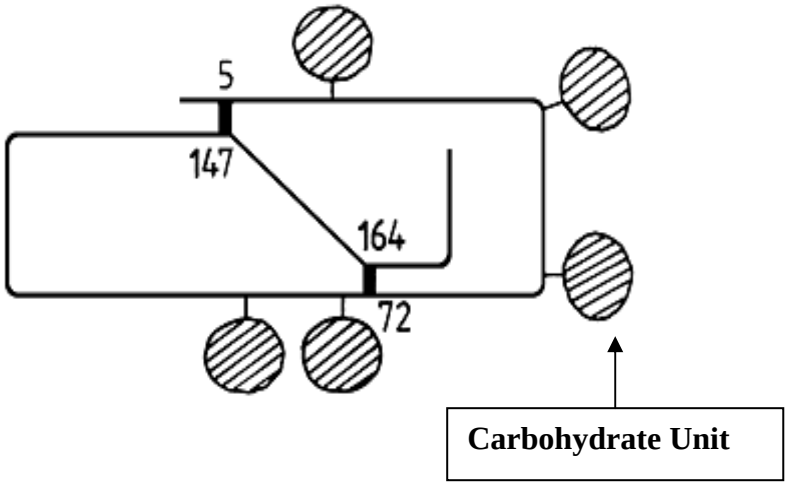


Fig. 2: Schematic drawing of alpha-acid glycoprotein (AGP)

Typical mobile phases are phosphate buffer pH 4-7 with a low percentage of organic modifier. The first choice of modifier is IPA. If this doesn't produce a separation then Acetonitrile, Ethanol, Methanol or THF should be tried. Changing the modifier, results in a temporary change in the protein structure. Column loading is of vital importance. Changing the pH has a critical effect on the selectivity, especially for amines. The most important tool in the method development is the pH, the reason is that by changing the pH the net charge of the chiral selectors as well as the charge of the solute can be changed, which affect the way the analytes interacts with the chiral selectors. AGP has a low isoelectric point of 2.7, this means that using column at pH 2.7 gives a net charge of zero of the chiral stationary phase, increasing the pH from 2.7 to 7 means that the degree of net negative charge of the chiral selectors increases. The charge distribution, at different pH is shown in Fig. 3. This gives the prerequisites for ionic binding of positively charge solutes resulting in a high affinity and high retention of the solute, a change of the net charge of the chiral selectors strongly affects the interaction between the solutes and the chiral stationary phase. It has been demonstrated that ionic binding of amines to the AGP column is a very important type of interaction for retention of this category of compounds. The solutes are also retained by hydrophobic interaction and hydrogen bonding. The relative influence of the different types of binding forces depends of the natures of the solute. A good starting point for amines is 10 mM sodium acetate buffer at pH 4.5, for acids 10mM phosphate at pH 7.0 would be a good choice.

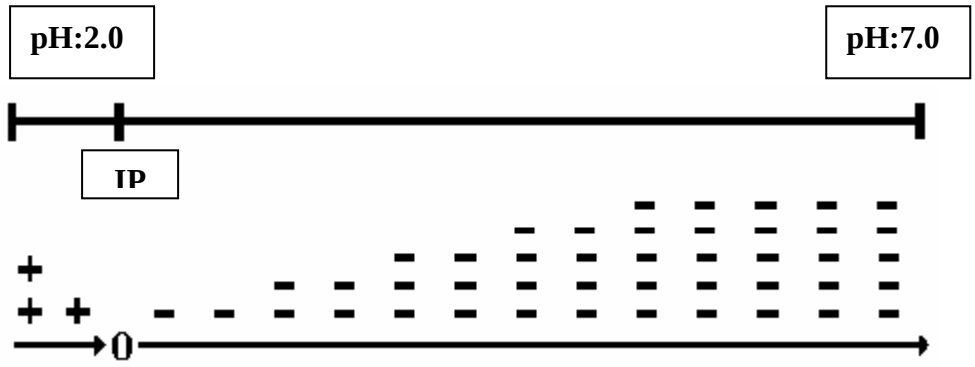


Fig. 3: Net charge of AGP at different pH

Charged modifiers may be used when organic modifiers have failed. These result in a permanent change in the protein structure and should be used with caution. They include Butyric acid, Octanoic acid, Decanoic acid, Dimethyloctylamine. Ethylene Glycol, 1-, 2- butanol and Sodium chloride, have also been used. Temperature affects the separation, as temperature increases the retention time and separation factors decrease.

For a compound to be resolved on an AGP column it requires the following properties:

1. Ring close to chiral centre
2. At least one hydrogen bonding site
3. The distance between the ring and the H-bonding site should not be greater than 3 atoms (unless there is more than one).

AGP columns may be regenerated by washing with 25% aqueous IPA

2. Literature Review

The literature reviews regarding duloxetine suggest that various analytical methods were reported for its determination as drug, in pharmaceutical formulation and in various biological fluids. Analytical methods for the determination of the impurities and enantiomeric purity were also reported.

A literature survey revealed that a few HPLC methods were reported for determination of duloxetine in bulk drug, pharmaceutical formulation and other biological fluids [22-26]. Only a few enantioselective chromatographic separation methods for duloxetine and its *(R*)-enantiomer have been described in the literature. Chiral separations have been mainly focused on capillary electrophoreses with modified cyclodextrins [27-30].

Jing Yang, Xiumei Lu, Yujin Bi, Feng Qin and Famei Li have reported HPLC method for determining the duloxetine and its *(R)*-enantiomer using hydroxypropyl- -cyclodextrin as chiral selector with resolution 1.3, analysis time about 60 min, and a vancomycin chiral stationary phase with resolution of isomers 1.7, analysis time approximately 20 min. The developed method was applied for enantiomeric purity of duloxetine bulk drug substance and can be used for the quality control of duloxetine, as well as in subsequent pharmacological studies [31].

V. P. Rane and D. B. Shinde have reported development and validation of chiral LC method for the enantiomeric separation of duloxetine on amylose based stationary phase. The analyte was chromatographed on Chiralpak AD-H column by isocratic elution with hexane-ethanol-diethyl amine (80:20:0.2, v/v/v) at a flow rate of 1.0 mL/min. The resolution was found to be not less than 2.8 in optimized method. The developed method can be used for the determination of *(R)*-enantiomer during quality control of the duloxetine. Authors have also tried chiral-AGP column using mobile phase ammonium acetate buffer (pH 3.5; 10 mM)-ethanol (65:35, v/v) but they could not achieved enantiomeric separation of duloxetine on chiral-AGP column [32].

3. Aim of Present Work

As per preceding discussion in the literature review, a few chromatographic methods are reported for the enantiomeric separation of the duloxetine hydrochloride. So far to our present knowledge, there is no validated, simple, rapid and accurate reverse phase chiral HPLC method for the determination of *(R)*-enantiomer in duloxetine hydrochloride was available as well as no successful report on AGP column in literature. In day-to-day routine laboratory activities, most of the HPLC methods are in reverse phase. It is tedious to convert HPLC instrument from reverse phase to normal phase and from normal phase to reverse phase for single analysis. Therefore, our aim is to develop simple, rapid and accurate reverse phase HPLC method on Chiral AGP column, which can be used comfortably in day-to–day activities.

Our work deals with the systematic method development studies such as, effect of organic modifier, effect of pH of buffer, effect of concentration of buffer and effect of column temperature. This work also deals with the validation of the developed method for the determination of the *(R)*-enantiomer in duloxetine hydrochloride. The developed method is recommended for routine monitoring of process development and quality control analysis.

4. Experimental

4.1 Chemicals

Duloxetine and its *(R)*-enantiomer were kindly supplied by Process Research Department of Cadila Healthcare Limited, Ankleshwar, India. HPLC grade acetonitrile was purchased from S.D.Fine, India. Analytical Reagent grade sodium acetate and acetic acid were purchased from Qualigens. HPLC grade water was obtained from Milli-Q water purification system.

4.2 Instrumentation

A Agilent 1100 series (Germany) HPLC system equipped with degasser auto sampler, auto injector, thermostatic compartment and photo diode array detector was utilized for method development and validation. The out put signal was monitored and processed using Agilent Chemstation software.

4.3 Mobile Phase Preparation

The mobile phase was sodium acetate buffer (pH 3.8; 10 mM)-acetonitrile (93:07, v/v). The buffer solution was prepared by dissolving 0.82 g sodium acetate in water and diluted up to the mark in 1000 mL volumetric flask with the water. pH was set 3.8 by glacial acetic acid. The buffer solution was filtered through a 0.45µm nylon membrane (Millipore Pvt. Ltd. Banglore, India). The final mobile phase was prepared by mixing 930 mL of buffer solution and 70 mL of acetonitrile in 1000 mL mobile phase bottle and degassed in an ultrasonic bath (Spincotech Pvt. Ltd., Mumbai).

4.4 Diluent Preparation

Mobile phase used as a diluent.

4.5 Sample Preparation

Stock solution of *(R)*-enantiomer (100 µg/mL) and duloxetine (4 mg/mL) were prepared by dissolving the appropriate amount of the substances in mobile phase. The analyte concentration of duloxetine was fixed as 0.4 mg/mL. Working solution was also prepared in mobile phase.

4.6 Chromatographic Conditions

The chiral column used for the separation was 150 mm x 4.0 mm, Chiral-AGP (Chromtech make) packed with 5 µm particles. The mobile phase was sodium acetate buffer (pH 3.8; 10 mM)-acetonitrile (93:07, v/v). The flow rate was set at 1.0 mL/min. The column was maintained at 20° C, and the detection was carried out at a wavelength of 220 nm. The injection volume was 5 L.

4.7 Method Validation

4.7.1 System Suitability

The racemic mixture containing equal quantity of *(R)*-enantiomer and duloxetine was injected in the equilibrated chromatographic system. The system suitability parameters such as, resolution (Rs), symmetry (S), retention factor (k), separation factor ( ) and efficiencies (N) were obtained.

4.7.2 Precision

Precision of the method is the degree of agreement among the individual test results when the procedure is applied repeatedly to multiple sampling of a homogenous sample. The precision of the method was checked by analyzing six replicate samples of duloxetine (at 0.4 mg/mL) spiked with 0.5 % of *(R)*-enantiomer and calculating the percentage relative standard deviation of retention time and peak area. The intermediate precision was also evaluated at different day by performing six successive injections.

4.7.3 Limit of Detection and Limit of Quantification

Limit of detection (LOD) and Limit of quantification (LOQ) were determined at a signal-to-noise ratio of 3 and 10. LOD and LOQ were achieved by injecting a series of dilute solutions of *(R)*-enantiomer.

The precision of the developed method for *(R)*-enantiomer at LOQ was checked by analyzing six test solutions of *(R)*-enantiomer prepared at the LOQ level and calculating the percentage relative standard deviation of retention time and peak area. The accuracy of the method was checked for *(R)*-enantiomer at LOQ level by analyzing three replicate samples of duloxetine spiked with *(R)*-enantiomer at LOQ level and calculating the percentage relative standard deviation.

4.7.4 Linearity of (R)-enantiomer

Detector response linearity was assessed by preparing calibration sample solutions of *(R)*-enantiomer covering from 400 ng/mL to 3000 ng/mL (400, 1000, 1500. 2000, and 3000 ng/mL), in mobile phase from *(R)*-enantiomer stock solution. The regression curve was obtained by plotting peak area versus concentration, using the least squares method. Linearity was checked for three consecutive days in the same concentration range from the same stock solution. The percentage relative standard deviation of the slope and Y-intercept of the calibration curve was calculated.

4.7.5 Accuracy

The standard addition and recovery experiments were conducted to demonstrate accuracy of the method. The study was carried out in triplicate for the determination of recovery of *(R)*-enantiomer at 0.4, 0.5 and 0.6 % of the Duloxetine target analyte concentration. The recovery of *(R)*-enantiomer was calculated from the slope and Y-intercept value range of 400-3000 ng/mL (slope and Y-intercept values obtained in the linearity study).

4.7.6 Robustness

The robustness of a method was determined by altering experimental conditions and chromatographic resolution between Duloxetine and *(R)*-enantiomer was evaluated. To study the effect of flow rate, pH of buffer, concentration of buffer and column temperature on the resolution of enantiomers, it was changed by varying 0.1 units, 0.1 units, -2 to +2 mM and –1 to +1 °C respectively, while the other chromatographic parameters were held constant as stated in section 2.4.

4.7.7 Solution stability

Stability of Duloxetine in solution at analyte concentration was studied by keeping the solution in tightly capped volumetric flask at room temperature on a laboratory bench for 3 days. Content of *(R)*-enantiomer was checked for 6, 12, 24 and 48 h.

5. Result and Discussion

5.1 Development and Optimization of the HPLC Method

The aim of this study is to separate the enantiomers of duloxetine hydrochloride with optimum resolution within short time using Chiral-AGP column. The racemic sample solution was used for the method development. The wavelength of the detection was set 220 nm based on the UV spectra of duloxetine (Fig. 4)

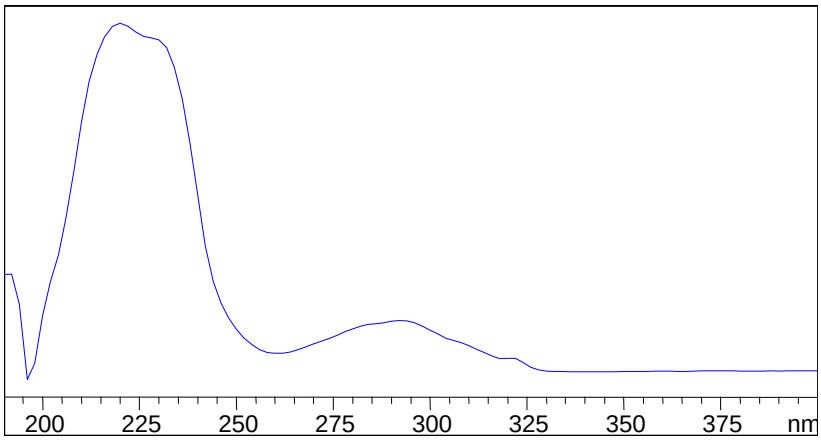


Fig. 4: UV spectra of Duloxetine

Duloxetine is a weak base (secondary amine) in nature. Therefore, to separate the pair of enantiomers on Chiral AGP column, it is better to work at acidic pH range, which was also evidenced by experiment at pH: 7.0 (Fig. 5) and pH: 4.5 (Fig. 6).

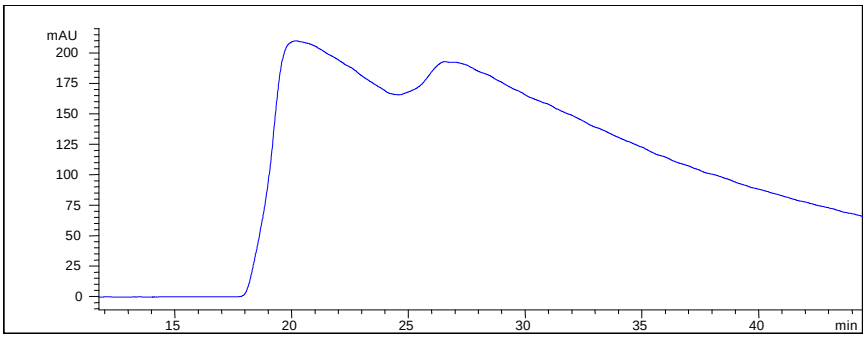


Fig. 5: 10 mM Phosphate (Na_2HPO_4) buffer (pH: 7.0) : IPA [90:10]
Flow: 0.8mL/min, 220nm, 30°C column temp.

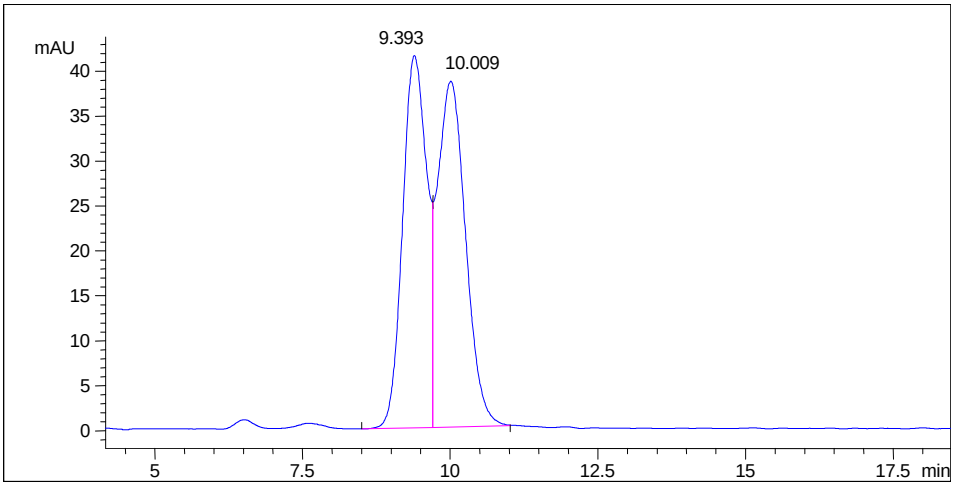


Fig. 6:10mM Sodium Acetate buffer pH : 4.5:IPA [90:10]
Flow: 0.8 mL/min, 30°C column temp.

The mobile phase parameters affecting the retention and resolution of the duloxetine enantiomers on the Chiral-AGP column are; the type and concentration of organic modifier, pH and concentration of the buffer, and column temperature. Each of these parameters were studied to evaluate their influence on the separation of the duloxetine enantiomers. The systematic experimental strategy was applied to develop and optimize the enantiomeric separation of duloxetine on the Chiral-AGP column.

5.1.1 Effect of Organic Modifier

Two organic modifiers, acetonitrile and isopropyl alcohol in the range of 5-10 % were tried with sodium acetate buffer (pH 4.5; 10 mM) as a mobile phase at a flow rate of 0.8 mL/min. It can be seen from the Fig. 7 that, when the percentage of organic modifier decreased from 10 to 5 retention and resolution were increased. Thus, less retention and high enatioselectivity were observed for 7 % concentration of organic modifier. The optimum resolution and good peak shape was found on using acetonitrile as compared to isopropyl alcohol at equivalent volume based mobile phase composition.

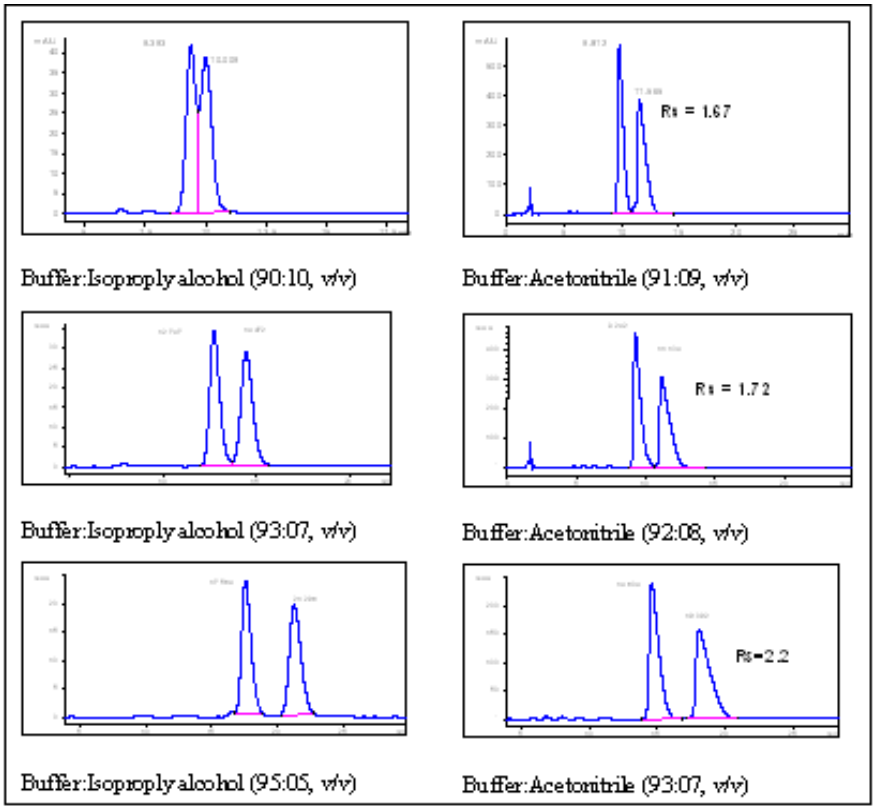


Fig. 7: Chromatograms of different organic modifier compositions

5.1.2 Effect of pH of Buffer

An important factor in chiral chromatography is the pH of the buffer that effects the binding affinity of both chiral selector and the analyte. The pH, mobile phase has shown to have strong influence on the retention and enatioselectivity of basic, acidic and non-protolytic compounds [33].

The effect of pH of the buffer was investigated on the resolution and retention of enantiomers of duloxetine. The other chromatographic parameters; buffer concentration (10 mM), acetonitrile (7 %), column temperature (30° C), flow rate (1.0 mL/min) and wavelength (220 nm) were maintained constant, while pH of the buffer was scanned from 3.2 to 5.2. The plot of the pH versus retention factor (Fig. 8) was drawn to understand the effect of pH on the retention and separation of the enantiomers of duloxetine.

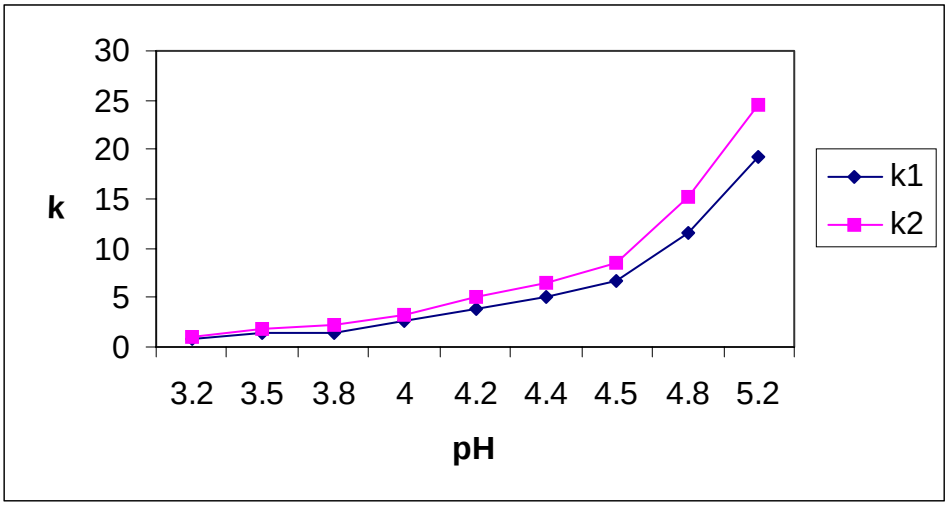


Fig. 8: Effect of pH of buffer (Plot of pH versus retention factor, k)

It can be seen from the Fig. 8 that as the pH was raised from 3.2 to 5.2, retention of the enantiomers increased. Resolution and selectivity also increased with higher mobile phase pH, however, baseline separation within short time, peak shape and adequate resolution were optimal at a pH 3.8 and 4.0. Few of the representative chromatograms of the effect of pH are shown in Fig. 9, 10, 11 and 12. The selectivity and resolution between the two enantiomers at different buffer pH values are presented in Table 2.

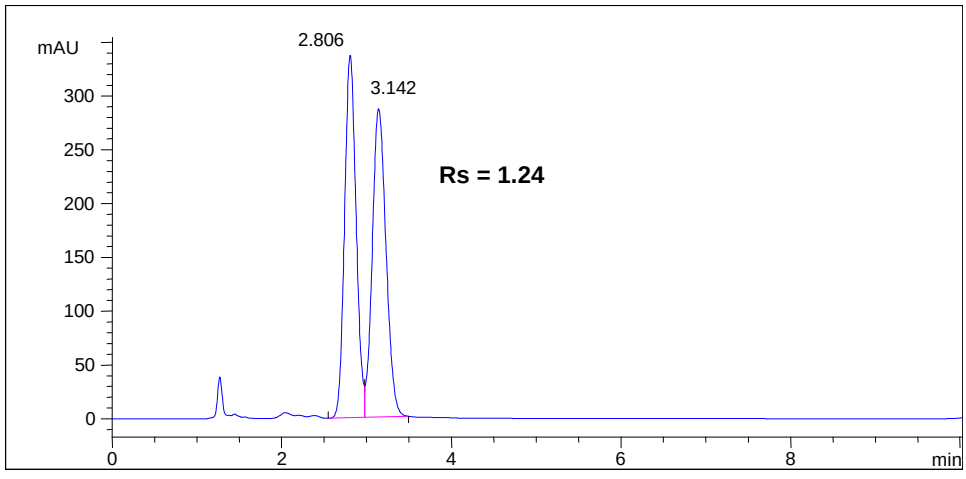


Fig. 9: 10mM Sodium phosphate (NaH_2PO_4) buffer pH: 3.2:ACN [93:07] Flow: 1.0 mL/min

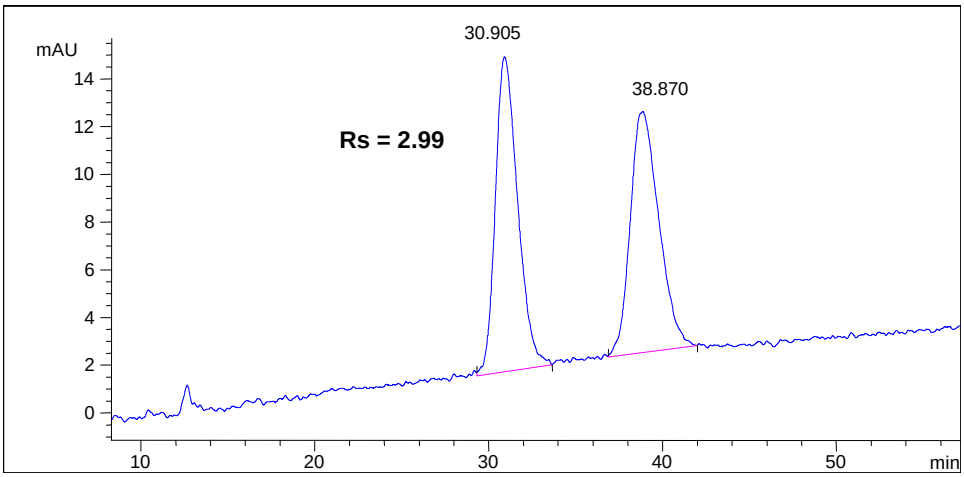


Fig. 10: 10 mM Sodium Acetate(CH_3COONa) buffer pH : 5.2:ACN [93:07] Flow: 1.0 mL/min

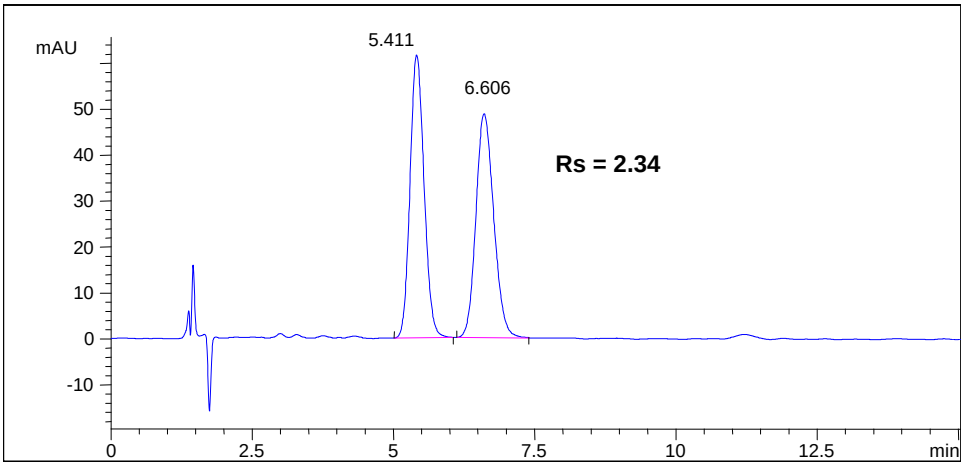


Fig. 11: 10 mM Sodium Acetate(CH_3COONa) buffer pH : 4.0:ACN [93:07] Flow: 1.0 mL/min

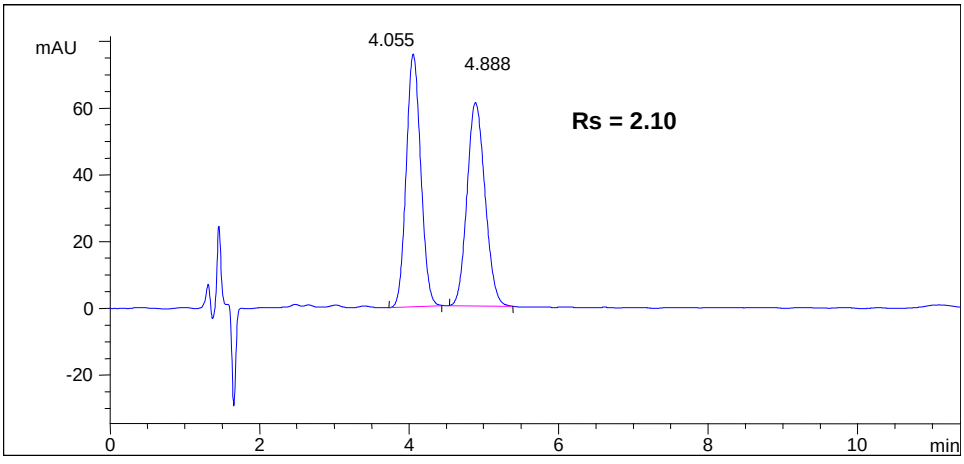


Fig. 12: 10 mM Sodium Acetate(CH_3COONa) buffer pH : 3.8:ACN [93:07] Flow: 1.0 mL/min

Table 2: Selectivity and Resolution Between Both the Enantiomers at Different pH Value of the Buffer.

Buffer pH	Retention factor (k)		Selectivity ( )	Resolution (Rs)
	(*R*)-enantiomer	Duloxetine		
3.2	0.84	1.06	1.26	1.24
3.5	1.37	1.75	1.28	1.70
3.8	1.37	2.19	1.60	2.11
4.0	2.54	3.32	1.31	2.34
4.2	3.89	5.03	1.29	2.34
4.4	5.09	6.53	1.28	2.45
4.5	6.65	8.50	1.28	2.41
4.8	11.51	15.10	1.31	3.20
5.2	19.23	24.44	1.27	2.99

5.1.3 Effect of Concentration of Buffer

Effect of concentration of sodium acetate buffer in the range of 5-25 mM was investigated. While the other chromatographic parameters; pH of the buffer (pH 4.0), acetonitrile (7%), column temperature (30° C), flow rate (1.0 mL/min) and wavelength (220 nm) were maintained constant. The results of selectivity and resolution between both the enantiomers at different mobile phase buffer concentration are summarized in Table 3.

Table 3: Selectivity and Resolution Between Both the Enantiomers at Different Mobile Phase Buffer Concentration.

Buffer concentration (mM)	Retention factor (k)		Selectivity ( )	Resolution (Rs)
	(*R*)-enantiomer	Duloxetine		
5	2.36	3.03	1.28	1.96
10	2.54	3.32	1.31	2.34
15	2.82	3.63	1.29	2.24
20	2.76	3.54	1.28	2.22

It can be seen that the optimum resolution and retention were achieved at the 10 mM concentration of sodium acetate buffer. The chromatograms obtained for different concentration of sodium acetate buffer are shown in Fig. 13, 14, 15 and 16.

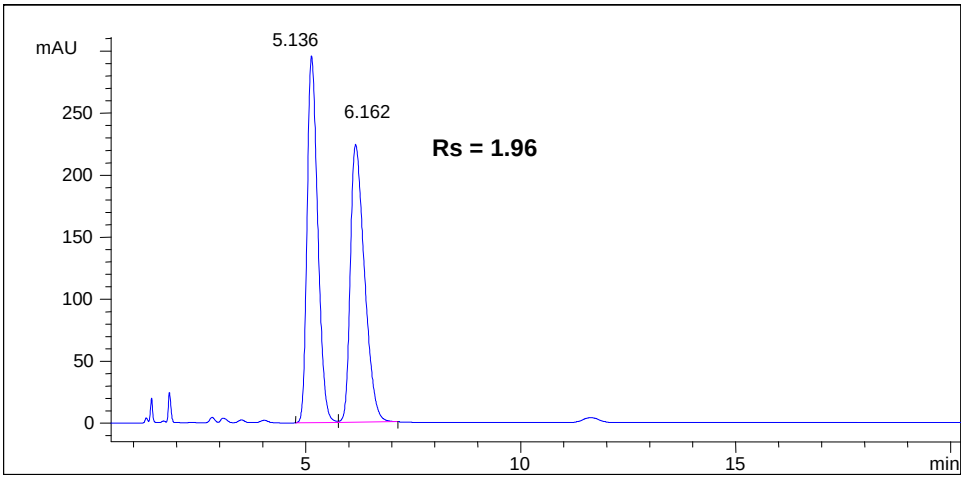


Fig. 13: Chromatogram of 5 mM buffer concentration

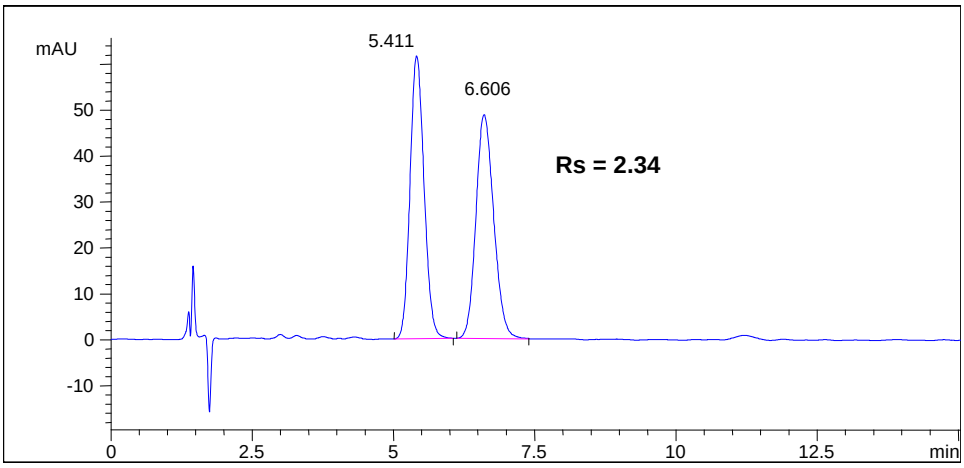


Fig. 14: Chromatogram of 10 mM buffer concentration

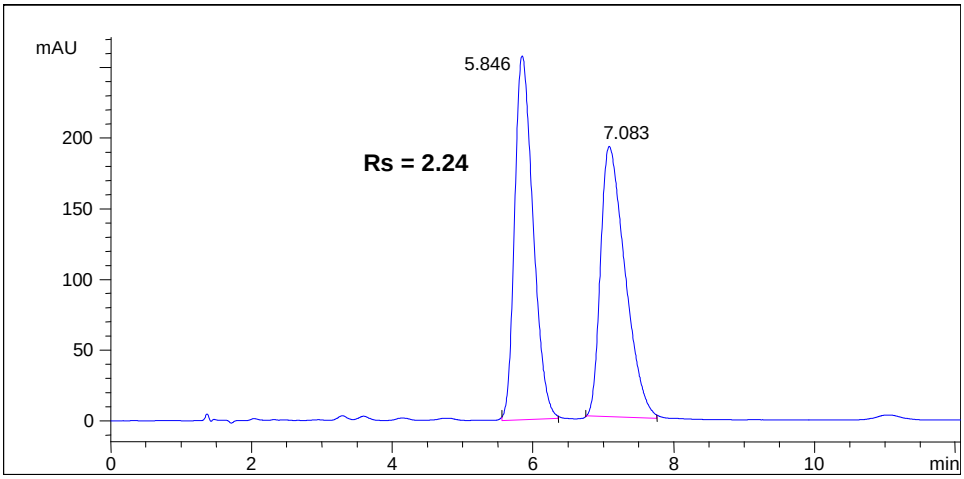


Fig. 15: Chromatogram of 15 mM buffer concentration

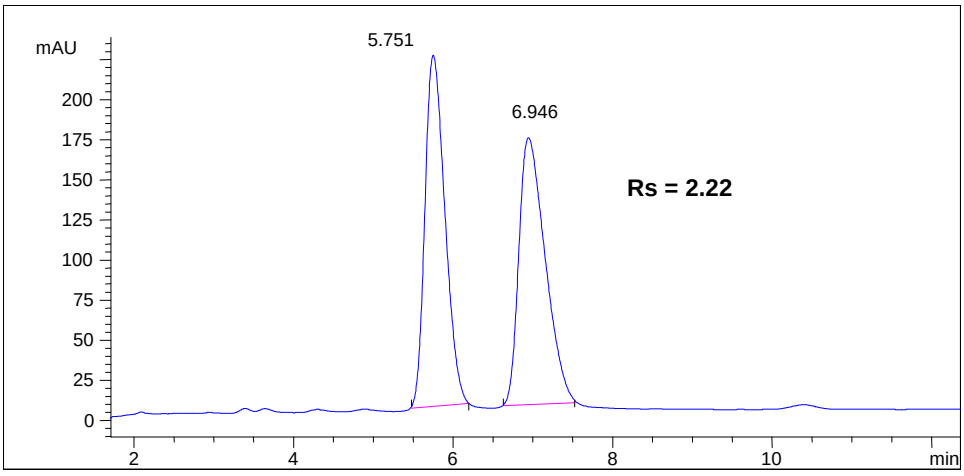


Fig. 16: Chromatogram of 20 mM buffer concentration

5.1.4 Effect of Column Temperature

Column temperature has been reported to influence enantiomer retention, enantioselectivity and resolution [33, 34]. The effect of column temperature was studied from 20° C to 40° C with 5° C interval. While other chromatographic parameters; mobile phase consisting of sodium acetate buffer (pH 4.0; 10 mM)-acetonitrile (93:07, v/v), flow rate (1.0 mL/min) and wavelength (220 nm) were maintained constant. The plot of the column temperature versus retention factor (Fig. 17) was drawn to understand the effect of column temperature on the retention and separation of the enantiomers of duloxetine.

It can be seen from the Fig. 17 that retention and selectivity decreased with increasing column temperature. Few of the representative chromatograms of the effect of column temperature are presented in Fig. 18, 19 and 20. The selectivity and resolution between the two enantiomers at different column temperature values are summerized in Table 4. The resolution between two enantiomers also increased as column temperature decreased (Table 4).

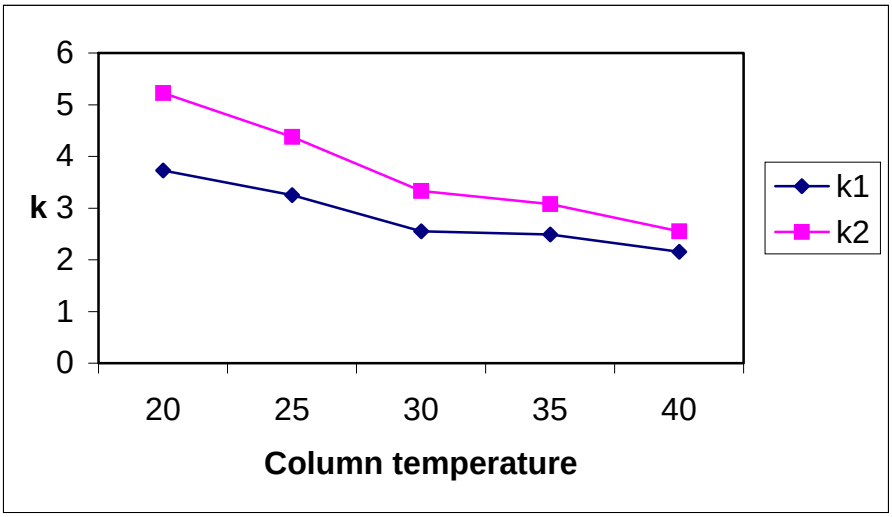


Fig. 17: Effect of column temperature (Plot of column temperature versus retention factor, k)

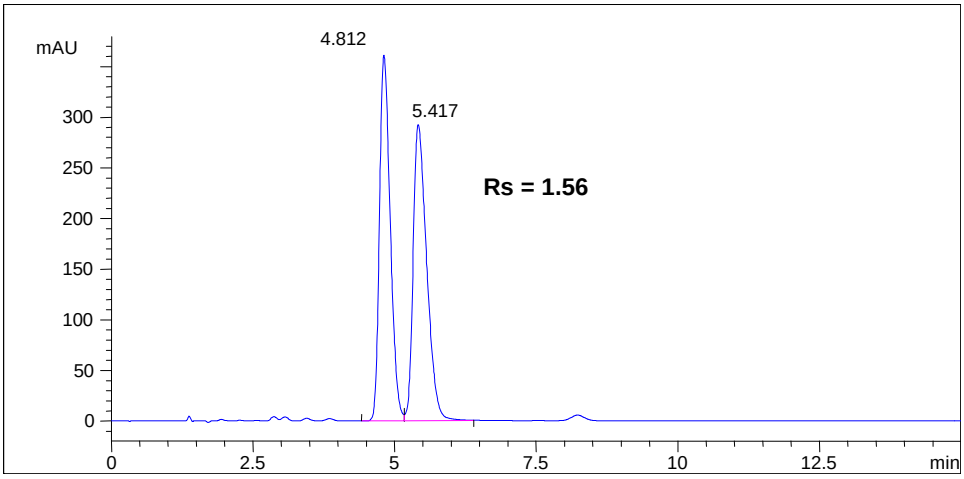


Fig. 18: Chromatogram of 40° C column temperature

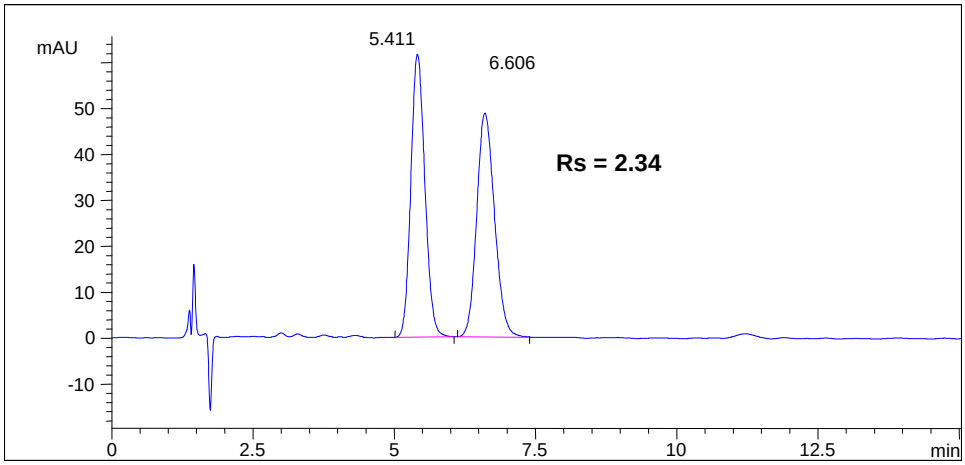


Fig. 19: Chromatogram of 30° C column temperature

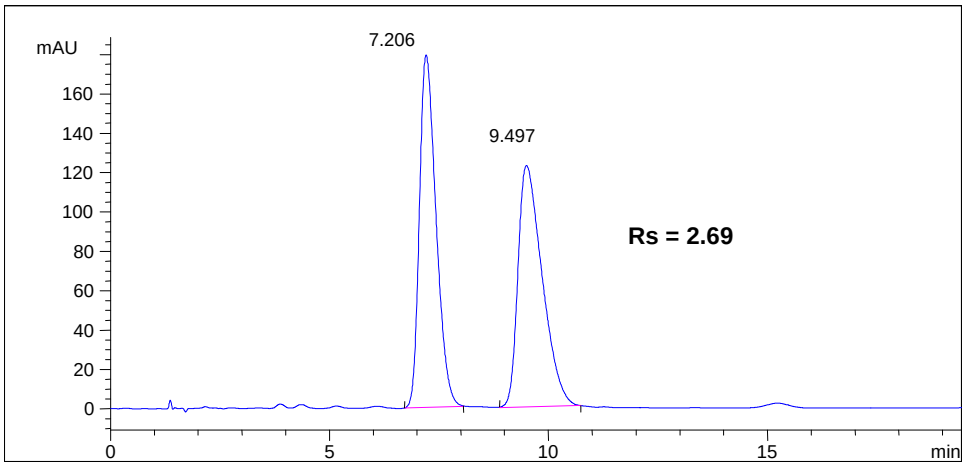


Fig. 20: Chromatogram of 20° C column temperature

Table 4: Selectivity and Resolution Between Both the Enantiomers at Different Column Temperature.

Column temperature (°C)	Retention factor (k)		Selectivity ( )	Resolution (Rs)
	(*R*)-enantiomer	Duloxetine		
20	2.73	5.23	1.40	2.69
25	3.25	4.38	1.35	2.50
30	2.55	3.33	1.31	2.34
35	2.49	3.08	1.24	1.92
40	2.16	2.55	1.18	1.56

In the frame of the optimization process the temperature dependency of retention and enantioselectivity was also studied by plotting Van't Hoff plot of ln versus 1/T for the temperature range 20 to 40° C (Fig. 21).

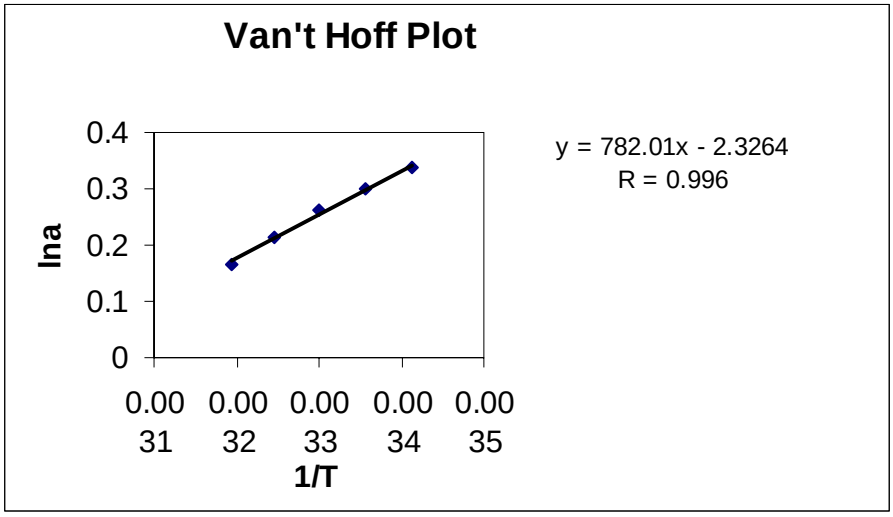


Fig. 21: Van't Hoff Plot for the separation of duloxetine enantiomers

The apparent enantioselectivity ( ) can be expressed by the Gibbs Hemholtz equation:

(G) = RTln (1)

where - (G) is the difference in the Gibbs free energy between diastereomeric complexes formed upon interaction with the chiral selector and the individual enantiomers of a racemic analyte.

Eq. 1 can be expressed as,

ln = - (H)/RT + (S)/R (2)

where (H) and (S) are the enthalpy and entropy differences respectively, between the two enantiomers when they interact with the stationary phase. R is the gas constant (8.314 J/mol K) and T is absolute temperature.

The Van't Hoff plot (Fig. 21) showed linear relation with correlation coefficient greater than 0.995. It suggests that correlation thermodynamic parameters and retention mechanism are temperature independent. The slope (- (H)/R) and intercept ((S)/R) were used to calculate (H) and (S). The obtained values of (H) was – 6501.61 J/mol, (S) was – 19.34 J/mol and (G) was – 834.99 J/mol (at 20° C). The negative value of (G) was due to contribution of negative (H) value. Therefore, it can be said that transfer of solute from the mobile phase to stationary phase and the separation are enthalpically driven.

5.1.5 Optimum Separation

The influence of each chromatographic parameter was systematically studied. The optimal parameters from all the studies have been taken to finalize the condition of the method. The study on effect of organic modifier suggested that acetonitrile is good as a organic modifier. The study on effect of buffer pH concluded that pH should be 3.8 or 4.0. The study on the concentration of buffer said that 10 mM is the ideal concentration. The study on effect of column temperature showed that resolution is higher at 20° C. The optimized separation for the enantiomers of duloxetine on Chiral-AGP column is shown in Fig. 22. Which was achieved using mobile phase consisting of sodium acetate buffer (pH 3.8; 10 mM)-acetonitrile (93:07, v/v). The flow rate was 1.0 mL/min. Column temperature was maintained 20° C. The enantiomers were baseline resolved with a runtime less than 8 min. Interestingly, distomer was eluted prior to eutomer in the developed method.

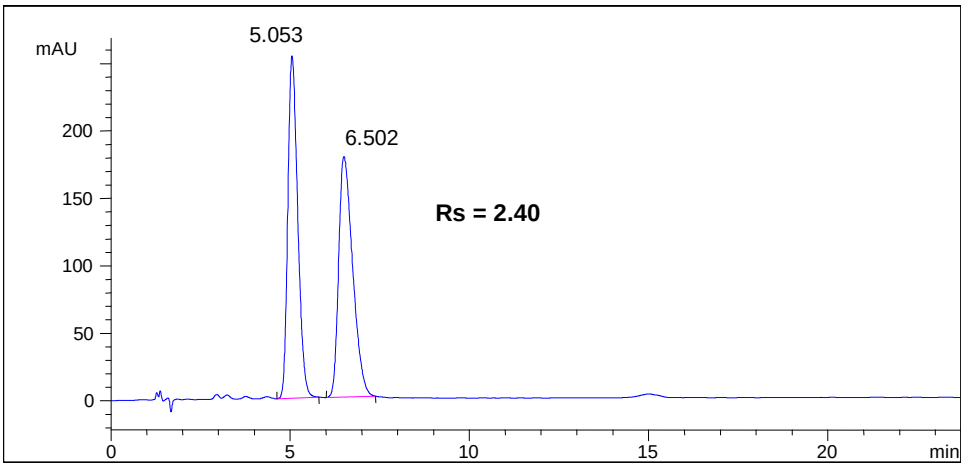


Fig. 22: 10 mM Sodium Acetate (CH_3COONa) buffer pH : 3.8:ACN [93:07] Flow: 1.0 mL/min, (Racemic in Mobile Phase), T = 20° C

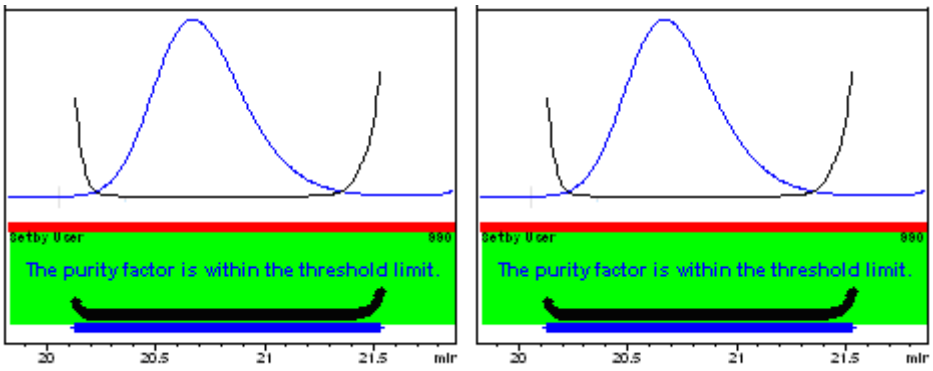
5.2 Results of Method Validation

5.2.1 Results of System Suitability

The system suitability results summarized in Table 5, which showed that resolution between both the enantiomers was not less than 2.2. Peak purity of both the enantiomers was not less than 0.999/990 using PDA detector. Report of peak purity is presented in Fig. 23.

Table 5: System Suitability Data

System suitability parameters	(*R*)-enantiomer (n =3)	Duloxetine (n=3)
Retention factor (k)	2.37	3.33
Resolution (Rs)	2.38	
Symmetry (S)	0.80	0.79
Selectivity ( )		1.41



(a) **(b)**

Fig. 23: Peak purity report of (a) *(R)*-enantiomer and (b) Duloxetine

5.2.2 Results of Precision

In the precision study, the percentage relative standard deviation (RSD) was less than 0.05 % for the retention time and 0.5 % for the peak area of both the enantiomers indicating good precision of the method. Data of precision study are summarized in Table 6. In the intermediate precision study, results showed that RSD values were in the same order of magnitude than those obtained for repeatability and data are presented in Table 7.

Table 6: Result of Precision Study

Precision Data				
Sr. No	***(R)*-enantiomer**		**Duloxetine**	
	RT	**Area**	**RT**	**Area**
1	5.133	62.32	6.224	15731.20
2	5.133	62.35	6.225	15757.10
3	5.131	62.61	6.221	15794.60
4	5.133	62.12	6.224	15732.50
5	5.131	62.62	6.221	15747.00
6	5.133	62.06	6.223	15743.70
Average	5.13	62.35	6.22	15751.02
SD	0.0010	0.2351	0.0017	23.4169
% RSD	**0.02**	**0.38**	**0.03**	**0.15**

Table 7: Results of Intermediate Precision Study

Intermediate Precision Data				
Sr. No	***(R)*-enantiomer**		**Duloxetine**	
	RT	**Area**	**RT**	**Area**
1	5.181	63.95	6.291	15985.00
2	5.179	64.55	6.287	16010.70
3	5.18	63.87	6.288	15982.60
4	5.179	64.24	6.289	15996.00
5	5.186	64.84	6.293	16020.60
6	5.183	64.57	6.293	15997.10
Average	5.18	64.34	6.29	15998.67
SD	0.0027	0.3798	0.0026	14.6993
% RSD	**0.05**	**0.59**	**0.04**	**0.09**

5.2.3 Result of Limit of Detection and Limit of Quantification

The LOD and LOQ concentration were estimated to be 150 and 400 ng/mL for *(R)*-enantiomer, when the signal-to-noise ratio of 3 and 10 were used as the criteria. The method precision for *(R)*-enantiomer at LOQ was 1.4 % RSD (Table 8). The recovery of *(R)*-enantiomer at LOQ was 93 % in the spiked Duloxetine samples. It can be said that LOQ value of the *(R)*-enantiomer is quite law than the specification limit. The method is precise and accurate at LOQ level.

Table 8: Results of Precision study at LOQ level

Sr. No	***(R)*-enantiomer at LOQ level**	
	RT	**Area**
1	5.195	9.85
2	5.196	9.74
3	5.192	9.54
4	5.192	9.63
5	5.194	9.64
6	5.189	9.47
Average	5.19	9.65
SD	0.0025	0.1351
% RSD	**0.05**	**1.40**

5.2.4 Result of Linearity of (R)-enantiomer

The described method was linear in the range of 400-3000 ng/mL for *(R)*-enantiomer. The calibration curve was drawn by plotting the peak area verses its corresponding concentration with a correlation coefficient of 0.999. The equation of the calibration curve for *(R)*-enantiomer was $y = 31.402x - 3.4162$. Linearity was checked for *(R)*-enantiomer over the same concentration range for three consecutive days (Fig. 24, 25 and 26). The percentage relative standard deviation of the slope and Y-intercept of the calibration curve were 0.25 and 4.4, respectively. The data are summarized in Table 9. It can be observed that method is linear within the studied range.

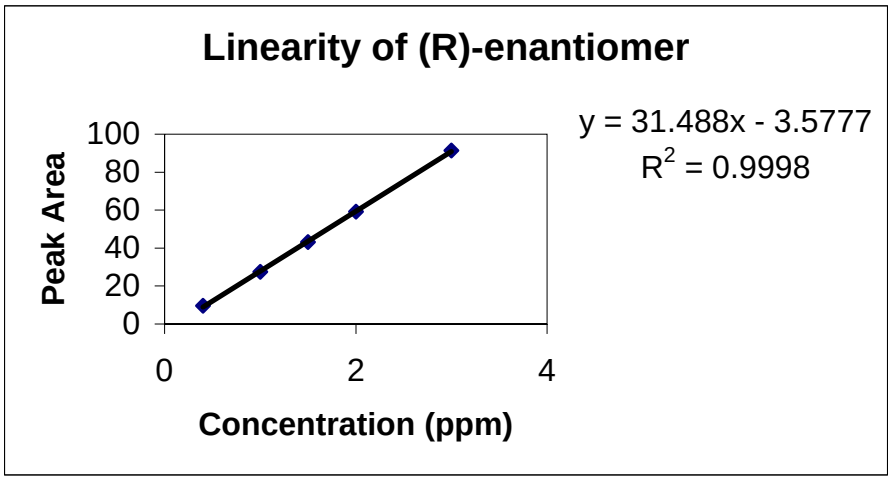


Fig. 24: Linearity of (R)-enantiomer (400 to 3000 ng/mL), **for Day 1**

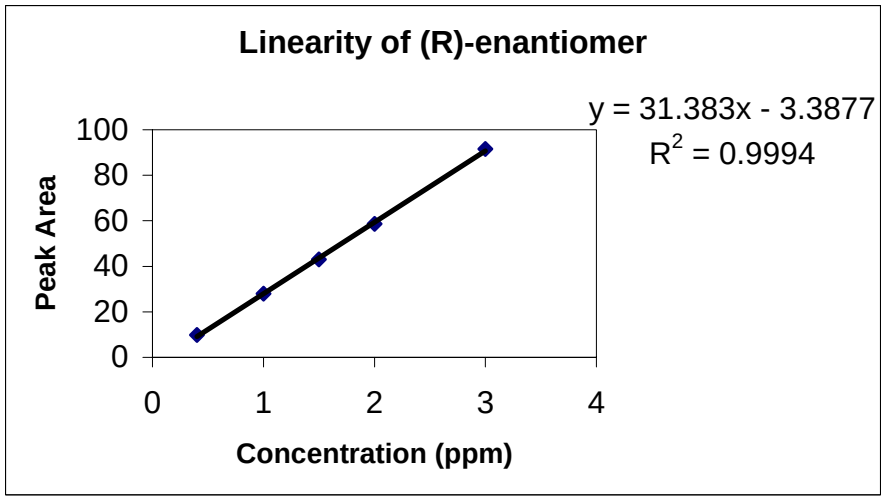


Fig. 25: Linearity of (R)-enantiomer (400 to 3000 ng/mL), **for Day 2**

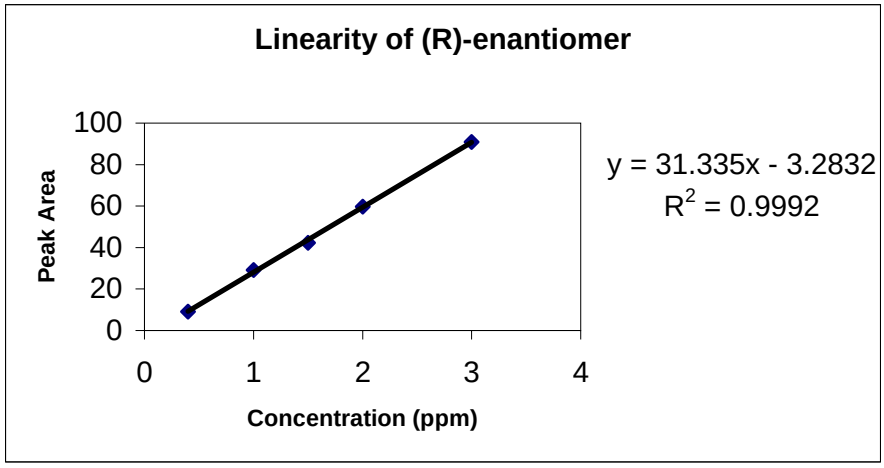


Fig. 26: Linearity of (R)-enantiomer (400 to 3000 ng/mL), **for Day 3**

Table 9: Results of Linearity Study

Linearity	Equation of Calibration curve	
	Slope	Intercept
Day 1	31.488	3.5777
Day 2	31.383	3.3877
Day 3	31.335	3.2832
Average	31.402	3.4162
SD	0.0782	0.1493
% RSD	0.2492	4.3705

5.2.5 Result of Accuracy Study

In order to determine the accuracy of the method standard addition and recovery experiments were conducted in triplicate at 0.4, 0.50 and 0.6 % of analyte concentration, i.e. 1600, 2000 and 2400 ng/mL of *(R)*-enantiomer. Recovery was calculated from slope and Y-intercept of the calibration curve obtained in linearity study. The data are presented in Table 10. It can be seen from the data that method is very accurate for the determination of the *(R)*-enantiomer in bulk drug.

Table 10: Recovery Results of *(R)*-enantiomer in Bulk Sample

Added (ng)	Recovered (ng)	% Recovery	% RSD
1600	1676	104.7	1.7
2000	2098	104.9	0.7
2400	2495	103.9	0.5

n = 3 determinations

5.2.6 Result of Robustness Study

The chromatographic resolution between both enantiomers was used to evaluate the method robustness under modified conditions. Sufficient resolution between Duloxetine and *(R)*-enantiomer was obtained under all separation conditions tested, which was demonstrated sufficient robustness. Results are summarized in Table 11. It can be seen from the data that method is robust for its intended use.

Table 11: Results of Robustness Study

Parameter	Resolution between two enantiomers
Flow rate (mL/min)	
0.9	2.41
1.0	2.38
1.1	2.29
pH of buffer	
3.7	2.31
3.8	2.38
3.9	2.40
Concentration of buffer (mM)	
8	2.34
10	2.38
12	2.35

Column temperature	
19	2.41
20	2.38
21	2.35

5.2.7 Result of Solution Stability

There was no significant change in the content of *(R)*-enantiomer observed during solution stability experiments. The data are presented in Table 12. It can be seen from the data that % bias of the area of *(R)*-enantiomer was within 2.0 %. Hence, Duloxetine sample solution is stable for at least 2 days.

Table 12: Result of Solution Stability

Time interval	***(R)*-enantiomer**		
	RT	**Area**	**% Bias of Area**
Initial	5.196	64.18	-
6 h	5.194	63.85	-0.51
12 h	5.192	64.49	0.48
24 h	5.193	63.95	-0.36
48 h	5.192	63.31	-1.36

6. Conclusion

A simple, rapid and accurate reverse phase chiral LC method has been developed and validated for the enantiomeric separation of Duloxetine on the Chiral-AGP column.

In the strategy of method development, the effect of organic modifier, the effect of pH of buffer, the effect of concentration of buffer and the effect of column temperature on the resolution and retention of enantiomers have been evaluated. The thermodynamic parameters (H), (S) and (G) have been determined.

The optimized chromatographic parameters gives baseline separation with resolution greater than 2.2 between the two enantiomers within 8 min. The method was completely validated showing satisfactory data for all the method validation parameters tested.

The developed method can be used for the quantitative determination of chiral impurity [*(R)*-enantiomer] in the bulk drug substance.

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Part-A

Application of the Chromatography for the Separation of Isomers of Some Pharmaceuticals

Section-II

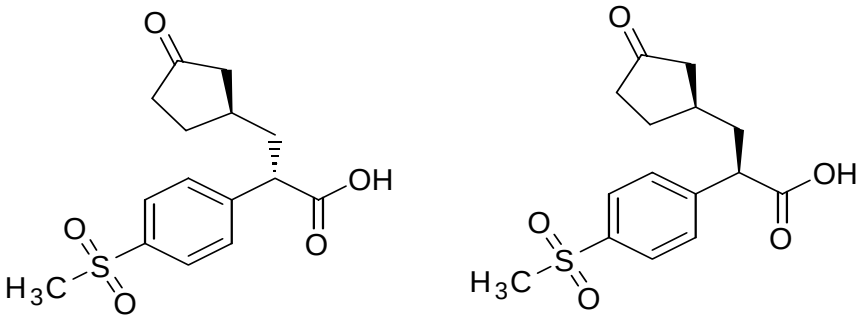
HPLC Separation of Two Diastereomers of 2-[4-(Methylsulfonyl)Phenyl]-3-(3(R)-Oxocyclopentyl) Propanoic Acid

1. Introduction

Approximately, 25 % of commercially available chiral drugs are either racemates or diastereomers & biological activity of chiral molecule substantially differs from its racemates or its stereoisomers is well documented [1, 2]. Thus, the separation of each isomer in such mixtures is usually critical to both the pharmaceutical industry as well as to the regulatory agencies to assure the safety and efficacy of the drug [3, 4]. Consequently, the industry regulators require the drug sponsors to provide assay methods capable of resolving and quantitating the stereoisomers to assess the stereo chemical integrity of the drug substance and drug product [5].

In line of our focus of research in part-A, chromatographic separation of different isomers, we have attempted separation of diastereomers in this section. Diastereomers (or diastereoisomers) are stereoisomers that are not enantiomers (non-superimposable mirror images of each other). Diastereomers can have different physical properties and different reactivity. In another definition diastereomers are pairs of isomers that have opposite configurations at one or more (but not all) of the chiral centers so they are not mirror images of each other [6]. In simpler terms two stereoisomers are said to be diastereoisomers if they are not mirror images of each other and one or more stereogenic centers differ between the two stereoisomers.

The new set of diastereomers was selected for the present study, the selected compound is 2-[4-(methylsulfonyl)phenyl]-3-(3*(R)*-oxocyclopentyl)propanoic acid (Fig. 1). It has novel skeleton and an interesting molecule for generation of pharmacologically active compounds. Its molecular formula is $C_{15}H_{18}O_5S$ having molecular weight 310.37 g/mole.



(S, R) ***(R, R)***

Fig. 1: Chemical Structure of 2-[4-(methylsulfonyl)phenyl]-3-(3*(R)*-oxocyclopentyl)propanoic acid

C-alkylation of ethyl 2-[4-(methylthio)phenyl]acetate with 2*(S)*-iodomethyl-8,8-dimethyl-6,10-dioxaspiro[4.5]decane and consecutive hydrolysis and oxidation yielded two diastereomers of 2-[4-(methylsulfonyl)phenyl]-3-(3*(R)*-oxocyclopentyl)propanoic acid [7]. As shown in Fig. 1, the title compound contains two chiral centers, one with fixed configuration *(R)* and an another chiral center is racemic therefore, two diastereomers *(R,R)* and *(S,R)* are possible. The one diastereomer of title compound can be converted to another diastereomer by crystallization induced dynamic resolution. To monitor this conversion process, suitable and precise chromatographic method was extensively needed.

The coversion of undesired isomer to desired isomer is also very important to reduce waste from green chemistry angle. The green chemistry consists of chemicals and chemical processes designed to reduce or eliminate negative environmental impacts. The use and production of these chemicals may involve reduced waste products, non-toxic components, and improved efficiency.

Green chemistry is a highly effective approach to pollution prevention because it applies innovative scientific solutions to real-world environmental situations. The 12 Principles of Green Chemistry, originally published by Paul Anastas and John Warner [8] provide a road map for chemists to implement green chemistry.

This study describes development of HPLC method for separation of the two diastereomers of 2-[4-(methylsulfonyl)phenyl]-3-(3*(R)*-oxocyclopentyl)propanoic acid on a non-chiral column. The method development involved the selection and optimization of the stationary phase, mobile phase, column temperature etc. The optimized method was subjected to partial validation. The method was validated for the parameters, such as, system suitability, peak homogeneity, precision, limit of detection, limit of quantification, linearity, robustness and finally solution stability.

2. Literature Review

The literature review regarding chromatographic separation of diastereomers was revealed that various analytical methods were reported for different set of diastereomers in bulk drug and pharmaceutical formulation using chiral and non-chiral column.

The high performance liquid chromatography is a powerful and widely used technique for the separation and quantitation of diastereomers [9-17]. The use of chiral columns to separate the stereoisomers using HPLC is very common, following are the examples, where author used chiral column for the separation of stereoisomers.

- **Kumar N, Windisch V, Trivedi P** and **Golebiowski C.** have reported use of -cyclodextrin chiral column for the separation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor drug substance diastereomers, and their analogues [12].
- **Kunath A, Theil F.** and **Wagner J.** have reproted Diastereomeric and enantiomeric separation of monoesters prepared from meso-cyclopentanediols and racemic carboxylic acids on a silica phase and on amylose and cellulose chiral stationary phases [13].
- **Wu C, Akiyama A** and **Straub IA.** have reported high-performance liquid chromatographic reversed-phase and normal-phase separation of diastereomeric -ketoamide calpain inhibitors on Nucleosil Chiral-2 column [11].
- **Gopal D, Grinberg N, Dowling T, Prepall H, Bicker G,** and **Tway P.** have used quinine as an additive to the mobile phase to enhance separation of diastereomers of pharmaceutical compound on a diol column in normal phase mode [14].
- **Akapo S, McCrea C, Gupta J, Roach M** and **Skinner W.** have used Chiral-AGP column as protein based chiral stationary phase in reversed phase mode to analyze formoterol stereoisomers [16].

In light of frequent use of chiral column, separation of diastereomers using non-chiral column is certainly noteworthy. However, there are few reports describing use of non-chiral column in reversed phase mode to separate diastereomers.

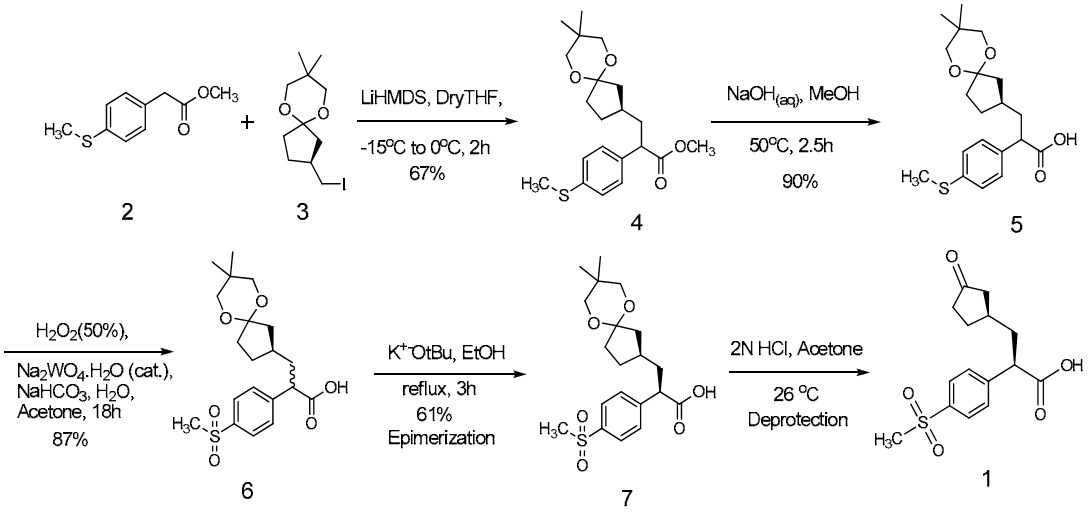
- **Rauwald HW** and **Beli A** have reported high-performance liquid chromatographic separation and determination of diastereomeric anthrone-C-glucosyls in Caps aloes [9].
- **Kirby DA, Miller CL** and **Rivier JE** have reproted separation of neuropeptie Y diastereomers by high-performance liquid chromatography and capillary zone electrophoresis [10].
- **Wang T.** hasreported High-performance liquid chromatographic separation of -methyl ADC-13 enolphosphate diphenyl ester and its -methyl diastereomers [15].
- **Wu C, Akiyama A** and **Straub IA.** have reported high-performance liquid chromatographic reversed-phase and normal-phase separation of diastereomeric -ketoamide calpain inhibitors [11].

There is no direct report available for the HPLC separation of diastereomers of the compound of interest, 2-[4-(methylsulfonyl)phenyl]-3-(3*(R)*-oxocyclopentyl)propanoic acid on non-chiral column.

3. Aim of Present Work

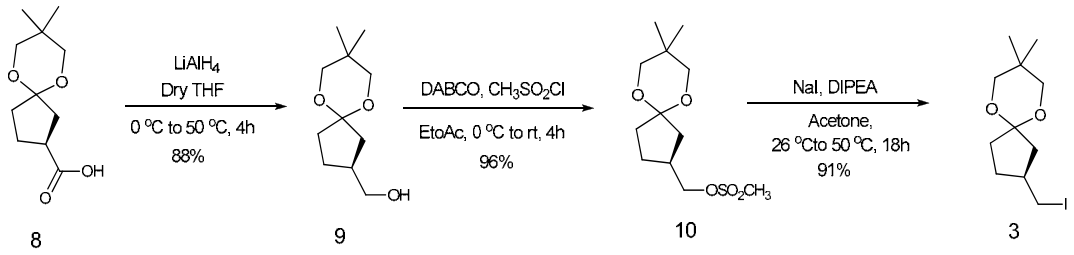
As per preceding discussion, separation method for the stereoisomers is very much needed by pharmaceutical industries and regulatory agencies. The aim of present study was to develop HPLC method for the separation of new set of diastereomers on non-chiral column. The selected compound is new moiety, which may further modify to generate pharmacologically active compound for the drug discovery program. The title compound, 2-[4-(methylsulfonyl)phenyl]-3-(3*(R)*-oxocyclopentyl)propanoic was synthesized by chemist as per scheme 1. The mixture of two diastereomers (Fig.1) needed for the present study was obtained by de-protection of the compound **6** with 2N HCl, Acetone at 26°C.

Scheme 1



C-alkylation of ethyl 2-[4-(methylthio)phenyl]acetate (**2**) with 2*(S)*-iodomethyl-8,8-dimethyl-6,10-dioxaspiro[4.5]decane (**3**) followed by consecutive hydrolysis and oxidation yielded two diastereomers of 2-[4-(methylsulfonyl)phenyl]-3-(3*(R)*-oxocyclopentyl)propanoic acid [7]. The iodo compound (**3**) was in turn synthesized from chiral acid (**8**) ([ ]$_D$ = +16.6^o, c = 1.5, CH_3OH) (Scheme 2), which was synthesized and resolved by chemist according to the literature procedure [18, 19]. Additionally, chiral purity for iodo compound (**3**) was checked using chiral GC (Column: BetDEXtm [225+120], 60m, 0.25mm, thickness 0.25µm), which was found 100 % chirally pure. The GC chromatograms of the racemic iodo compound and chirally pure enantiomer are shown in Fig. 2 and 3 respectively.

Scheme 2



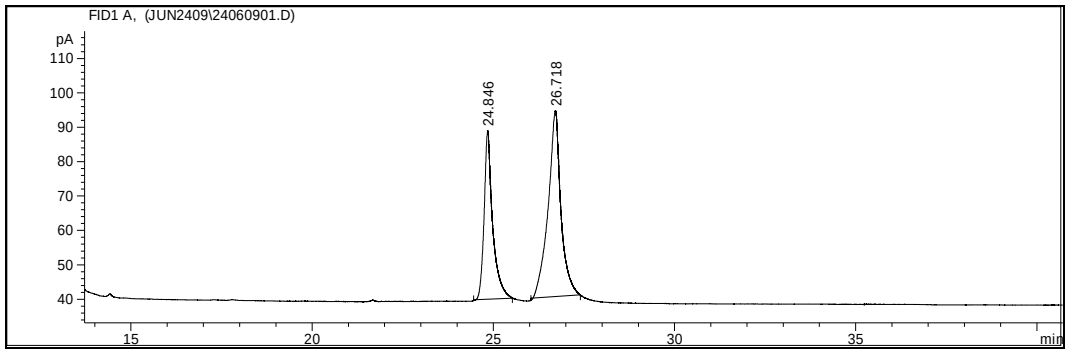


Fig. 2: Chiral GC chromatogram of racemic iodo compound

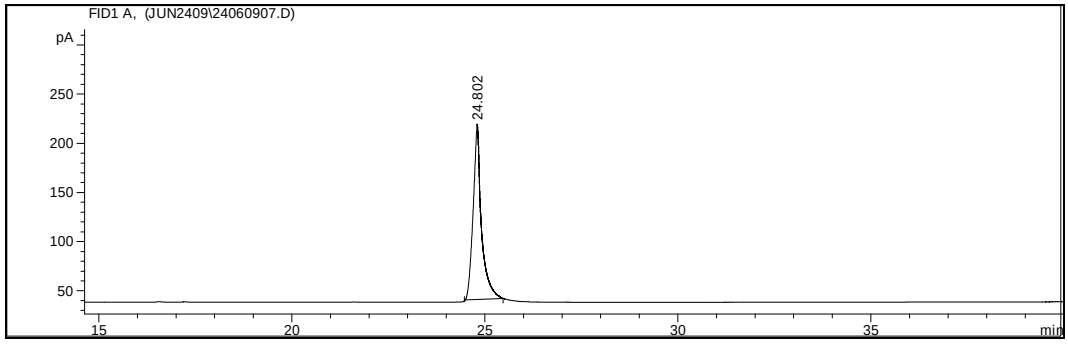


Fig. 3: Chiral GC chromatogram of chirally pure iodo compound

Although, title compound (Fig. 1) contains two chiral centers, which may have theoretically four isomers. However, since one of the chiral center of cyclopentanone ring, attached to 3-position of propanoic acid is fixed. The another chiral center at 2-position of propanoic acid was generated during synthesis is racemic. Therefore, one chiral center has fixed configuration *(R)* and another chiral center is racemic, which results two diastereomers *(S,R)* and *(R,R)*. The chemical method being employed in epimerization process from (**6**) to (**1**) was crystallization induced dynamic resolution.

To understand extend of crystallization induced dynamic resolution method and to monitor epimerization process, the chemist needed suitable chromatographic method. The present study describes development and validation of the HPLC separation method for new set of two diastereomers, which can be utilized to monitor epimerization process.

4. Experimental

4.1 Chemicals

HPLC grade Triflouroacetic acid was purchased from Merck (Germany), while Acetonitrile was purchased from S.D. Fine Chemicals (India). Water used throughout the course of this study was purified by Barnstead (USA) water purification system. Chemistry Department of Zydus Research Centre, Ahmedabad, India, kindly supplied diastereomeric mixture of the title compound.

4.2 Instrumentation

HPLC system used was an Agilent Technology (1100 series, Germany), equipped with degasser, quaternary pump, auto sampler, auto injector, thermostatic compartment and photo diode array detector. The out put signal was monitored and processed using Agilent Chemstation software. Peak homogeneity was checked by Shimadzu LCMS 2010A (Japan) using positive electron spray ionization mode.

4.3 Mobile Phase Preparation

The mobile phase was 0.05 % triflouroacetic acid in water-acetonitrile (85:15, v/v). The 0.05 % triflouroacetic acid solution was prepared by dissolving 500 L triflouroacetic acid with water and prepared 1000 mL solution and filtered through a 0.45µm nylon membrane (Millipore Pvt. Ltd. Banglore, India). The final mobile phase was prepared by mixing 850 mL of buffer solution and 150 mL of acetonitrile in 1000 mL mobile phase bottle and degassed in an ultrasonic bath (Spincotech Pvt. Ltd., Mumbai).

4.4 Diluent Preparation

Mobile phase used as a diluent.

4.5 Sample Preparation

Stock solution of diastereomeric mixture of the title compound (1000 g/mL) was prepared by dissolving the appropriate amount of the substance in mobile phase. The analyte concentration of each diastereomer was fixed as 100 g/mL. Working

solutions for method validation was prepared by subsequence dilution of the stock solution in mobile phase.

4.6 Chromatographic Conditions

The chromatographic conditions were optimized using a J'sphere-ODS-H80 (150 mm 4.6 mm, 4 m, YMC make, Japan) HPLC column. The mobile phase was 0.05 % triflouroacetic acid in water-acetonitrile (85:15, v/v). The flow rate was set at 1.0 mL/min. The column was maintained at 30° C, and the detection was carried out at 228 nm. The injection volume was 5 L. The reverse phase columns ODS-AQ (250 mm 4.6 mm, 5 m, YMC make, Japan) and Kromasil-C_{18} (150 mm 4.6 mm, 5 m) were also employed during method development.

4.7 Method validation

4.7.1 System suitability and Peak Homogeneity

System suitability test is an integral part of chromatographic methods and is used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability parameters such as, resolution (Rs), symmetry (S), retention factor (k), separation factor ( ) and efficiencies (N) were determined. Peak homogeneity of both diastereomers were confirmed using PDA and MS detector.

4.7.2 Precision

Precision of the method is the degree of agreement among the individual test results when the procedure is applied repeatedly to multiple sampling of a homogenous sample. The precision of the method was checked by analyzing six replicate samples of both diastereomers at the analyte concentration (100 g/mL) and calculating the percentage relative standard deviation of retention time and peak area.

4.7.3 Limit of Detection and Limit of Quantification

Limit of detection (LOD) and Limit of quantification(LOQ) were determined at a signal-to-noise ratio of 3 and 10. LOD and LOQ were achieved by injecting a series of dilute solutions of both the diastereomers.

The precision of the developed diastereoselective method for both the diastereomers at LOQ was checked by analyzing six test solutions prepared at the LOQ level and calculating the percentage relative standard deviation of retention time and peak area.

4.7.4 Linearity

Detector response linearity was evaluated for both the diastereomers by determining seven working solutions from 0.5 (LOQ) to 200 g/mL, (0.5, 1, 2, 5, 10, 50, 100 and 200 g/mL), prepared in mobile phase. Peak area and concentration of diastereomers were subjected to regression analysis to calculate calibration equation and correlation coefficient.

4.7.5 Robustness

The robustness of a method is the ability of the method to remain unaffected by small changes in parameters such as flow rate, mobile phase composition and column temperature. To determine robustness of the method, experimental conditions were purposely altered and chromatographic resolution between two diastereomers was evaluated.

The flow rate of the mobile phase was 1.0 mL/min. To study the effect of flow rate on the resolution of diastereomers, it was changed by 0.1 units from 0.9 to 1.1 mL/min. The effect of change in percent acetonitrile on resolution was studied by varying from – 1 to + 1 % while the other mobile phase components were held constant as stated in *Chromatographic conditions*. The effect of concentration of the trifloroacetic acid (in water) on resolution was studied at 0.04 % and 0.06 % instead of 0.05 %, while the other mobile phase components were held constant as stated in *Chromatographic conditions*. To study the effect of column temperature on the resolution of diastereomers, it was maintained at 25° C and 35° C instead of 30° C.

4.7.6 Solution Stability

Stability of sample solution at analyte concentration was studied by keeping the solution in tightly capped volumetric flask at room temperature on a laboratory bench for 2 days. Sample solution was analyzed for 6, 12, 24 and 48 h.

5. Result and Discussion

Development and Optimization of the HPLC Method

The objective of this study was to separate the two diastereomers of the title compound, using a non-chiral column, since diastereomers possess different physical properties. The sample solution of mixture of two diastereomers was used for the method development. The wavelength of the detection was set 228 nm based on the UV spectra of both the diastereomers (Fig. 4 and Fig. 5)

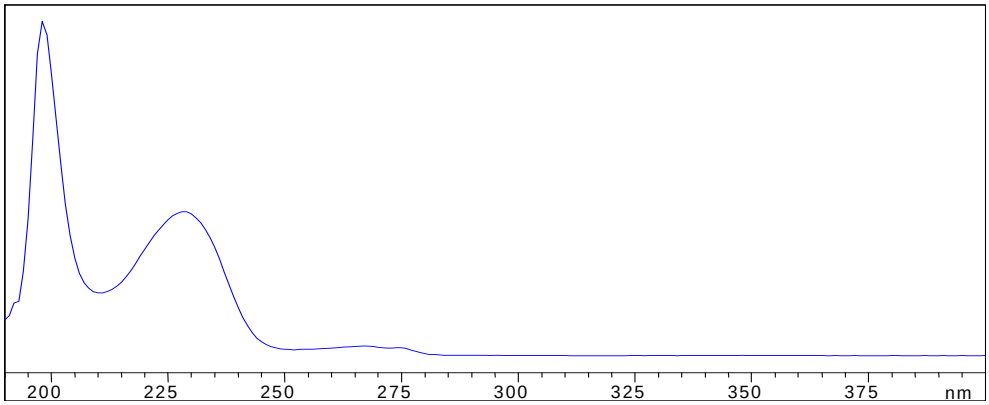


Fig. 4: UV spectra of the Diastereomer-1

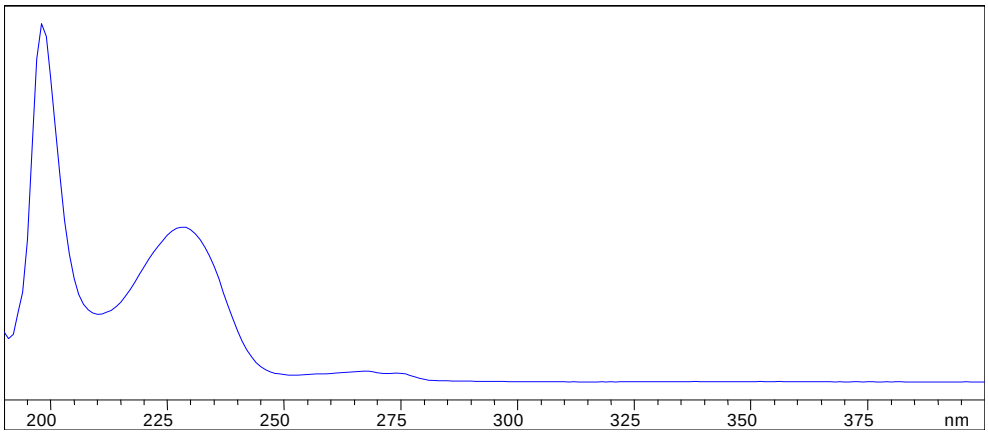


Fig. 5: UV spectra of the Diastereomer-2

To develop the suitable HPLC method for the separation of diastereomers, different mobile phases and stationary phases were employed. For this, different columns were used, namely: YMC ODS-AQ, Kromasil-C_{18} and YMC J'sphere-ODS-H80.

The compound of interest has free carboxylic acid group. Therefore, ionization of compound may play an important role in retention and as a result in the separation of both the diastereomers. To investigate effect of pH of the mobile phase, couples of experiments were performed using YMC ODS-AQ column. This column had hydrophilic endcappings, which could allow polar eluent to penetrate between the C_{18} chains to enhance solute-stationary phase interactions. Stronger retention and often different selectivity are encountered on ODS-AQ compared with conventionally endcapped ODS [20]. Few of trials are discussed here.

The initial attempt was made using phosphate buffer (pH 7.0; 20 mM), but separation was not achieved by using either methanol or acetonitrile as a organic modifier in different composition. Mixture of diastereomers was eluted before 2 minutes as a single peak (Fig. 6).

Trial Condition 1

Column: YMC ODS-AQ, 150 x 4.6 mm, 5 m

Mobile phase: Phosphate buffer (pH 7.0; 20 mM)-methanol (70:30, v/v)

Flow Rate: 1.0 ml/min

Column Temperature: 30°C

Wavelength of detection: 228 nm

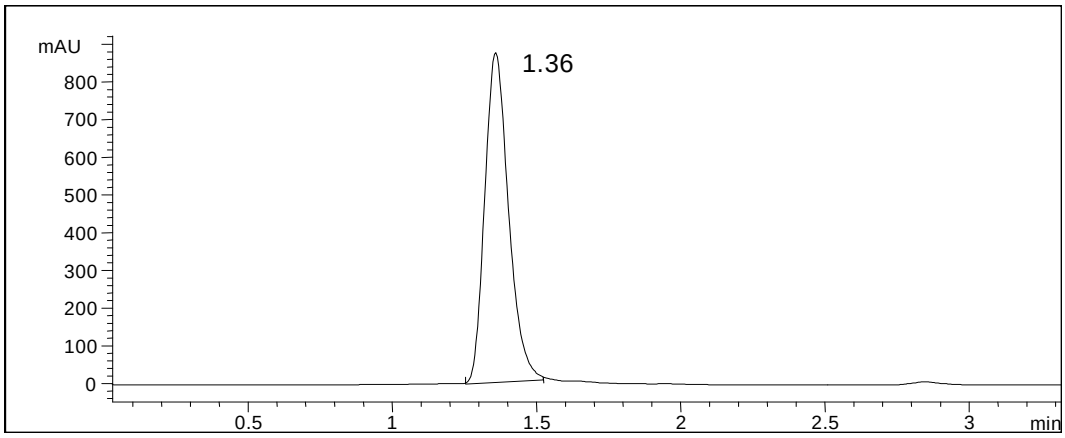


Fig. 6: Chromatogram of trial condition 1

It can be seen from the chromatogram that column could not discriminate two diastereomers of the compound of interest at pH 7.0 of the buffer. Theoretically, compound may be in ionized form at this pH and the ionized form of the compound

nature is polar and more loving to the mobile phase. Since, compound was not retaining in the column because of the mobile phase pH, stationary phase could not find sufficient time to discriminate both the diastereomers. Therefore, mobile phase should use towards acidic pH.

In further attempt, acetate beffer (pH 4.5; 20 mM) and phosphate buffer (pH 3.0; 20 mM) were tried. The separation of diastereomers was observed but it was not up to baseline (Fig. 7 and Fig. 8).

Trial Condition 2

Column: YMC ODS-AQ, 150 x 4.6 mm, 5 m

Mobile phase: Acetate buffer (pH 4.5; 20 mM)-methanol-acetonitrile (80:15:5, v/v/v)

Flow Rate: 1.0 ml/min

Column Temperature: 30°C

Wavelength of detection: 228 nm

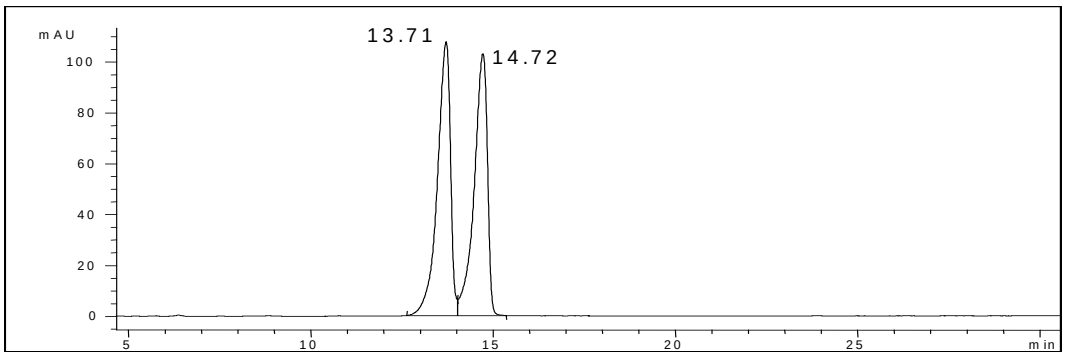


Fig. 7: Chromatogram of trial condition 2

Trial Condition 3

Column: YMC ODS-AQ, 150 x 4.6 mm, 5 m

Mobile phase: phosphate buffer (pH 3.0; 20 mM)-methanol (70:30, v/v);

Flow Rate: 1.0 ml/min

Column Temperature: 30°C

Wavelength of detection: 228 nm

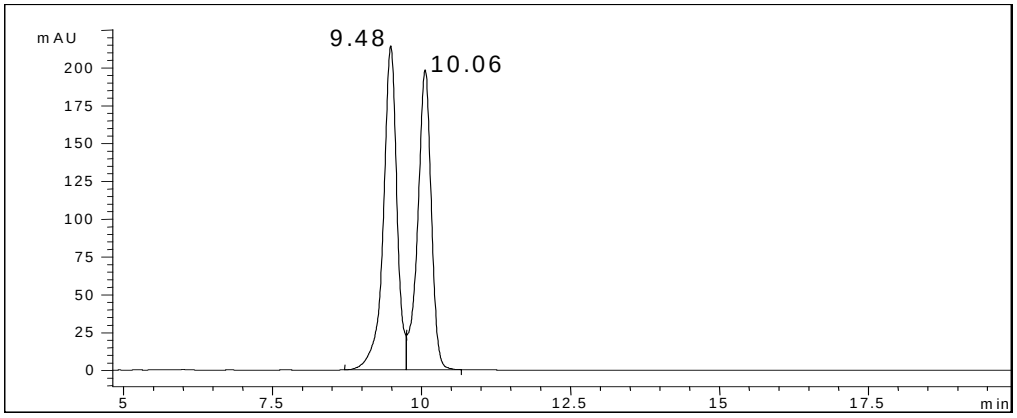


Fig. 8: Chromatogram of trial condition 3

It can be said from the chromatograms of trial condition 2 and 3 that separation of the diastereomers observed towards acidic pH. However, resolution is not base-to-base, therefore, buffer pH and mobile phase composition should be optimized to get desire resolution between two diastereomers.

To improve the separation, 0.05 % trifloroacetic acid in water-acetonitrile (85:15, v/v) was tried. The baseline separation was achieved with slight fronting in the peaks of both the diastereomers using YMC ODS-AQ and Kromasil-C_{18} column. After these optimizations, non-chiral column YMC J'sphere-ODS-H80 (150 mm 4.6 mm, 4 m) and mobile phase consisting of 0.05 % trifloroacetic acid in water-acetonitrile (85:15, v/v) were found to be appropriate allowing good baseline resolution of both the diastereomers (Fig. 9).

Optimized Condition

Column: YMC J'sphere-ODS-H80 (150 mm 4.6 mm, 4 m)

Mobile phase: 0.05 % trifloroacetic acid in water-acetonitrile (85:15, v/v)

Flow Rate: 1.0 ml/min

Column Temperature: 30°C

Wavelength of detection: 228 nm

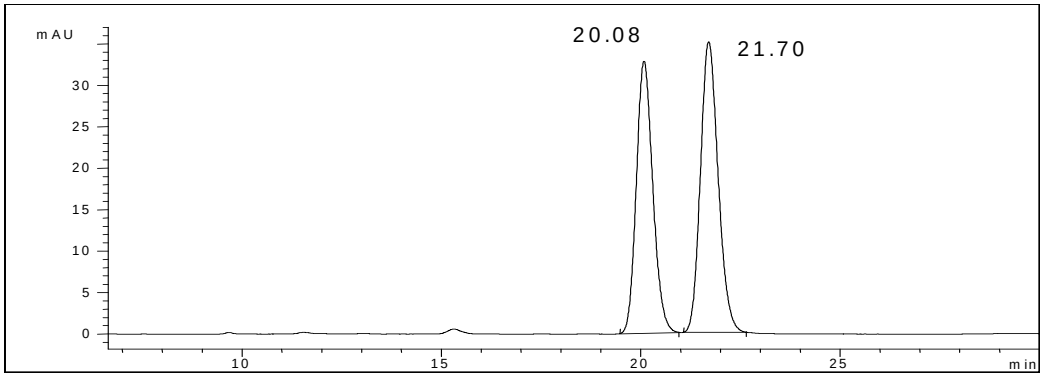


Fig. 9: Chromatogram of optimized condition

The optimized condition gave good peak shape, adequate separation and baseline resolution. System suitability parameters were also found within acceptable limit. Additionally, effect of the column temperature was also investigated on the separation of diastereomers. Experiment was carried out at 25, 30, 35, and 40° C column temperature while the other mobile phase components were held constant as stated in Optimized condition. The influence of temperature on the different parameters (retention and separation factors, peak symmetry, efficiencies, resolution) was summarized in Table 1. It was observed that an increase in temperature leads to a general decrease in the retention factors and resolution. Therefore, 30° C temperature seems suitable to achieve desirable diastereomeric separation.

Table 1: Effect of Column Temperature on System Suitability Parameters

System suitability parameters	25°C	30°C	35°C	40°C
Retention factor	k_1= 13.72 k_2= 14.91	k_1= 11.92 k_2= 12.94	k_1= 10.50 k_2= 11.40	k_1= 9.25 k_2= 10.03
Resolution	Rs=2.23	Rs=2.19	Rs=2.11	Rs=2.01
Numbers of theoretical plates	N_1=13111 N_2=13248	N_1=12677 N_2=13138	N_1=12649 N_2=12734	N_1=12260 N_2=12118
Symmetry	S_1=0.79 S_2=0.80	S_1=0.82 S_2=0.83	S_1=0.78 S_2=0.79	S_1=0.74 S_2=0.75
Selectivity	=1.09	=1.08	=1.08	=1.08

To gain an insight in the retention mechanism, the thermodynamic Van't Hoff relationship was studied by plotting ln k-values versus 1/T. Indeed, in HPLC, the partition coefficient (K) of a solute decreases when increasing the temperature [21].

$$\ln K = - \quad G°/RT = - \quad H°/RT + \quad S°/R \qquad (1)$$

where $G°$ is the Gibbs free energy for the solute-stationary phase interaction, R is the gas constant (8.314 J/mol K), $H°$ and $S°$, the enthalpy and entropy of transfer of the solute from the mobile to the stationary phase.

The distribution constant K is directly linked to the retention factor k, following:

$$k = \quad K \qquad (2)$$

with , the stationary to mobile phase volume ratio. Therefore, expression (1) becomes:

$$\ln k = - \quad H°/RT + \quad S°/R + \ln \qquad (3)$$

Experimentally, Van't Hoff plots were made for both diastereomers, varying temperature from 298 to 313° K. It is presented in Fig. 10.

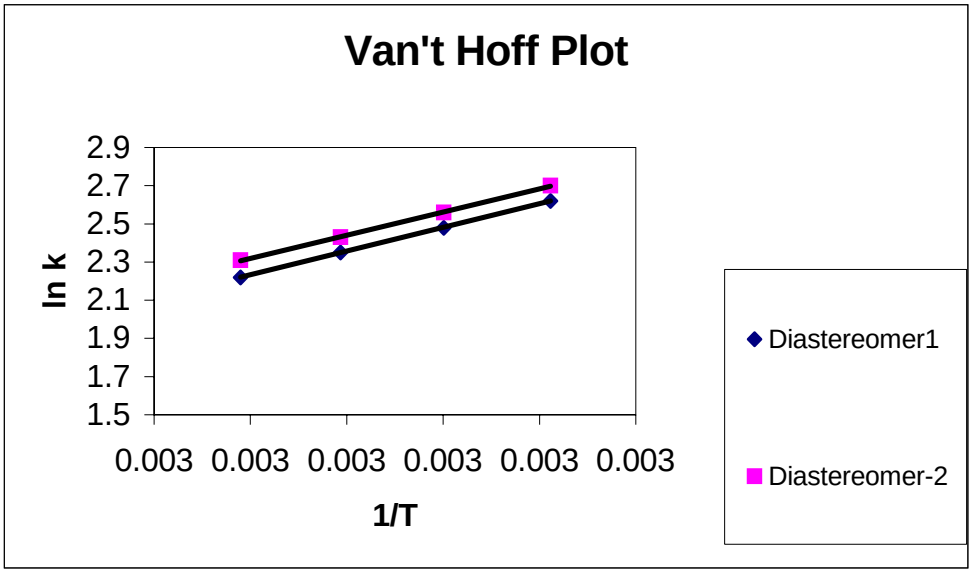


Fig. 10: Van't Hoff Plot of both the Diastereomers

Linearity equation for the Diastereomers-1: $y = 2481.3x - 5.7073$

Linearity equation for the Diastereomers-2: $y = 2426x - 5.4426$

A good linear correlation was observed over the studied range (r 0.999), indicating that the retention mechanism is independent of a change in temperature in the studied range (Table 2). From these linear plots, the standard enthalpy $H°$ and entropy $S°$ were calculated, using the slope ($- H°/R$) and the intercept ($S°/R + ln$), and results are summarized in Table 2. The enthalpy term represents the energy involved when the solute molecules break their interactions with the mobile phase and interact with, and enter, the stationary phase. These interactions result from intermolecular forces and are accompanied by the absorption or evolution of heat. It is seen that the Van't Hoff curve indicates a very large enthalpy value but conversely a very low entropy contribution means that the distribution in favor of the stationary phase is dominated by molecular forces. To clearify further, the solute is retained in the stationary phase as a result of molecular interactions and that the forces between the solute molecules with those of the stationary phase are much greater than the forces between the solute molecules and those of the mobile phase. Thus, the change in standard enthalpy is the major contribution to the change in standard energy (Table 2). It can be said, according to $G°$ negative values (Table 2), the solute transfer is enthalpically driven ($H° \quad T \ S°$) in this temperature range.

Table 2: Thermodynamic results and correlation coefficients of Van't Hoff plots for both diastereomers

Thermodynamic parameters	**Diastereomer-1**	**Diastereomer-2**
Standard enthalpy change, $H°$ (J/mol)	- 20629.53	- 20169.76
Standard entropy change, $S°$ (J/mol)	- 44.22	- 42.02
Standard free energy change, $G°$ (J/mol)[a]	- 7451.97	- 7647.80
Correlation coefficient (r)	0.999	0.999

[a] $T = 298$ K

5.2 Results of Method Validation

5.2.1 Results of System suitability and Peak Homogeneity

The system suitability results, such as, retention factor, resolution, numbers of theoretical plates, symmetry and selectivity for both diastereomers are summarized in Table 3, which showed that resolution between both the diastereomers was not less than 2.1.

Table 3: **System suitability data**

System suitability parameters	Diastereomer-1 (n =3)	Diastereomer-2 (n=3)
Retention factor (k)	11.92	12.94
Resolution (Rs)	2.19	
Numbers of theoretical plates (N)	12647	13138
Symmetry (S)	0.83	0.82
Selectivity ( )		1.09

PDA and MS detector confirmed peak homogeneity of both peaks. Peak purity of both the diastereomers was not less than 0.999/990 using PDA detector. Report of peak purity is presented as Fig. 11 and 12 for diastereomers-1 and 2, respectively.

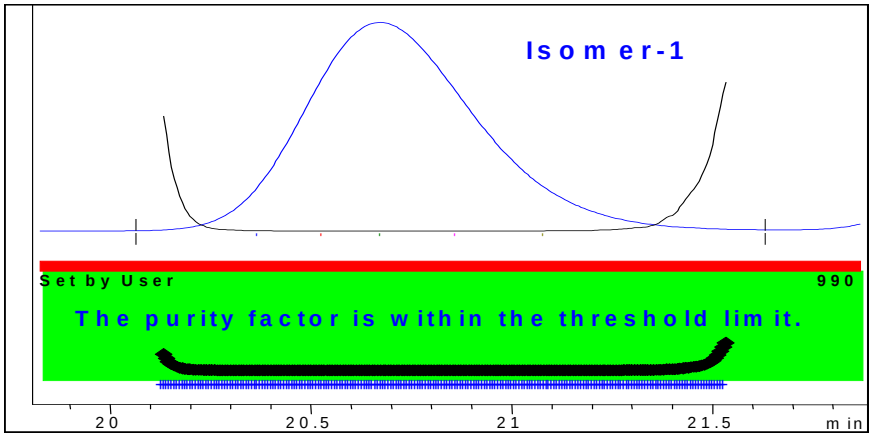


Fig. 11: Peak purity report of Diastereomer-1

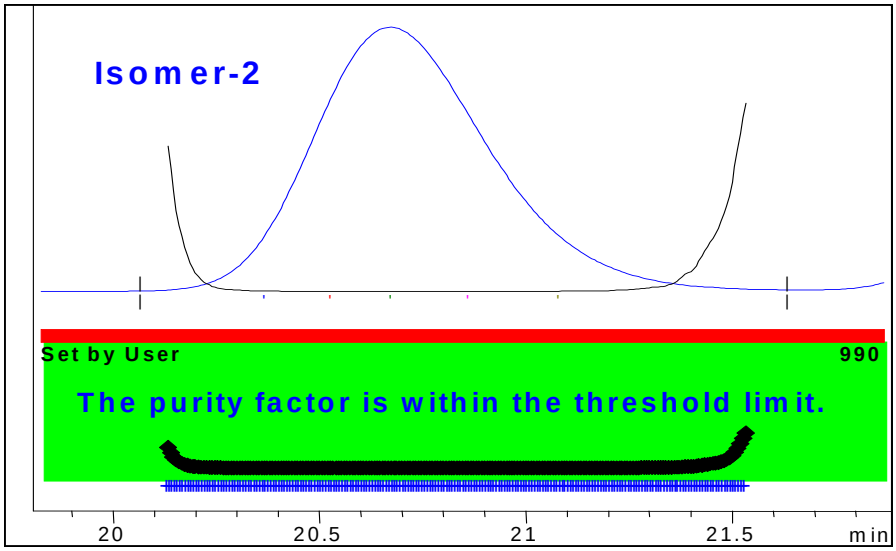


Fig. 12: Peak purity report of Diastereomer-2

The scanning (50-2000 mass unit) of both the peaks using MS detector was done to check the homogeneity. The LCMS total ion chromatogram of both diastereomers is presented in Fig. 13. The line spectra of diastereomer-1 and diastereomer-2 are shown in Fig. 14 and Fig. 15 respectively. The mass peaks of the title compound can be seen from the line spectra, corresponding to the M+1(311), M+NH_4 (328), M+Na (333) and M+K (349), which indicate homogeneity of the peaks.

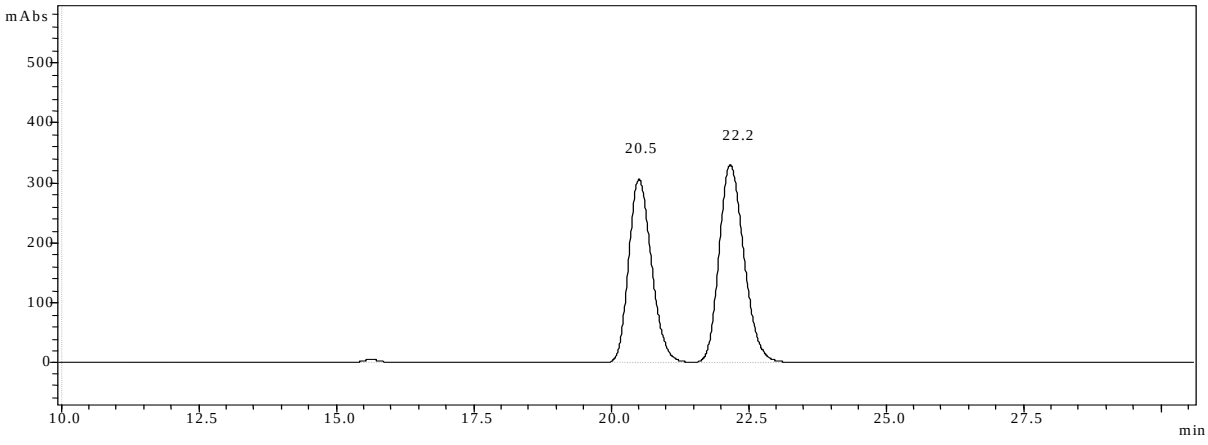


Fig. 13: LCMS total ion chromatogram of both the diastereomers.

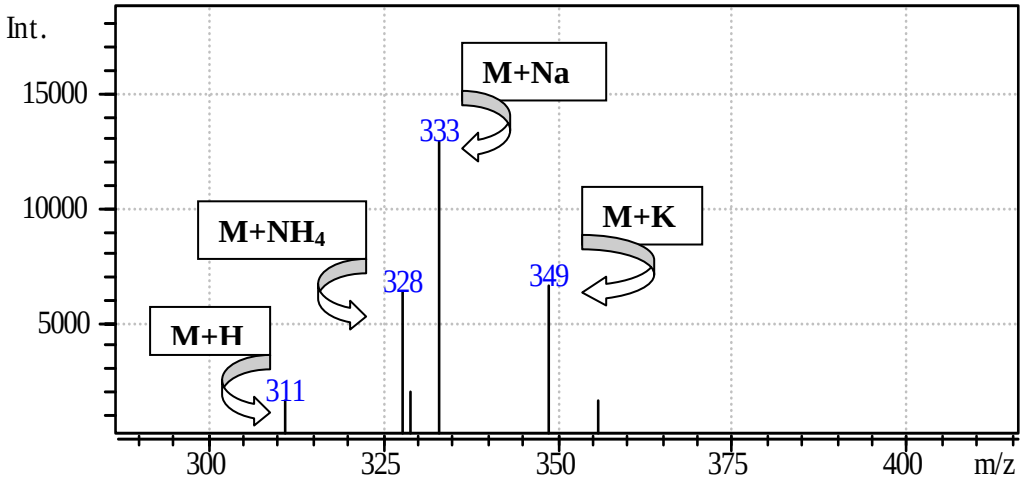


Fig. 14: Line spectra of Diastereomer-1

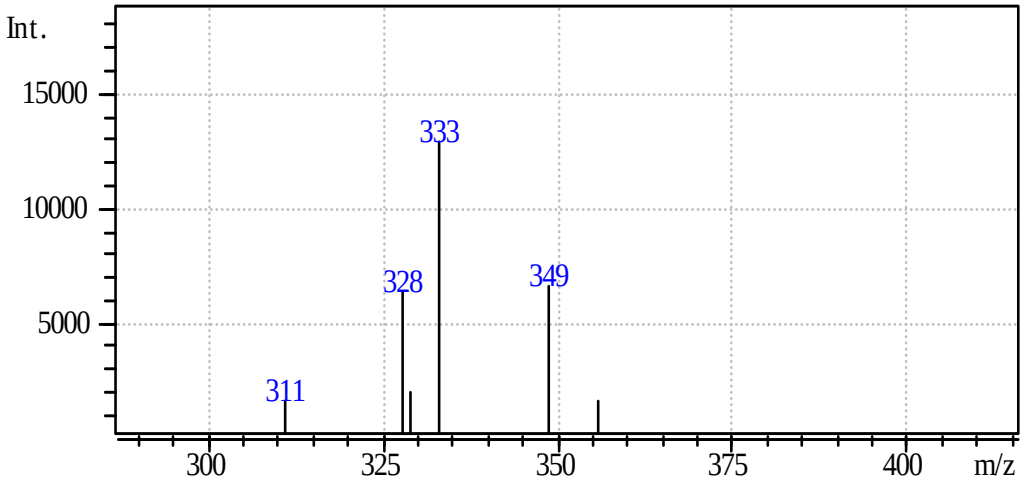


Fig. 15: Line spectra of Diastereomer-2

5.2.2 Results of Precision

In the precision study, the percentage relative standard deviation (RSD) of both the diastereomers was found to be 0.1 % for the retention time and 0.3 % for the peak area, indicating good precision of the method. Data of precision study are summarized in Table 4.

Table 4: Result of Precision Study

Precision at Test Concentration (100 ppm)				
Sr. No	**Diastereomer-1**		**Distereomer-2**	
	RT	**Area**	**RT**	**Area**
1	20.043	939.981	21.661	1081.985
2	20.053	945.922	21.672	1088.509
3	20.072	947.115	21.693	1091.014
4	20.083	945.693	21.704	1088.827
5	20.092	946.427	21.714	1090.215
6	20.085	944.720	21.699	1089.456
Average	20.071	944.98	21.691	1088.33
SD	0.019	2.573	0.020	3.242
% RSD	**0.097**	**0.27**	**0.093**	**0.298**

5.2.3 Result of Limit of detection and Limit of quantification

The LOD and LOQ concentration were estimated to be 0.15 and 0.50 g/ml for both the diastereomers, when the signal-to-noise ratio of 3 and 10 were used as the criteria. The method precision for diastereomer-1 and diastereomer-2 at LOQ was 1.8 and 1.5 % RSD, respectively. The data of the precision study at LOQ level for both diastereomers are summarized in Table 5.

Table 5: Results of Precision study at LOQ level

Precision at LOQ level (0.5 ppm)				
Sr. No	**Diastereomer-1**		**Distereomer-2**	
	RT	**Area**	**RT**	**Area**
1	20.135	4.714	21.721	5.350
2	20.139	4.751	21.777	5.305
3	20.152	4.932	21.765	5.409
4	20.130	4.720	21.782	5.337
5	20.167	4.726	21.769	5.196
6	20.151	4.713	21.754	5.253
Average	20.146	4.759	21.761	5.308
SD	0.014	0.086	0.022	0.075
% RSD	**0.068**	**1.801**	**0.101**	**1.419**

5.2.4 Result of Linearity

The described method was linear in the range of 0.5-200 g/ml for both the diastereomers. The calibration curve was drawn by plotting the peak area versus its corresponding concentration with a correlation coefficient of 0.999. The equation of the calibration curve for diastereomer-1 was $y = 9.3363x - 2.0705$ and for diastereomer-2 was $y = 10.732x - 2.4793$. The results showed good correlation between the peak area and concentration of both the diastereomers. The linearity plot of diastereomer-1 and diastereomer-2 are presented in Fig. 16 and Fig. 17 respectively.

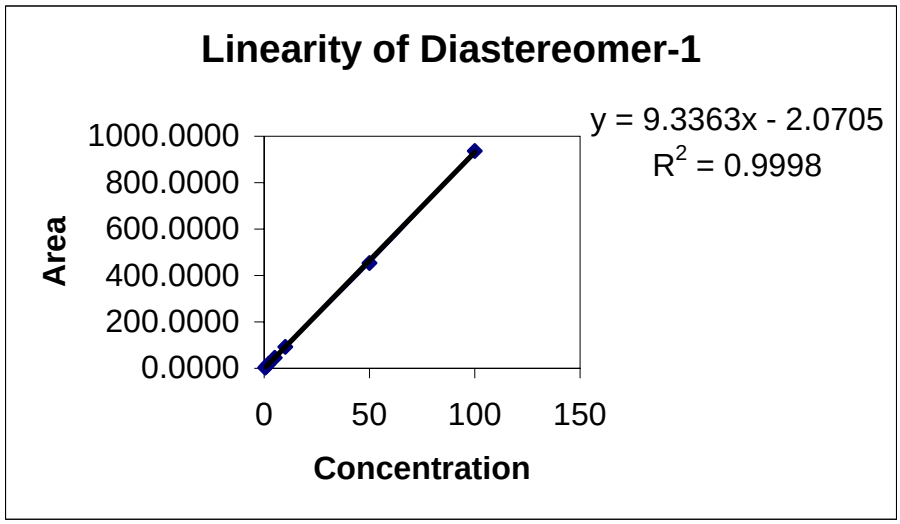


Fig. 16: Linearity of Diastereomer-1

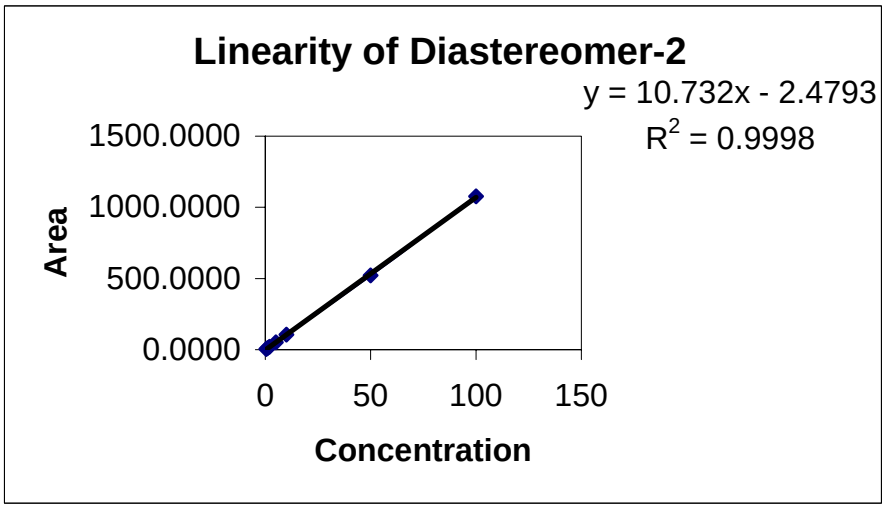


Fig. 17: Linearity of Diastereomer-2

The overlaid chromatograms of the linearity of the diastereomeric separation from 0.5 to 200 g/ml are presented in Fig. 18, while three-dimensional look is shown in Fig. 19.

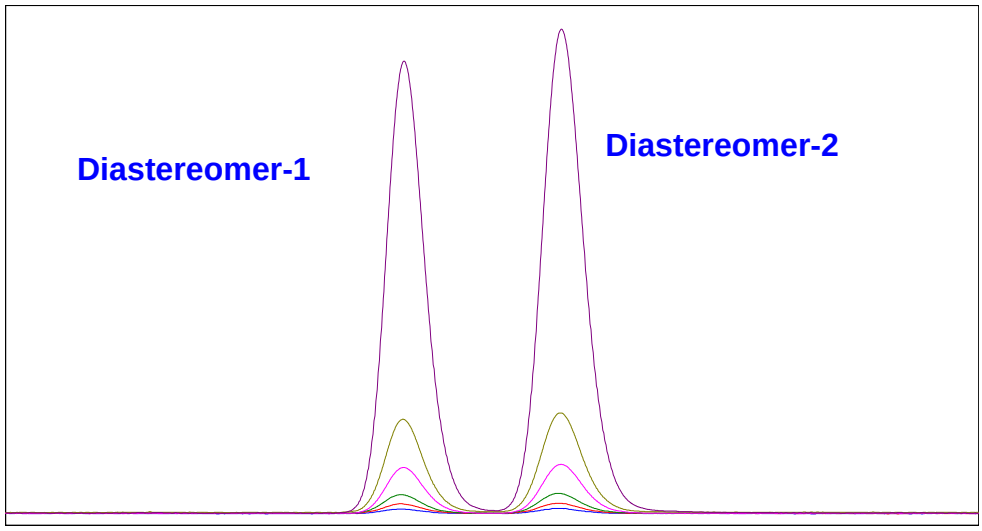


Fig. 18: Overlaid chromatograms of Linearity

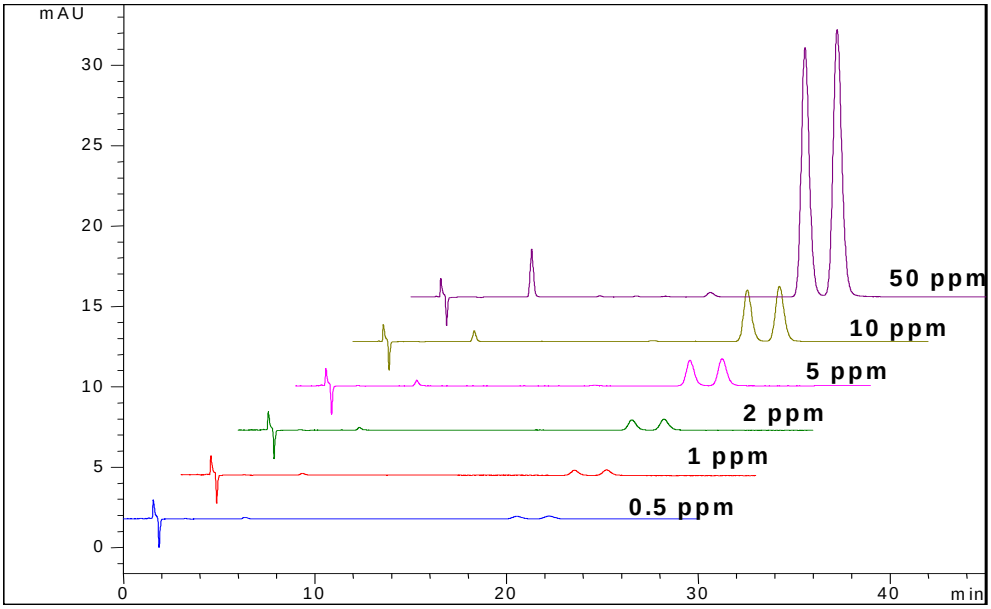


Fig. 19: Three-dimensional presentation of the chromatograms of Linearity

5.2.5 Result of Robustness Study

The chromatographic resolution between both diastereomers was used to evaluate the method robustness under modified conditions. Sufficient resolution between both the diastereomers was obtained under all separation conditions tested, which was demonstrated sufficient robustness. Results are summarized in Table 6. It can be seen from the data that method is robust for its intended use.

Table 6: Results of Robustness Study

Parameter	**Resolution between two diastereomers**
Flow rate (mL/min)	
0.9	2.20
1.0	2.19
1.1	2.09
Acetonitrile percentage in mobile phase	
14	2.28
15	2.19
16	1.98
Concentration of triflouroacetic acid (%) in water	
0.04	2.16
0.05	2.19
0.06	2.17
Column temperature	
25	2.23
30	2.19
35	2.11

5.2.6 Result of Solution Stability

There was no significant change in the peak area of both diastereomers was observed during solution stability experiments. The data are presented in Table 7. It can be seen from the data that % bias of the area was within 2.0 %. Hence, sample solution is stable for at least 2 days.

Table 7: Result of Solution Stability

Time interval	Diasteromer-1		Diasteromer-2	
	Peak Area	% Bias	Peak Area	% Bias
Initial	945.92	-	1085.94	-
6 h	944.85	- 0.11	1089.23	0.30
12 h	947.15	0.13	1082.26	- 0.34
24 h	941.32	- 0.49	1080.54	- 0.50
48 h	935.21	- 1.13	1075.32	- 0.98

6. Conclusion

A simple, suitable, linear, precise and economical HPLC method was described for the diastereomeric separation of the 2-[4-(methylsulfonyl)phenyl]-3-(3*(R)*-oxocyclopentyl)propanoic acid.

In the method development attempt, selection of the mobile phase and stationary phase was performed. The effect of column temperature on the resolution and retention of two diastereomers have been evaluated. The thermodynamic parameters (H), (S) and (G) have been determined.

The baseline separation was achieved on a non-chiral column (YMC J'shpere-ODS-H80 with resolution greater than 2.0 between the two diastereomers. The method was validated with respect to system suitability, precision, LOD, LOQ, linearity, robustness and solution stability. Satisfactory results were observed for all the parameters.

The proposed method can be used for monitoring conversion of one diastereomer to an another. The developed method can also be scaled up for the isolation of diastereomers using preparative HPLC.

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Part-A

Application of the Chromatography for the Separation of Isomers of Some Pharmaceuticals

Section-III

HPLC Separation of E/Z isomers of Acrivastine

1. Introduction

Acrivastine is a potent competitive histamine H_1-receptor antagonist. It is a second-generation, non-sedating antihistamine derived from the first-generation compound triprolidine. The drug lacks significant anticholinergic effects, and has a low potential to penetrate the central nervous system [1]. Due to its rapid onset of action, acrivastine will be particularly useful for 'on demand' therapy in patients with intermittent symptoms. It possesses specific antihistaminic activity for the treatment of allergic rhinitis with reduced potential for the sedative side effects that characterize triprolidine and other first-generation H_1-antihistamines [2].

The incidence of allergic disorders has been increasing dramatically throughout the world for the past several decades. Natural environmental allergens, air pollutants, stress, and xenobiotics have all been implicated in the genesis of these disorders [3, 4]. Although many allergic disorders are not considered life-threatening, they significantly reduce the quality of life for the patients and consume a great amount of healthcare resources. H_1-antihistamines have been extensively used for the treatment of allergic disorders, especially allergic rhinitis and urticaria [5].

The acrivastine is very example of geometric isomers. The terms cis and trans are from Latin, in which cis means "on the same side" and trans means "on the other side" or "across". The term "geometric isomerism" is considered an obsolete synonym of "cis-trans isomerism" by IUPAC. It is sometimes used as a synonym for general stereoisomerism (e.g., optical isomerism being called geometric isomerism); the correct term for non-optical stereoisomerism is diastereomerism.

The cis/trans system for naming isomers is not effective when there are more than two different substituents on a double bond. The E/Z notation should then be used. **Z** (from the German *zusammen*) means *together* and corresponds to the term *cis*; **E** (from the German *entgegen*) means *opposite* and corresponds to the term *trans*.

Whether a molecular configuration is designated E or Z is determined by the Cahn-Ingold-Prelog priority rules (higher atomic numbers are given higher priority). For each of the two atoms in the double bond, it is necessary to determine which of the two substituents is of a higher priority. If both of the substituents of higher priority are on the same side, the arrangement is *Z*; if they are on opposite sides, the arrangement is *E*.

1.1 Drug Profile

1.1.1 Description

Acrivatine is chemically, (*E*)-3-{6-[(*E*)-1-(4-methylphenyl)-3-pyrrolidin-1-yl-prop-1-enyl]pyridin-2-yl}prop-2-enoic acid (Fig. 1). Its molecular formula is $C_{22}H_{24}N_2O_2$ and molecular weight is 348.44 g/mole.

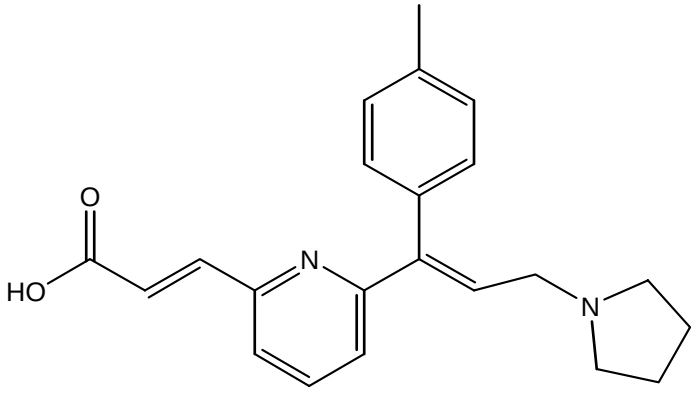


Fig. 1: Chemical Structure of Acrivastine

1.1.2 Mechanism of action:

Acrivastine is an analogue of triprolidine and it is considered to be relatively less sedating than traditional antihistamines; believed to involve competitive blockade of H1-receptor sites resulting in the inability of histamine to combine with its receptor sites and exert its usual effects on target cells [6].

1.1.3 Clinical Use

Temporary relief of nasal congestion, decongest sinus openings, running nose, itching of nose or throat, and itchy, watery eyes due to hay fever or other upper respiratory allergies .

1.1.4 Pharmacodynamics/Kinetics

Metabolism: Minimally hepatic

Time to peak: ~1.1 hours

Excretion: Urine (84%); feces (13%)

1.1.5 Dosage

Oral: Adults: 1 capsule 3-4 times/day

1.1.6 Adverse Reactions

>10%: Central nervous system: Drowsiness, headache

1% to 10%:

Cardiovascular: Tachycardia, palpitation

Central nervous system: Nervousness, dizziness, insomnia, vertigo, lightheadedness, fatigue

Gastrointestinal: Nausea, vomiting, xerostomia, diarrhea

Genitourinary: Dysuria

Neuromuscular & skeletal: Weakness

Respiratory: Pharyngitis, cough increase

Miscellaneous: Diaphoresis

<1%: Dysmenorrhea, dyspepsia

1.1.7 Drug Interactions

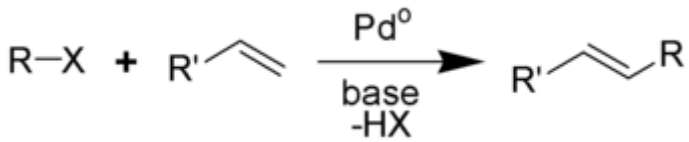
Decreased effect of guanethidine, reserpine, methyldopa, and beta-blockers. Increased toxicity with MAO inhibitors (hypertensive crisis), sympathomimetics, CNS depressants, ethanol (sedation)

1.1.8 U.S. Brand Names

Semprex®-D

1.2 Heck Reaction

The **Heck reaction** (also called the **Mizoroki-Heck reaction**) is the chemical reaction of an unsaturated halide (or triflate) with an alkene and a strong base and palladium catalyst to form a substituted alkene [7, 8]. Together with the other palladium-catalyzed cross-coupling reactions, this reaction is of great importance, as it allows to do substitution reactions on planar centers. It is named after the American chemist Richard F. Heck.



The reaction is performed in the presence of an organopalladium catalyst. The halide or triflate is an aryl, benzyl, or vinyl compound and the alkene contains at least one proton and is often electron-deficient such as acrylate ester or an acrylonitrile.The catalyst can be tetrakis(triphenylphosphine)palladium(0), palladium chloride or palladium(II) acetate. The ligand is triphenylphosphine or BINAP. The base is triethylamine, potassium carbonate or sodium acetate. Several reviews have been published [9, 10].

1.2.1 Stereoselective Reaction

This coupling reaction is stereoselective with a propensity for trans coupling as the palladium halide group and the bulky organic residue move away from each other in the reaction sequence in a rotation step. The Heck reaction is applied industrially in the production of naproxen and the sunscreen component octyl methoxycinnamate. The naproxen synthesis includes a coupling between a brominated naphthalene compound with ethylene [11]. In the recent literature, numbers of articles are publilshed regarding heack reaction which are revealed for formation of selective trans geometry of the product [12-27].

2. Literature Review

A through literature review, it has been observed that acrivastine has been determined and studied by several procedures; gas chromatography mass-spectrometric analysis [28], a sensitive radioimunoassay to measure plasma levels [29], two non-direct spectrofluori-metric methods [30,31] and several spectrophotometric procedures to measure acrivastine in urine and pharmaceuticals [30,32,33,34]. The high performance liquid chromatography method for the determination of acrivastine in capsules using ultraviolant detection [34, 35]. There are reports for the determination of the acrivastine in human urine and pharmaceutical by spectroflourimetric method [36] and by electrochemical method [37].

The literature survey revealed that there are very few articles regarding HPLC determination of the acrivastine. There is no report available regarding separation of E and Z isomers of the acrivastine. To get literature insight, reports based on high performance liquid chromatography are discussed below in detail.

T. G. Altuntas, S. S. Zanooz, D. Nebioglu, Quantitative determination of acrivastine and pseudoephedrine hydrochloride in pharmaceutical formulation by high performance liquid chromatography and derivative spectrophotometry. Journal of Pharmaceutical and Biomedical Analysis 17 (1998) 103-109.

In this study, author described quantitative determination of acrivastine and pseudoephedrine hydrochloride in their pharmaceutical capcules form by high performance liquid chromatography method. The separation was performed on a reversed phase LiChrosorb RP-C18 analytical column (200 x 4.6 mm i.d., 5 mm particle size) (Hichrome Ltd., Berkshire, UK). The mobile phase consisted of a mixture of water/acetonitrile/methanol/perchloric acid/n-octylamine (500:130:25:13:0.3 v/v). The mobile phase was prepared daily, filtered, sonicated before use, and delivered at a flow rate of 3 ml/min. The detector wavelength was set at 260 nm [34].

Xiaochen Gu, Hongtao Li, Kyle R. MacNair, F. Estelle R. Simons, Keith J. Simons. Simultaneous analysis of the H1-antihistamine acrivastine and the decongestant pseudoephedrine hydrochloride by high-performance liquid chromatography. Journal of Pharmaceutical and Biomedical Analysis 37 (2005) 663-667.

The author was used high-performance liquid chromatography (HPLC) for the simultaneous quantification of the H1-antihistamine acrivastine and the decongestant pseudoephedrine hydrochloride. Both compounds were detected at the wavelength of 214 nm. A Waters ® C18 Nova-Pak ® column (4 m, 3.9 mm ×150 mm) was used for the analysis. The mobile phase was composed of acetate buffer (0.12 M sodium acetate trihydrate, pH adjusted to 4.0 using concentrated acetic acid)/acetonitrile/methanol at a volumetric ratio of 45:47:8. The mobile phase was delivered at a flow rate of 0.8 mL/min [35].

3. Aim of Present Work

As, we can see in the preceding literature review, there are few HPLC methods reported for the determination Acrivastine. Additionally, there are no HPLC method reported for the separation of isomeric impurity, i.e. Z-isomer of Acrivastine from the Acrivastine. The aim of present work was to devise a suitable separation method and subsequently validate it.

As described in the introduction section, Acrivastine drug (Fig. 1) has two unsaturated bonds, one at the 2-psition of propenoic acid and second at 1-position of the propene attached to 6-position of pyridine ring. All the four substituents at both double bonds are different, therefore four geometric isomers are possible, i. e. (E, E), (E, Z), (Z, E) and (Z, Z). Since, during the synthesis of Acrivastine the unsaturated bond at 2-position of propanoic acid is constructed by 'Heck reaction' and the Heck reaction is known for the generation of trans geometry predominantly. Therefore, configuration of the unsaturated bond at 2-position of propenoic acid is 'E', while the configuration of the unsaturated bond at 1-position of propene attached to 6-position of pyridine ring may 'E' or 'Z'. The geometry of the one of the double bond is fixed, because of extensive precedences and mechanism of the Heck reaction. Therefore, two geometric isomers (E, E) and (E, Z) are possible during synthesis.

The isomer (E, E) is desired isomer (Acrivastine) as a drug, while (E, Z) isomer is undesired. Therefore, it is necessary to monitor amount of undesired isomer present in Acrivastine, for which suitable, precise and accurate chromatographic method is needed. We have attempted to develop method for the determination of undesired geometric isomer (E, Z) in Acrivastine, which can be used for the support of process research and quality control of Acrivastine. Now onward, we refer undesired (E, Z) isomer, as a 'Z-isomer' of Acrivastine The structure of the 'Z-isomer' is shown in Fig. 2.

The present work deals with the systematic method development for the separation of Acrivastine and its Z-isomer. The isolation and characterization of the Z-isomer and Acrivastine from the crude reaction mixture was attempted. The relative response factor of Z-isomer was determined. The method was validated for the parameters; eg. system suitability, specificity, linearity, accuracy, precision, limit of quantification and detection, robustness, intermediate precision and solution stability.

The developed method was recommended for routine monitoring of process development and quality control analysis.

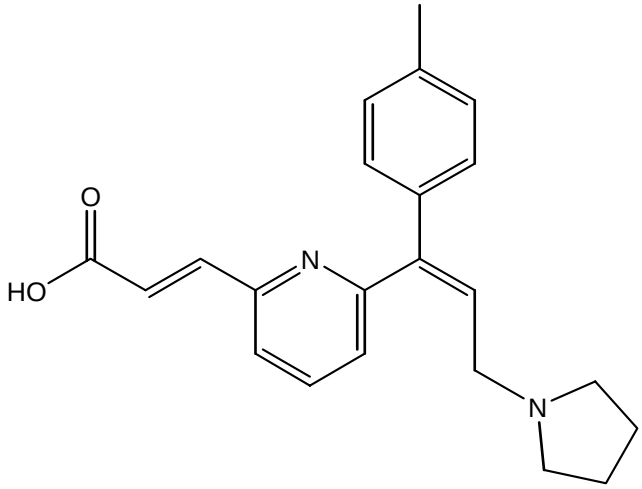


Fig. 2: Chemical Structure of Z-isomer of Acrivastine

4. Experimental

Materials and Instruments

Drug Sample

Process research department of Zydus Research Centre, Cadila Healthcare Ltd., Ahmedabad, kindly provided the crude reaction mixture containing acrivastine and its Z-isomer in the ratio of 30:70 and also provided pure sample of acrivastine drug substance.

Chemicals and Solvent Used

Acetonitrile - s d fine-CHEM Limited, Mumbai

Methanol - Merck, India

Trifluoro acetic acid – SIGMA-ALDRICH CHEMIE GmbH, Germany

Di-sodium hydrogen phosphate – Merck, India

Phosphoric acid – s.d. fine –CHEM

Potassium bromide - s d fine-CHEM Limited, Mumbai

Deuteriated methanol – 99.8+ atom %D

Water – HPLC grade water

Instruments used

HPLC – A Agilent 1100 series (Germany) HPLC system equipped with degasser auto sampler, auto injector, thermostatic compartment and photo diode array detector was utilized for method development and validation. The out put signal was monitored and processed using Agilent Chemstation software.

Preparative HPLC – SHIMADZU LC-8A, with class-VP software

FT-IR - SHIMADZU 8400S FT-IR, Shimadzu Make, Japan

Mass Spectrometry - Waters Quatromicro MSMS, Waters Make.

NMR – Bruker 300MHz

Freeze dryer - Sharp freeze -110, aapptec make

Rotavapour – BUCHI MAKE, Buchi rotavapor-R205,

Buchi vaccume controller V-805

Analytical balance – Mettler Toledo, AX 105 Delta Range

pH Meter - Metrohm, 780 pH-Meter

Sonicator – Bandelin electronic Heinrichstra Be 3-4 D-12207 Berlin, Germany

Vortexer – Spinix

Preparation of 0.05 % TFA in Water

Transferred accurately 500 µL of Trifluoro acetic acid into a 1000 mL volumetric flask containing approximately 700 mL water. Dissolved and make volume up to the mark with HPLC grade water, filter through 0.45 µm filter.

4.3 Preparation of Diluent [Water:Acetonitrile (70:30, v/v)]

Mixed accurately 350 mL of water and 150 mL of acetonitrile in 500 mL volumetric flask. After proper mixing, filter through 0.45 µm filter.

4.4 Sample Preparation (0.5 mg/mL)

Weighed accurately about 25 mg of Acrivastine sample into 50 mL of volumetric flask. Add 20 to 25 mL of diluent and sonicated to dissolve the solid. Diluted to volume upto the mark with diluent and mixed well.

4.5 Chromatographic Parameters

Elution	: Isocratic
Column	: Cosmosil packed column, 5C18-PAQ (250 x 4.6 mm, 5 µm)
Wave length	: 229 nm
Column oven temp.	: 30 C
Flow rate	: 1.0 mL / min.
Injection volume	: 5 µL
Run time	: 20 min
Mobile phase	: 0.05 % TFA in water: Acetonitrile (72:28, v/v)
Diluent	: Water:Acetonitrile (70:30)
Sample conc.	: 0.5 mg/mL

4.6 Preparative HPLC method parameters

Elution	: Isocratic
Column	: DAC, (250X50mm) Self packed column, YMC Make
Media used	: ODS-AQ, 10 m, 120A, YMC Make
Wave length	: 229 nm
Column temp.	: Ambient Temperature
Flow rate	: 80.0 mL / min.
Injection volume	: 5 mL
Mobile phase	: 0.05 % TFA in water: Acetonitrile (80:20, v/v)
Diluent	: DMF:Methanol (1:9)
Sample conc.	: 40 mg/mL
RT of main peak	: Approximately 38 min

Procedure:

About 200 to 250 mg crude reaction mixture was dissolved in minimum amount of DMF, sonicated to dissolve and then diluted with methanol to make volume of 5 mL, sample solution was filtered through 0.45 m filter. Sample solution was injected manually in equilibrated preparative HPLC system. The run was monitored using Class-VP software and main peak was collected in different fractions. After elution of the peaks of interest (~ 38 minutes), column switched over to 90 % Acetonitrile for 20 min to wash out other impurities and re-equilibrated for next injection.

The HPLC analysis of the collected fraction in the developed method was used to segregate fractions as per its purity. The fractions of less than 99 % purity was mixed and re-injected through pump in preparative HPLC system to get desire purity level. Again collected fractions were monitored in analytical HPLC system. Finally, all the fractions with more than 99.0 % purity were mixed and concentrated using rotavapor. Concentrated fractions were subjected to freeze drying to get solid material.

4.7 Characterization methods

The isolated materials using preparative HPLC were characterized by spectroscopic techniques such as, Mass, IR, NMR (H^1, C^{13}, DEPT).

4.7.1 Mass Spectrometry

The isolated compound was dissolved (about 100 ppm) in methanol and injected (2 L) through liquid chromatography flowed flow with methanol-water (90:10, v/v) at a flow rate 0.3 mL/min. The mass was operated in positive spary ionization (ESI+) mode with capillary voltage 3.5 KV, cone voltage 20V, source temperature 120°C, desolvation temperature 400°C and desolvation gas flow 800L/hr.

4.7.2 Infra-red Spectrocopy

The IR spectra were recorded in solid state using KBr pallet in the region 4000 cm^{-1} to 400 cm^{-1}. The operated parameters are; Numbers of scan:20, Resolution:4, Beam: internal, Mirror speed: 2.8 and Gain: auto.

4.7.3 Nuclear Magnetic Resonance

^1H (300 MHz) and ^{13}C (300 MHz) NMR spectra of isolated acrivastine and its Z-isomer was recorded on an Avance DPX- 300MHz spectrometer Bruker (Germany). The probe was a 5 mm BBO z-gradient probe, optimized for inverse detection. Spectra were recorded in deuteriated methanol (5-mm tubes) at 300 K. Sample concentration was 10 mg in 0.5 ml. The residual protonated resonance of the solvent (deuteriated methanol) was used as an internal chemical shift standard, which was related to tetramethylsilane with chemical shifts of 3.3 and 49.0 ppm, respectively, for ^1H and ^{13}C. Processing of the raw data were performed using Bruker XWin 3.5 Nmr software. The pulse conditions were 9.8μs (attenuation 0db) for ^1H and 7.10μs (attenuation -1.0db) for ^{13}C.

4.8 Relative Response Factor

Sample preparation:

Transferred an accurately weigh quantity of about 10 mg of acrivastine and its Z-isomer to 10 mL volumetric flasks, separately. Added about 7-8 mL of diluent to volumetric flask and sonicated to dissolve. Make volume up to the mark with diluent and mixed well to prepare 1000-ppm stock solution.

100 µL of above stock solution of acrivastine and its Z-isomer was taken in a single 20 mL volumetric flask. Make volume up to the mark with diluent and mixed well to prepare 5-ppm working solution.

Procedure:

Five replicate of above solution was injected in the equilibrated chromatographic system at 229 nm. Average area of acrivastine and its Z-isomer was used to calculate relative response factor.

4.9 Method validation

4.9.1 System Suitability and Specificity

The acrivastine and its Z-isomer were injected separately and together for the identification of the peak and specificity. The system suitability parameters such as, resolution (Rs), symmetry (S), retention factor (k), separation factor ( ) and efficiencies (N) were also evaluated.

Analysis sequence:

Analysis sequence for specificity test

Sr. No.	Sample	No. of Injections
1	Diluent	1
2	Mobile phase	1
3	Acrivastine	1
4	Z-isomer	1
5	Acrivastine + Z-isomer	3

The system suitability parameters were evaluated from chromatogram of acrivastine + Z-isomer for n=3.

4.9.2 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 prescribe conditions. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variance of a series of measurement. Here precision is determined by spiking the Z-isomer with acrivastine at 100 % specification level.

Preparation of Stock Solution of Z-isomer (50 ppm):

Transferred an accurately weighed quantity of about 10 mg of Z-isomer to10 mL volumetric flasks. Added about 5 mL of diluent to volumetric flask and sonicated to dissolve. Make volume up to the mark with diluent and mixed well to prepare 1000 ppm solution. Took 1 mL of this solution to 20 mL volumetric flask and diluted with diluent to prepare 50 ppm stock solution of Z-isomer.

Preparation of Acrivastine Solution for Area Correction:

Transferred an accurately weigh quantity of about 25 mg of acrivastine into 50 mL volumetric flask. Added 20.0 mL of diluent and sonicated to dissolve. Make up the volume up to mark with diluent and mixed well.

Preparation of Precision Solution (acrivastine with 100 % spiked Z-isomer):

Transferred an accurately weigh quantity of about 25 mg of API into 50 mL volumetric flask. Added 1.0 mL of 50 ppm stock solution of Z-isomer and 20.0 mL diluent, sonicated to dissolve. Make up the volume up to mark with diluent and mixed well.

Procedure:

Acrivastine sample solution was injected for the area correction of Z-isomer. The five sample sets of precision solution were prepared and 5 µL was injected from each set on an equilibrated chromatographic system. The % RSD was calculated for area and retention time of acrivastine and its Z-isomer.

4.9.3 Limit of Detection and Limit of Quantification

Limit of detection (LOD) and Limit of quantification (LOQ) were determined at a signal-to-noise ratio of 3 and 10. LOD and LOQ were achieved by injecting a series of dilute solutions of Z-isomer.

The precision of the developed method for Z-isomer at LOQ was checked by analyzing six test solutions of Z-isomer prepared at the LOQ level and calculating the percentage relative standard deviation of retention time and peak area. The accuracy of the method was checked for Z-isomer at LOQ level by calculating recovery of the Z-isomer from the sample of acrivastine spiked with Z-isomer at LOQ level.

Preparation of Stock Solution of Z-isomer (10 ppm):

Transferred an accurately weighed quantity of about 10 mg of Z-isomer to10 mL volumetric flasks. Added about 5 mL of diluent to volumetric flask and sonicated to dissolve. Make volume up to the mark with diluent and mixed well to prepare 1000 ppm solution. Took 1 mL of this solution to 100 mL volumetric flask and diluted with diluent to prepare 10 ppm stock solution of Z-isomer.

Preparation of solutions for LOD and LOQ

Sr. No.	Concentration	Preparation of solution
1	3.0 ppm	3.0 mL of stock solution (10 ppm) diluted to 10 mL
2	2.0 ppm	2.0 mL of stock solution (10 ppm) diluted to 10 mL
3	1.5 ppm	1.5 mL of stock solution (10 ppm) diluted to 10 mL
4	1.0 ppm	5.0 mL of stock solution (10 ppm) diluted to 50 mL
5	0.5 ppm	5.0 mL of 1.0 ppm solution diluted to 10 mL
6	0.2 ppm	5.0 mL of 1.0 ppm solution diluted to 25 mL
7	0.1 ppm	5.0 mL of 0.2 ppm solution diluted to 10 mL
8	0.05 ppm	5.0 mL of 0.1 ppm solution diluted to 10 mL

The above solutions were injected on an equilibrated chromatographic system and measure signal to noise ratio for Z-isomer.

Preparation of Acrivastine Solution for Area Correction

As per section *4.9.2 Precision.*

Preparation of Precision and Accuracy Solution at LOQ Level

Transferred an accurately weigh quantity of about 25 mg of API into 50 mL volumetric flask. Added 1.0 mL of 10 ppm stock solution of Z-isomer and 20.0 mL diluent, sonicated to dissolve. Make up the volume up to mark with diluent and mixed well.

The six replicates of for precision study was injected, while single run for accuracy determination at LOQ level was injected. The recovery was calculated using slope and Y-intercept value obtained from the linearity study.

4.9.4 Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration of analyte in the sample. The linearity of Z-isomer was evaluated from LOQ to 300 % concentration at specification level, i. e. 0.2 – 3.0 ppm for three consecutive days.

Preparation of Z-isomer Solution for Linearity Study:

The linearity solutions were prepared at concentration of 3, 2, 1.5, 1, 0.5, 0.2 ppm as per the procedure described in the section *4.9.3 Limit of detection and Limit of quantification.*

Procedure:

5 µL of each solution 0.2, 0.5, 1.0, 1.5, 2.0 and 3.0 was injected on an equilibrated chromatographic system. Area for each concentration was calculated and linearity was plotted for area against the concentration (ppm) for Z-isomer. The correlation coefficient was calculated for area against concentration. The equation for the linearity curve was also determined. Same procedure repeated at second and third day with fresh solution. The percentage relative standard deviation for the slope and Y-intercept was calculated.

4.9.5 Accuracy/Recovery

The accuracy of an analytical method is the closeness of test results obtained by the method to the true value. The standard addition and recovery experiments were conducted to demonstrate accuracy of the method. The study was carried out in triplicate for the determination of recovery of Z-isomer at 50 %, 100 % and 150 %

level with respect to the specification limit for Z-isomer. The recovery of Z-isomer was calculated from the slope and Y-intercept value range of 0.2-3.0 ppm (slope and Y-intercept values obtained in the linearity study).

Preparation of Stock Solution of Z-isomer (10 ppm):

As per section *4.9.3 Limit of Detection and Limit of Quantification*

Preparation of Acrivastine Stock Solution (1000 ppm):

Transferred an accurately weigh quantity of about 25 mg of acrivastine into 25 mL volumetric flask. Added 15.0 mL of diluent and sonicated to dissolve. Make up the volume up to mark with diluent and mixed well.

Preparation of Acrivastine Solution for Area Correction:

Transferred an accurately 5.0 mL of acrivastine stock solution in 10 mL volumetric flask. Added diluent to make up the volume up to mark and mixed well.

Preparation of Recovery Solution (acrivastine with 50 % spiked Z-isomer):

Transferred an accurately 5.0 mL of acrivastine stock solution(1000 ppm) and 0.5 mL of Z-isomer stock solution(10 ppm) in 10 mL volumetric flask. Added diluent to make up the volume up to mark and mixed well.

Preparation of Recovery Solution (acrivastine with 100 % spiked Z-isomer):

Transferred an accurately 5.0 mL of acrivastine stock solution(1000 ppm) and 1.0 mL of Z-isomer stock solution(10 ppm) in 10 mL volumetric flask. Added diluent to make up the volume up to mark and mixed well.

Preparation of Recovery Solution (acrivastine with 150 % spiked Z-isomer):

Transferred an accurately 5.0 mL of acrivastine stock solution(1000 ppm) and 1.5 mL of Z-isomer stock solution(10 ppm) in 10 mL volumetric flask. Added diluent to make up the volume up to mark and mixed well.

Procedure:

5 µL of acrivastine and each recovery solution of Z-isomer were injected on equilibrated chromatographic system. The peak area of Z-isomer was determined. The corrected area of Z-isomer was used for the calculation of recovery at each level.

4.9.6 Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage. The following parameters were changed to establish the robustness of the method. The resolution between acrivastine and Z-isomer was determined to evaluate robustness.

(a) Flow Rate Variation:

The flow rate of the mobile phase was changed to 0.9 mL/min and 1.1 mL/min from 1.0 mL/min.

(b) Mobile Phase Composition Variation:

The mobile phase composition of 0.05% triflouro acetic acid:acetonitrile was changed to (71:29, v/v) and (73:27, v/v) from (72:28, v/v).

(c) Column Oven Temperature Variation:

The temperature of the column oven was changed to 28° C and 32° C from 30° C.

4.9.7 Intermediate Precision:

Intermediate precision expresses variations; different days, different analysts, different equipments, different laboratories, etc. The objective of intermediate precision validation is to verify that the influence on the test results of operational and environmental variables of the analytical method.

The precision test was carried out using different laboratory, different instrument, and different column on different day to establish intermediate precision of the method.

Sample preparation and procedure:

Sample preparation and procedure were same as shown in the section *4.9.2 Precision* The % Bias was determined between original condition and changed condition.

4.9.8 Solution Stability

Stability of solution of acrivastine spiked with Z-isomer at specification level was studied by keeping the solution in tightly capped volumetric flask at room temperature on a laboratory bench for 2 days. Area of Z-isomer was checked for 12, 24 and 48 h.

Preparation of Stock Solution of Z-isomer (50 ppm):

As per section *4.9.2 Precision.*

Preparation of Test Solution (acrivastine with 100 % spiked Z-isomer)

As per section *4.9.2 Precision.*

Procedure:

5 µL of test solution was injected on equilibrated chromatographic system at every time point and area of the Z-isomer and acrivastine was determined. The % bias of area was calculated against initial value.

5. Result and Discussion

5.1 Method Development

The aim of this study is to develop chromatographic method for the separation of E/Z isomers of acrivastine. Structure of acrivastine and its Z-isomer are shown in Fig. 1 and Fig. 2 respectively in introduction section. The crude reaction mixture containing 30/70 ratios of E/Z isomers was used for the method development purpose. The wavelength for the detection was selected base of the UV spectra of both the isomers. The UV profile was obtained by scanning peaks from 200 to 400 nm using diode array detector. The UV spectra of acrivastine and its Z-isomer are presented in Fig. 3 and 4 respectively. It can be seen from the spectra that both isomers have first *max* at about 200 nm but it is not an ideal wavelength for the good chromatography. The second *max* of acrivastine is at 229nm, where Z-isomer has also reasonable absorbance. However, it is difficult to detect two different compounds at single wavelength with equal response. Therefore, 229nm wavelength was selected for the detection and relative response factor of Z-isomer was evaluated for accurate determination.

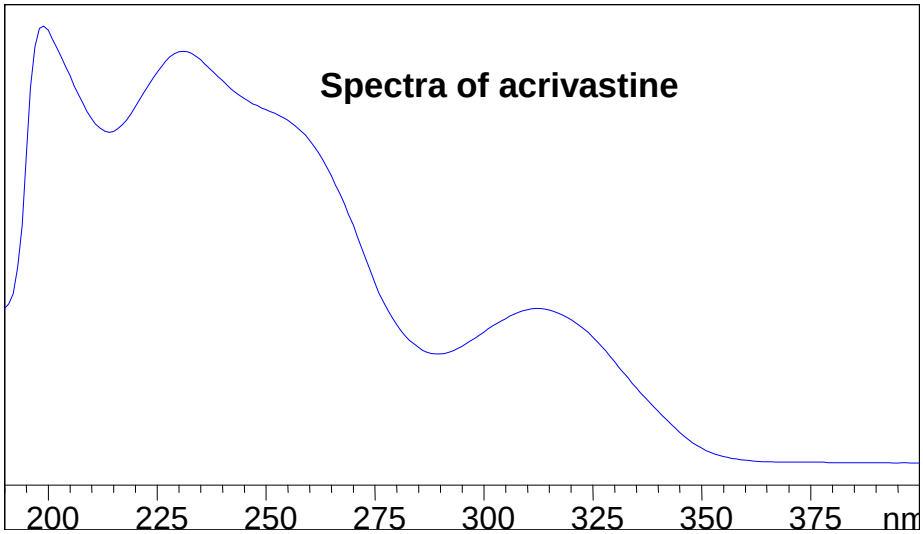


Fig. 3: UV spectra of Acrivastine

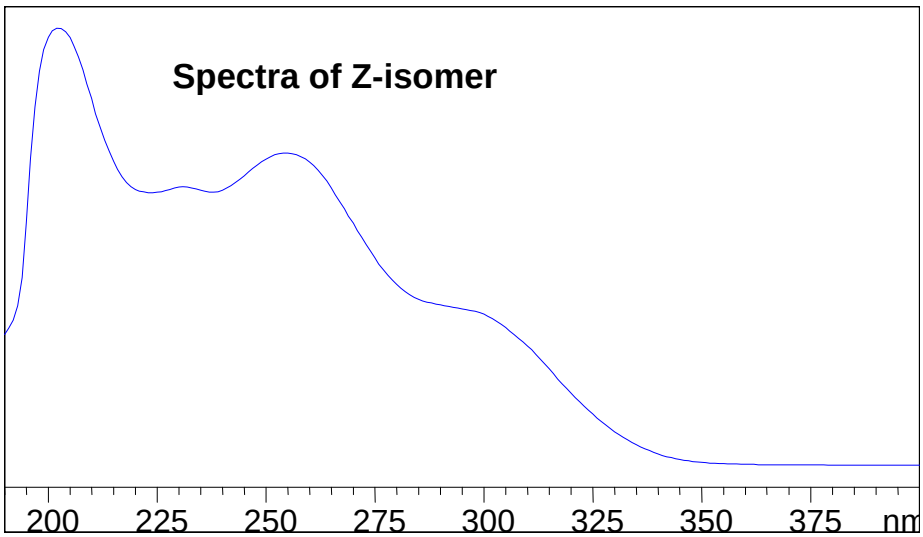


Fig. 4: UV spectra of Z-Isomer

HPLC is a widely used technique for the separation of geometric isomers. Now a day's, variety of columns having different chemistry are available for the HPLC separation. The method development was initiated in J'sphere, ODS (150 x 4.6 mm, 4 µm) column as a stationary phase. Column was kept under control temperature 30° C. Quaternary pump was used to flow 1.0 mL/min. UV detector was operated at 229 nm. As per the common development practice, method development was initiated with Water: Acetonitrile as a mobile phase in isocratic mode. Based on the observation of trials, different mobile phase components and stationary phases were tried to get desire separation goal. The detail of trials and its observation are described below.

Trial Condition 1:

Column: J'sphere, ODS (150 x 4.6 mm, 4 µm)

Mobile phase: Water:Acetonitrile (75:25, v/v)

Flow Rate: 1.0 ml/min

Column Temperature: 30°C

Chromatogram: Fig. 5

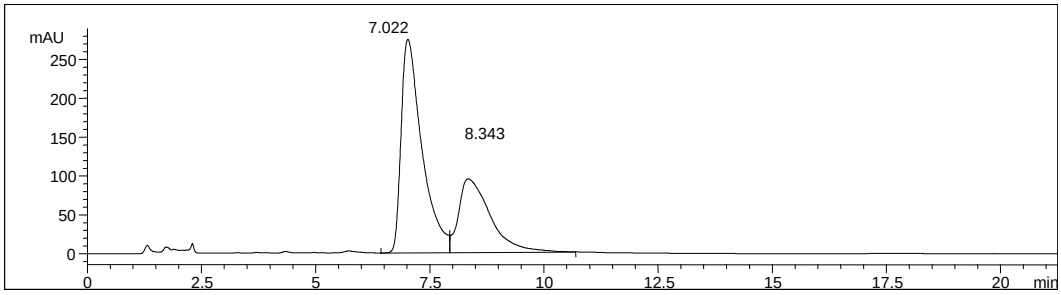


Fig. 5: HPLC chromatogram of trial Condition 1

Observation:

It can be seen from the Fig. 5 that there was indication of separation. The peak shape was not good and there was no baseline resolution. Therefore, some buffer or ion-pairing reagent should be tried.

Trial Condition 2:

Column: J'sphere, ODS (150 x 4.6 mm, 4 µm)

Mobile phase: Phosphate buffer (pH:6.8, 20mM):Acetonitrile (75:25, v/v)

Flow Rate: 1.0 ml/min

Column Temperature: 30°C

Chromatogram: Fig. 6

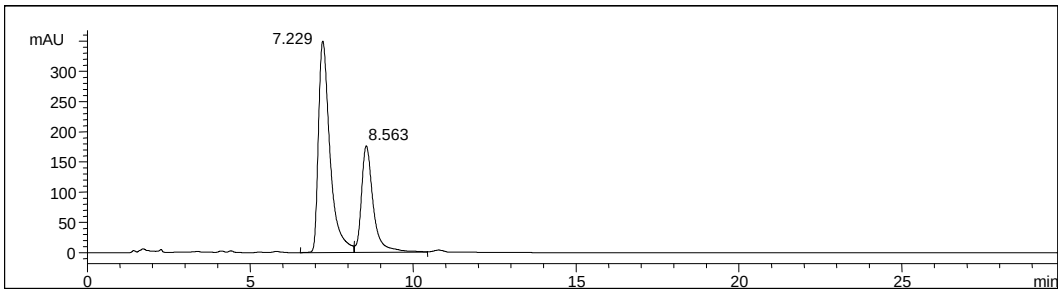


Fig. 6: HPLC chromatogram of trial Condition 2

Observation:

It was observed from chromatogram of trial condition 2 there was improvement in the separation but till the resolution and peak shapes were not up to the mark.

Trial Condition 3:

Column: J'sphere, ODS (150 x 4.6 mm, 4 µm)

Mobile phase: 0.05 % Triflouro acetic acid:Acetonitrile (75:25, v/v)

Flow Rate: 1.0 ml/min

Column Temperature: 30°C

Chromatogram: Fig. 7

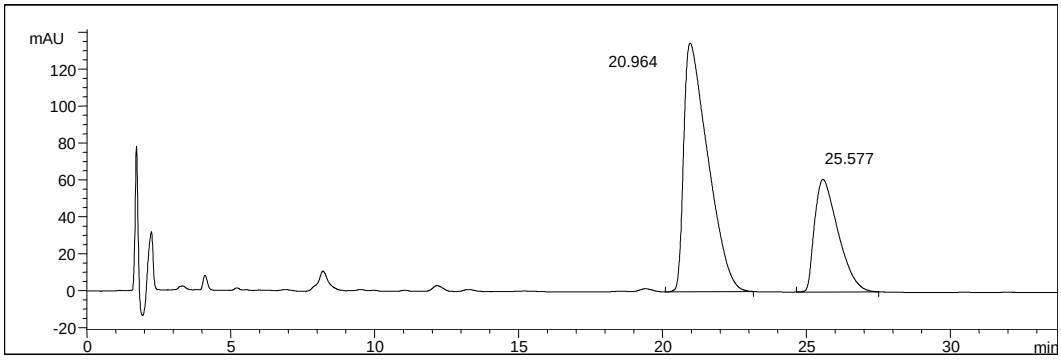


Fig. 7: HPLC chromatogram of trial Condition 3

Observation:

From the Fig. 7, it can be said that baseline separation achieved using 0.05 % triflouro acetic acid but retention was much. Peak broadening was observed for both the isomers.

Based on the above three trials, it can be concluded that 0.05 % triflouro acetic acid and acetonitrile are preferable as a mobile phase components. To improve peak shapes and to reduce retention, different stationary phases should be tried.

Trial Condition 4:

Column: YMC C8 (250 x 4.6 mm, 5 µm)

Mobile phase: 0.05 % Triflouro acetic acid:Acetonitrile (75:25, v/v)

Flow Rate: 1.0 ml/min

Column Temperature: 30°C

Chromatogram: Fig. 8

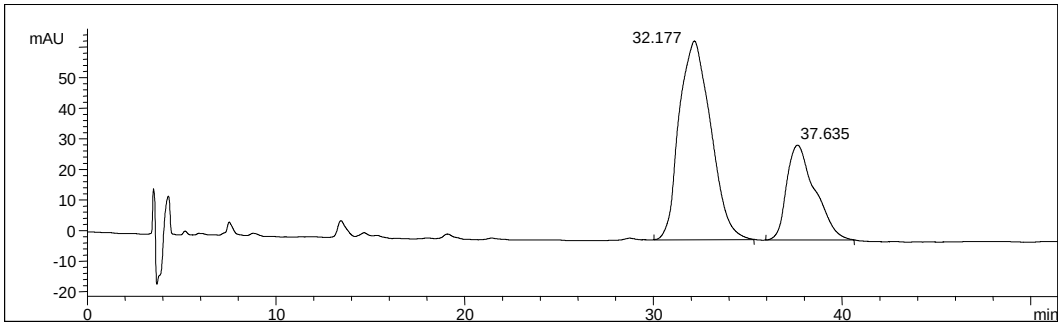


Fig. 8: HPLC chromatogram of trial condition 4

Observation:

The peak shapes were not good and long retention.

Trial Condition 5:

Column: Kromasil, C18 (250 x 4.6 mm, 5 µm)

Mobile phase: 0.05 % Triflouro acetic acid:Acetonitrile (75:25, v/v)

Flow Rate: 1.0 ml/min

Column Temperature: 30°C

Chromatogram: Fig. 9

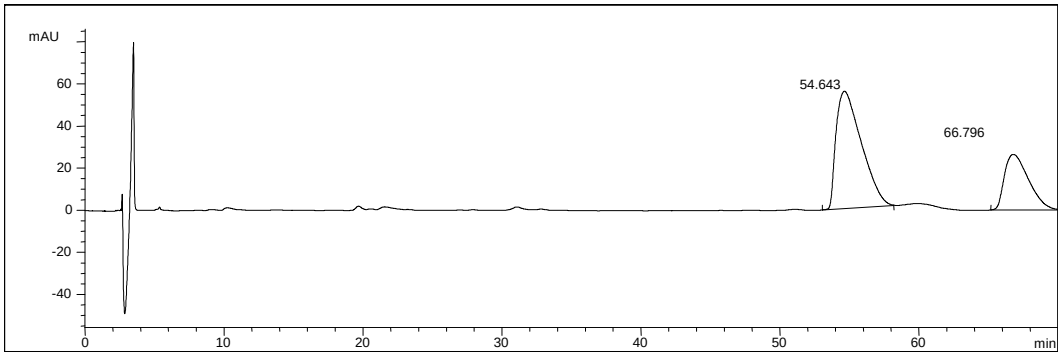


Fig. 9: HPLC chromatogram of trial condition 5

Observation:

The peak shapes were broad and retention was too long.

Trial Condition 6:

Column: YMC ODS-AQ (150 x 4.6 mm, 5 µm)

Mobile phase: 0.05 % Triflouro acetic acid:Acetonitrile (75:25, v/v)

Flow Rate: 1.0 ml/min

Column Temperature: 30°C

Chromatogram: Fig. 10

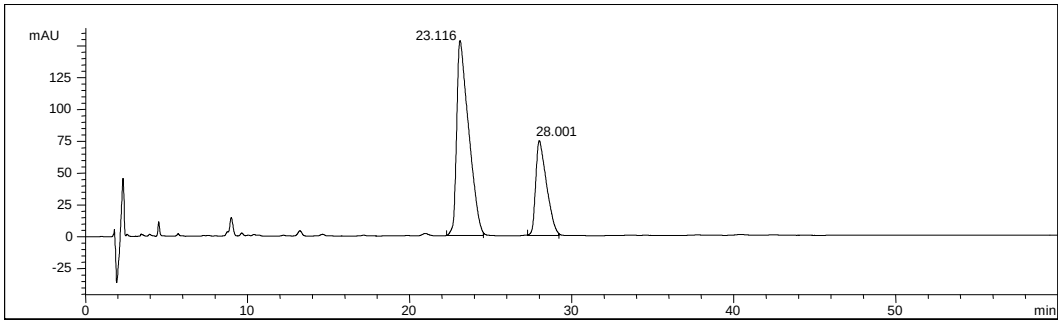


Fig. 10: HPLC chromatogram of trial condition 6

Observation:

It can be seen from the Fig. 10 that there was adequate baseline resolution and peak shape was also improved in this column. Still, retention can be optimized using different brand of AQ column.

Trial Condition 7:

Column: Cosmosil packed column, 5C18-PAQ (250 x 4.6 mm, 5 µm)

Mobile phase: 0.05 % Triflouro acetic acid:Acetonitrile (75:25, v/v)

Flow Rate: 1.0 ml/min

Column Temperature: 30°C

Chromatogram: Fig. 11

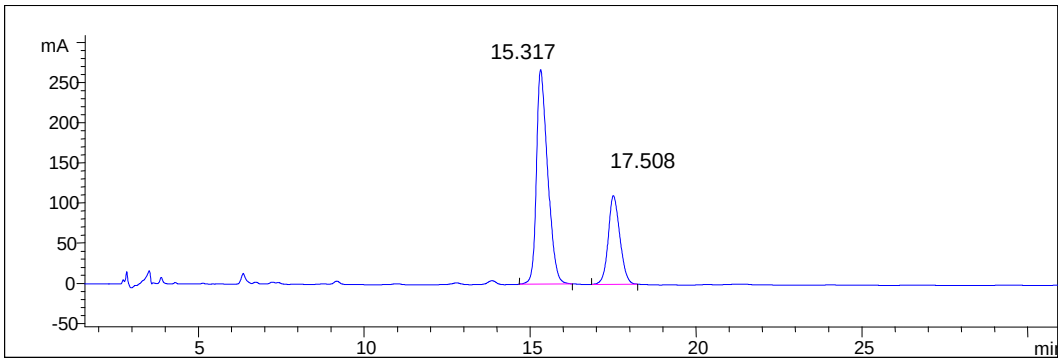


Fig. 11: HPLC chromatogram of trial condition 7

Observation:

It can be observed from the chromatogram of trial condition 7 that both isomers were well separated with resolution 3.56, peak shapes were also good. Therefore, Cosmosil packed 5C18-PAQ column was found to be suitable for desire separation. Now, mobile phase composition should be optimized to reduce retention with maintaining adequate resolution.

Trial Condition 8:

Column: Cosmosil packed column, 5C18-PAQ (250 x 4.6 mm, 5 µm)

Mobile phase: 0.05 % Triflouro acetic acid:Acetonitrile (70:30, v/v)

Flow Rate: 1.0 ml/min

Column Temperature: 30°C

Chromatogram: Fig. 12

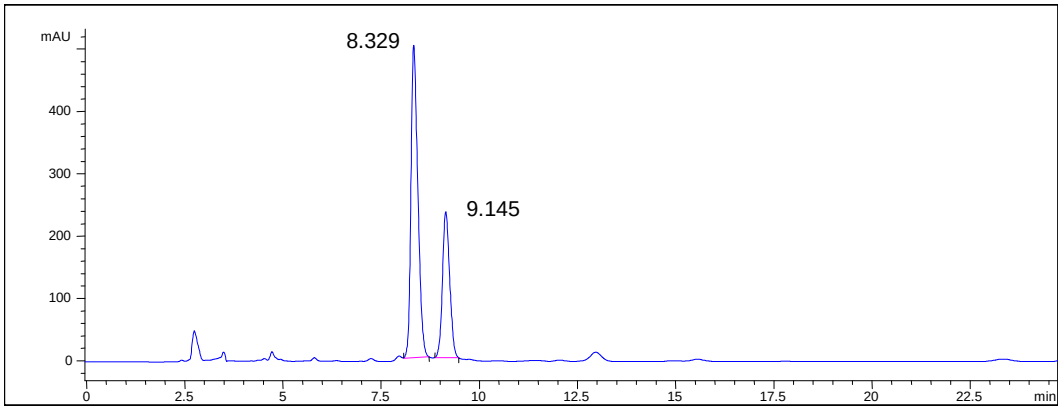


Fig. 12: HPLC chromatogram of trial condition 8

Observation:

It can be seen from Fig. 12 that both isomers were well separated; with resolution 2.56. The both the isomers were eluted within 10 minutes and peak shapes were also good. However, to be in safer side for baseline resolution, 28 % composition of acetonitrile should be tried instead of 30 %.

Trial Condition 9:

Column: Cosmosil packed column, 5C18-PAQ (250 x 4.6 mm, 5 µm)

Mobile phase: 0.05 % Triflouro acetic acid:Acetonitrile (72:28, v/v)

Flow Rate: 1.0 ml/min

Column Temperature: 30°C

Chromatogram: Fig. 13

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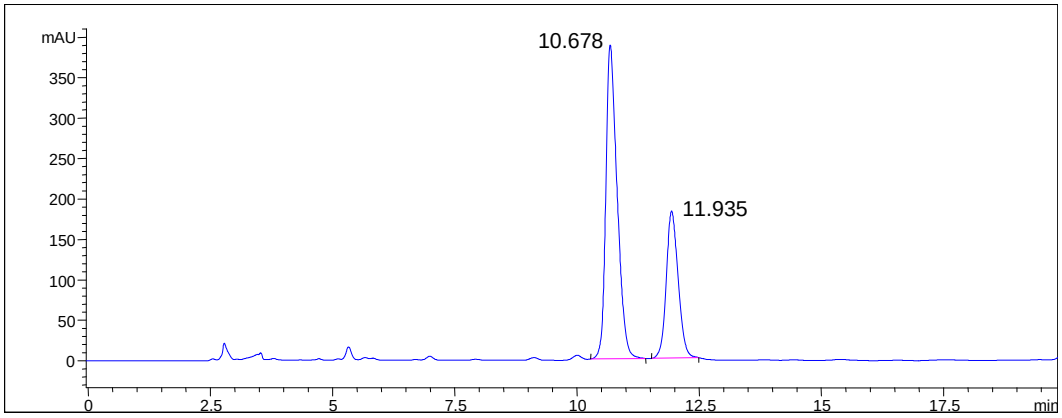


Fig. 13: HPLC chromatogram of trial condition 9

Observation:

It can be observed from Fig. 13 that acrivastine and its Z-isomer were well separated; with resolution 2.98. The both the isomers were eluted within 12 minutes and peak shapes were symmetric. The retention was also ideal and selectivity was higher than the trial condition 8.

It can be concluded from the entire method development discussion that trial condition 9 was optimal among all tested trial conditions. It was finalized for further validation study. Chromatographic parameters are as below.

Elution	: Isocratic
Column	: Cosmosil packed column, 5C18-PAQ (250 x 4.6 mm, 5 µm)
Wave length	: 229 nm
Column oven temp.	: 30 C
Flow rate	: 1.0 mL / min.
Injection volume	: 5 µL
Run time	: 20 min
Mobile phase	: 0.05 % TFA in water: Acetonitrile (72:28, v/v)
Diluent	: Water:Acetonitrile (70:30)
Sample conc.	: 0.5 mg/mL

5.2 Preparative HPLC Isolation

About 1.0 to 1.5 g reaction mixture was purified by preparative HPLC. The fractions collected manually at five different points of the peak and purity was checked by analytical HPLC. It has been observed that fraction collected at the apex of peak was pure more than 99.0 %, rest all fractions were below 99.0 % purity in all injection run.

Therefore, all the fractions below 99.0 % purity were mixed and re-injected for the further purification. The peak was collected in three parts and its purity was checked by analytical HPLC. The purity of all the three factions was found more than 99.0 %. To get the solid material, fractions were concentrated and then dried using freeze-dryer. After two days drying cycle, white solid material was observed. The 100 mg Z-isomer and 65 mg acrivastine was obtained having purity greater than 99.0 %. These materials were subjected to complete characterization by spectroscopic techniques.

5.3 Characterization of Isomers

The isolated samples of Acrivastine and its Z-isomer were subjected to complete identification and characterization. The samples were prepared at analyte concentration and injected in equilibrated chromatographic system. The retention time and UV spectrum obtained in the PDA detection was matched with that of crude reaction mixture for identification. The purity of the Acrivastine and its Z-isomer was found 99.26 and 99.02 % respectively.

The mass spectra obtained for Acrivastine and its Z-isomer are shown in Fig. 14 and 15 respectively. The mass spectrum showed peak at 349.56 m/z corresponds to the M+1 in positive ESI mode, which confirmed molecular weight of both isomers.

The presence of the different functional group was confirmed by IR spectroscopy. Thus, α,β-unsaturated carboxylic acid appeared at 3416 cm-1 in Acrivastine as broad band, whereas in its Z-isomer, it appeared at 3379 cm-1. Similarly, the carbonyls in both cases appeared at 1618 and 1686 cm-1 respectively. The IR bands observed at different wave number are presented in Table 1. The IR spectra are shown is Fig. 16 and 17.

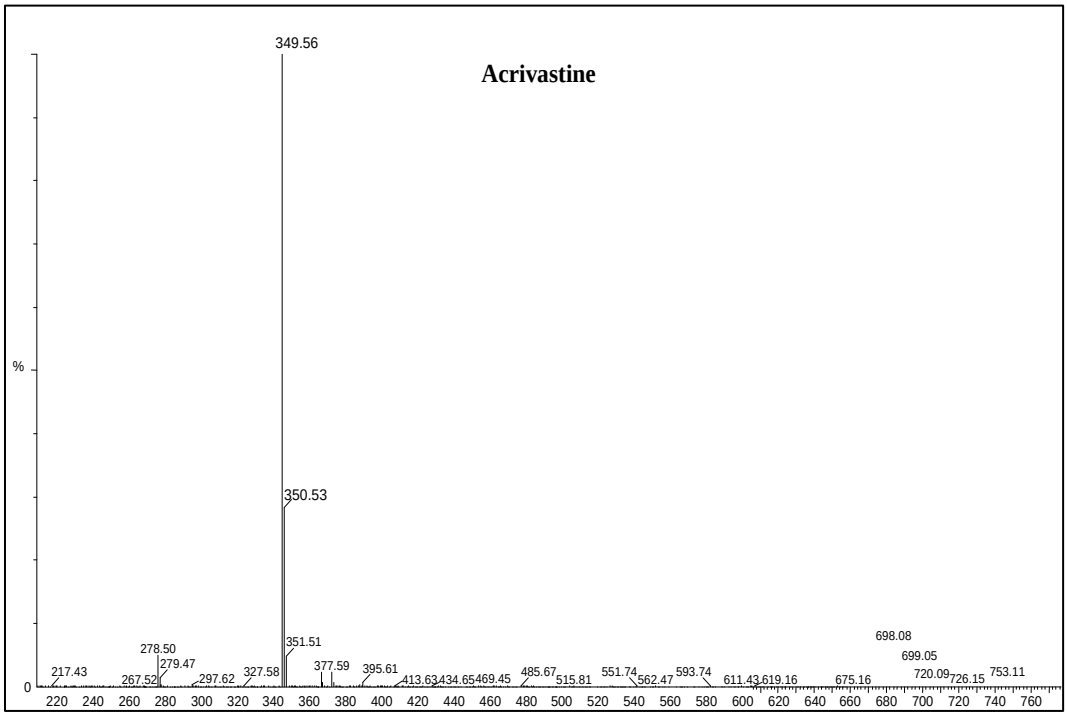


Fig. 14: Mass spectrum of Acrivastine

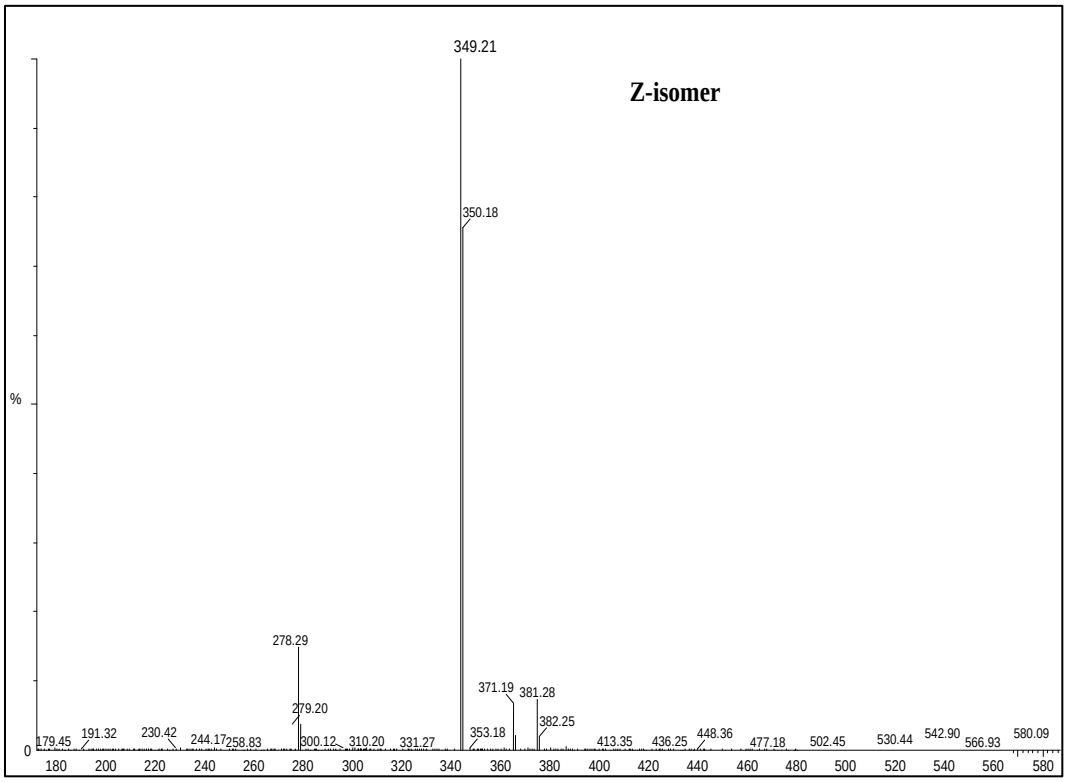


Fig. 15: Mass spectrum of Z-isomer of Acrivastine

Table 1: IR bands of at different wave number

Wave number (cm^{-1})	
Acrivastine	**Z-isomer**
3416, 1618, 1564, 1445, 1375 1298, 1157	3379, 3038, 1686, 1630, 1566, 1452, 1410, 1302, 1202, 1136

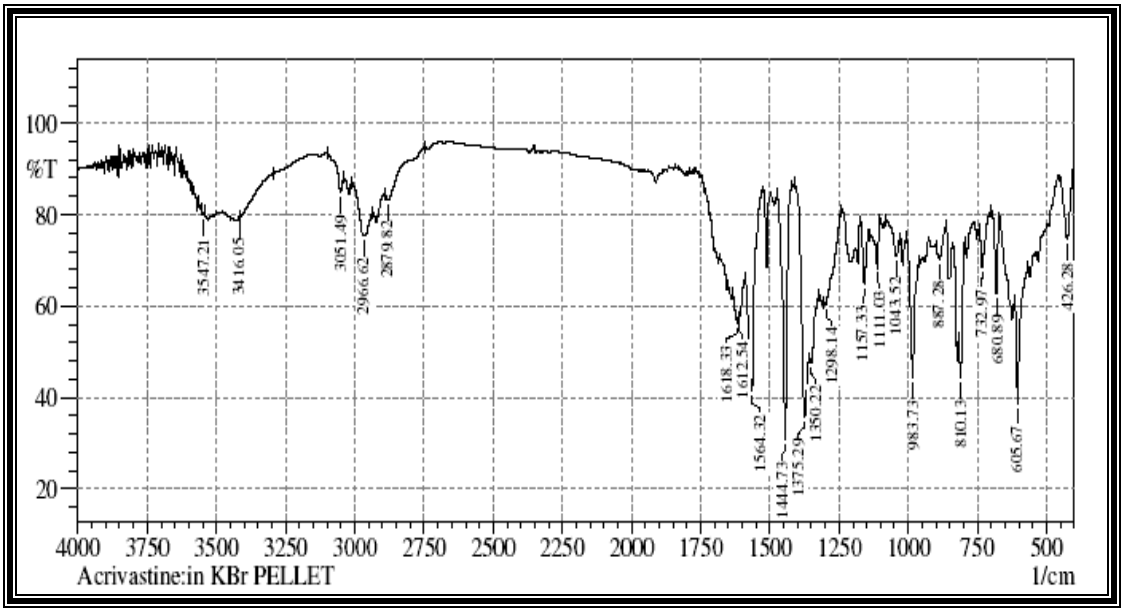


Fig. 16: IR spectrum of Acrivastine

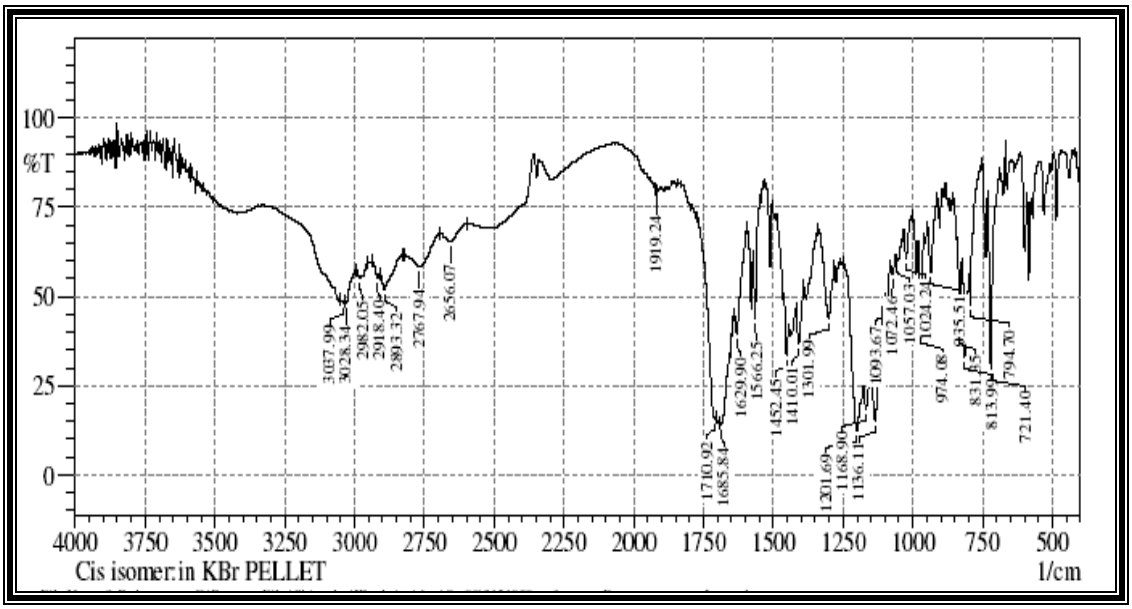


Fig. 17: IR spectrum of Z-isomer of Acrivastine

The widely used and acceptable technique for the structure elucidation and confirmation is NMR. The NMR spectra was obtained using 300 MHz instrument for ^{1}H, ^{13}C and DEPT in deuteriated methanol. The peak observed in ^{1}H and ^{13}C NMR are complied in Table 2 and 3 for acrivastine and Table 4 and 5 for its Z-isomer respectively. The NMR spectra of acrivastine for ^{1}H, ^{13}C and DEPT are shown in Fig. 18, 19 and 20, while for Z-isomer are shown in Fig. 21, 22 and 23.

Acrivastine

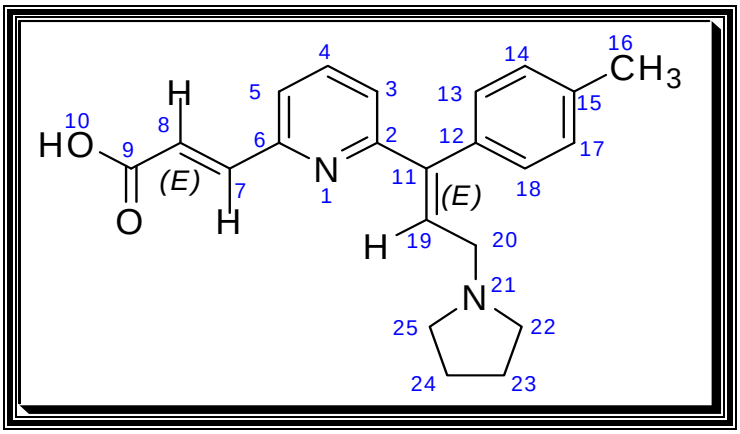


Table 2: ^{1}H-NMR of Acrivastine

Chemical Shift (ppm)	Proton No.
7.61 (t, 1H, ArH)	4
7.43 (d, J=15.6 Hz, 1H, CH)	7
7.36 (d, J=7.5 Hz, 1H, ArH)	5
7.32 (d, J=8.1 Hz, 2H, ArH)	13, 18
7.20 (t, 1H, ArH)	3
7.13 (d, J=7.8 Hz, 2H, ArH)	14, 17
7.09 (d, J=15.6 Hz, 1H, CH)	8
6.78 (d, J=7.7 Hz, 1H, CH)	19
3.83 (d, J=7.2 Hz, 2H, CH_2)	20
3.31 – 3.26 (m, 4H, CH_2 x 2)	22, 25
2.40 (s, 3H, CH_3)	16
2.01 (bs, 4H, CH_2 x 2)	23, 24

Table 3: ^{13}C-NMR of Acrivastine

Chemical Shift (ppm)	Nature of Carbon	Assignment
175.07	Quaternary	9
157.37, 155.5		2, 6
148.04		11
139.63		15
134.88		12
138.58, 139.80	Methine	4, 7
131.07		5
130.76, 130.61		13, 14, 17, 18
122.45, 123.50, 123.68		3, 8, 19
54.55	Methylene	20, 22, 25
24.02		23, 24
21.30	Methyl	16

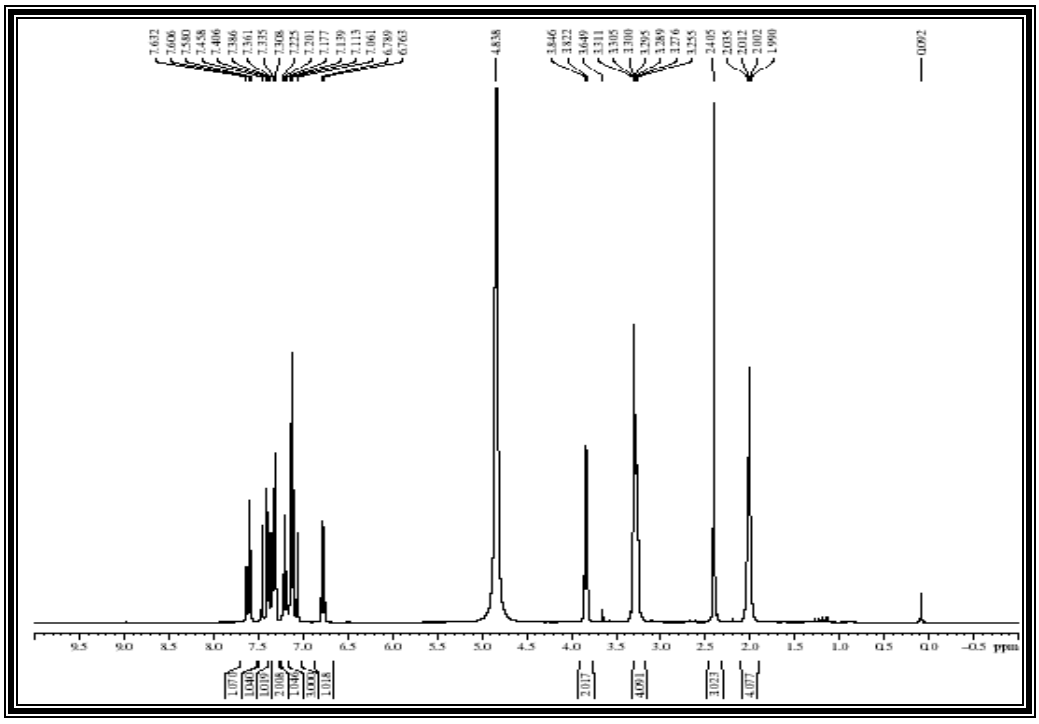


Fig. 18: ^{1}H-NMR of Acrivastine

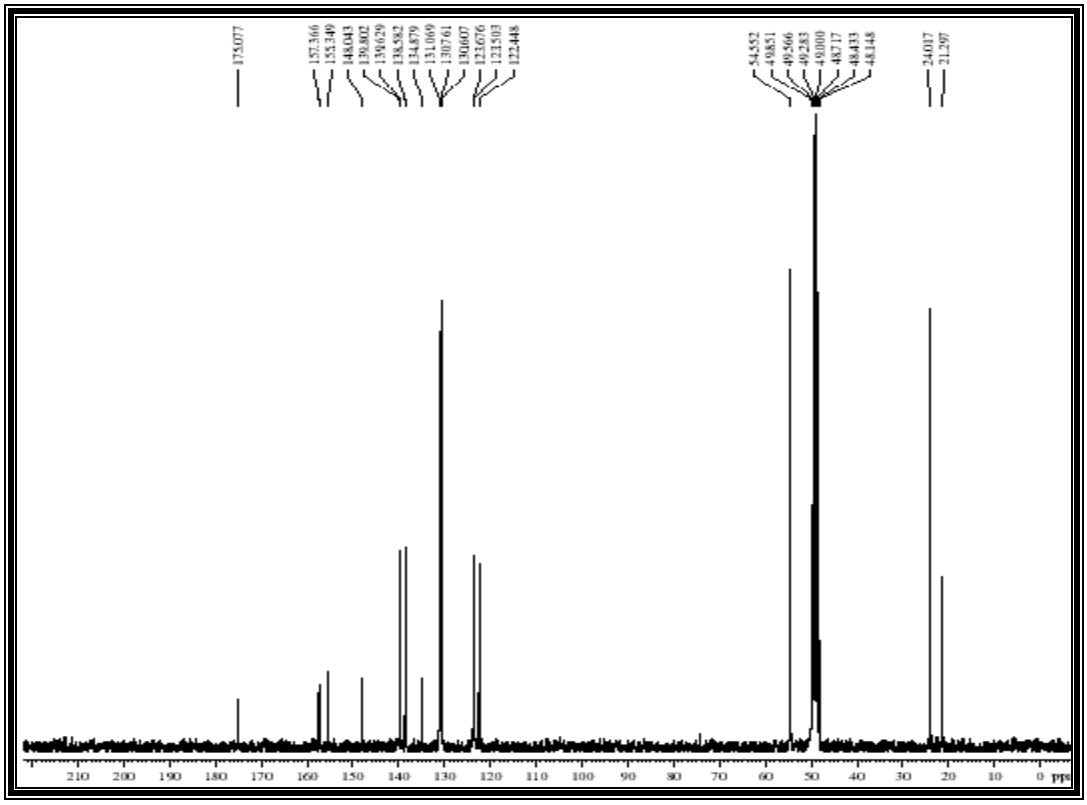


Fig. 19: ^{13}C-NMR of Acrivastine

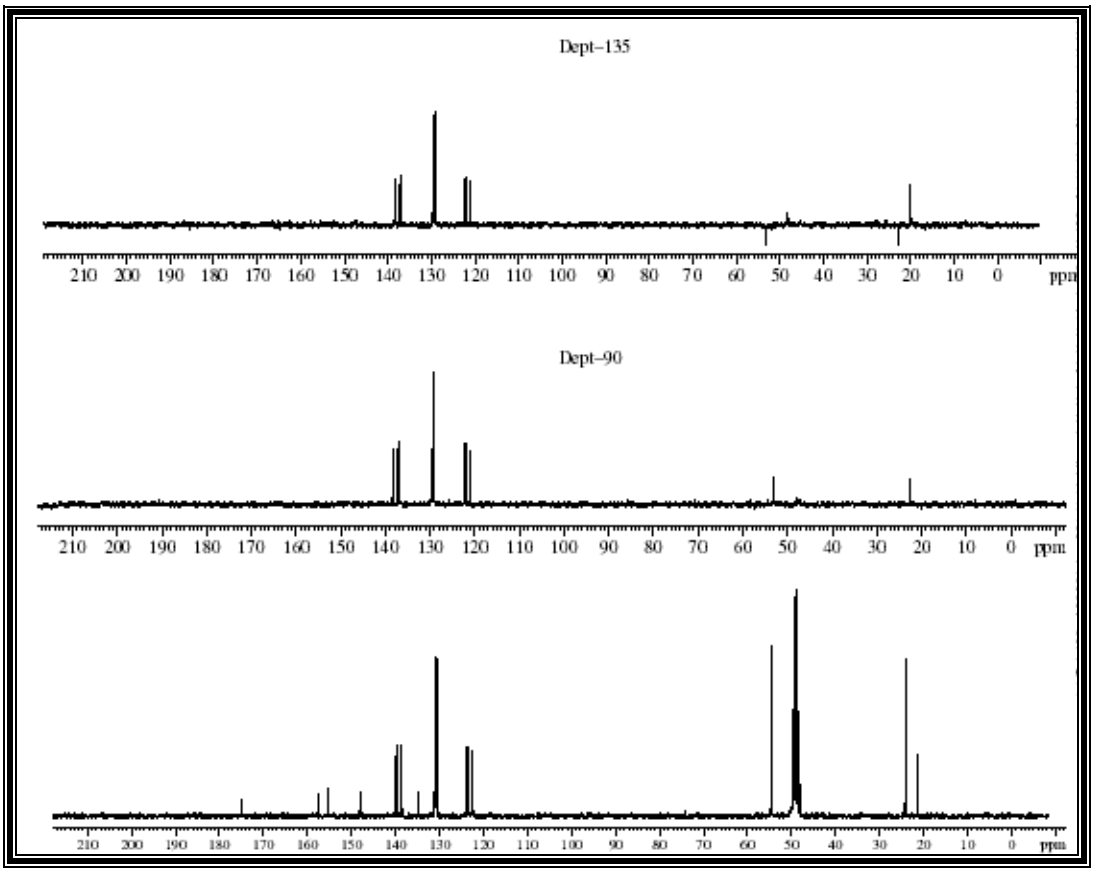


Fig. 20: ^{13}C-DEPT of Acrivastine

Z-isomer of Acrivastine

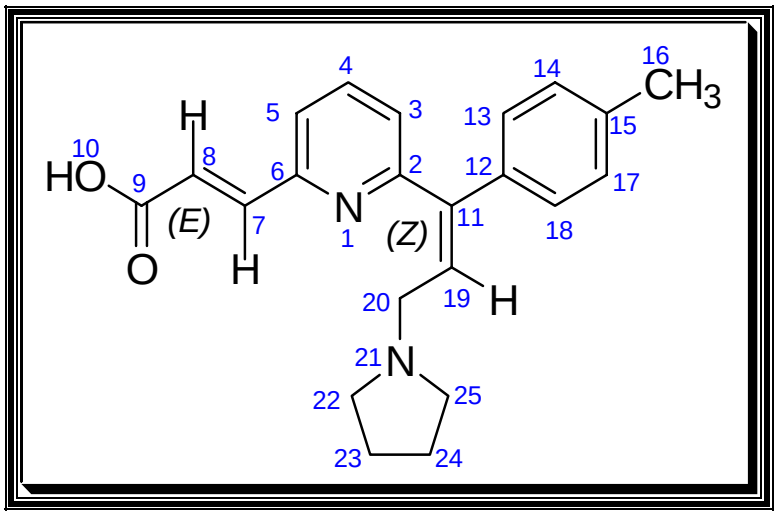


Table 4: ^{1}H-NMR of Z-isomer

Chemical Shift (ppm)	Proton No.
7.88 (t, 1H, ArH)	4
7.76 (d, J=15.8 Hz, 1H, CH)	7
7.69 (d, J=7.8 Hz, 1H, ArH)	5
7.19(s, 4H, ArH)	13, 14, 17, 18
7.18 (d, J=6.9 Hz, 1H, ArH)	3
6.88 (d, J=15.8 Hz, 1H, CH)	8
6.25 (t, J=7.5 Hz, 1H, CH)	19
4.00 (d, J=6.9 Hz, 2H, CH_2)	20
3.67 (bs, 2H, CH_2)	22, 25
3.20 (bs, 2H, CH_2)	
2.33 (s, 3H, CH_3)	16
2.09 (bs, 4H, CH_2 x 2)	23, 24

Table 5: ^{13}C-NMR of Z-isomer

Chemical Shift (ppm)	Nature of Carbon	Assignment
175.04	Quaternary	9
157.35, 155.36		2, 6
148.61		11
139.62		15
134.89		12
139.85, 138.54	Methine	7, 4
131.04, 130.77, 130.62		5, 13, 18, 14, 17
123.66, 123.52, 122.43		8, 3, 19
54.57	Methylene	20, 22, 25
24.02		23, 24
21.30	Methyl	16

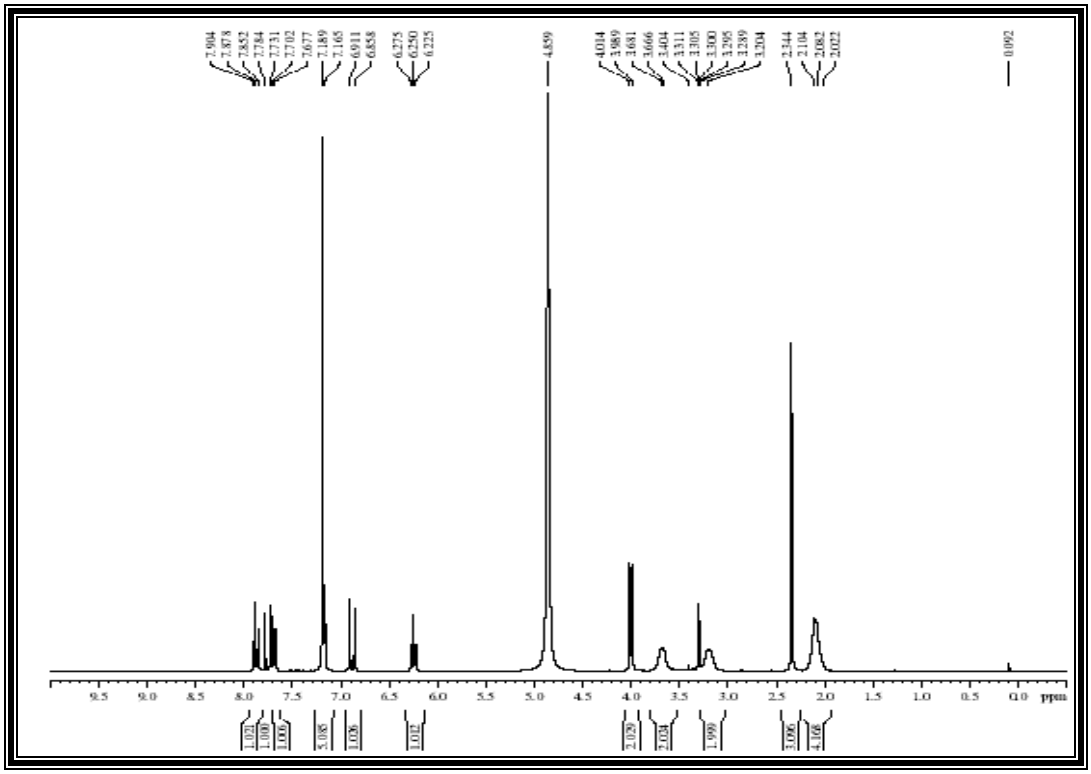


Fig. 21: 1H Spectrum of Z-isomer

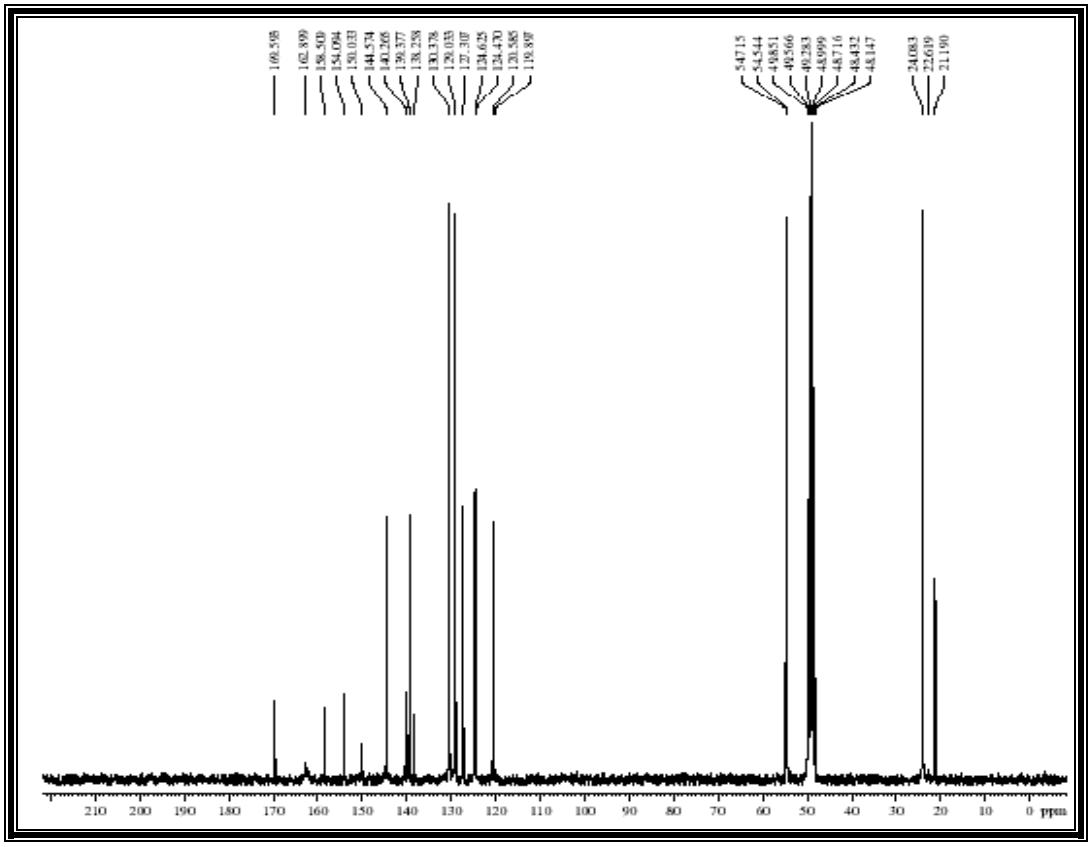


Fig. 22: ^{13}C Spectrum of Z-isomer

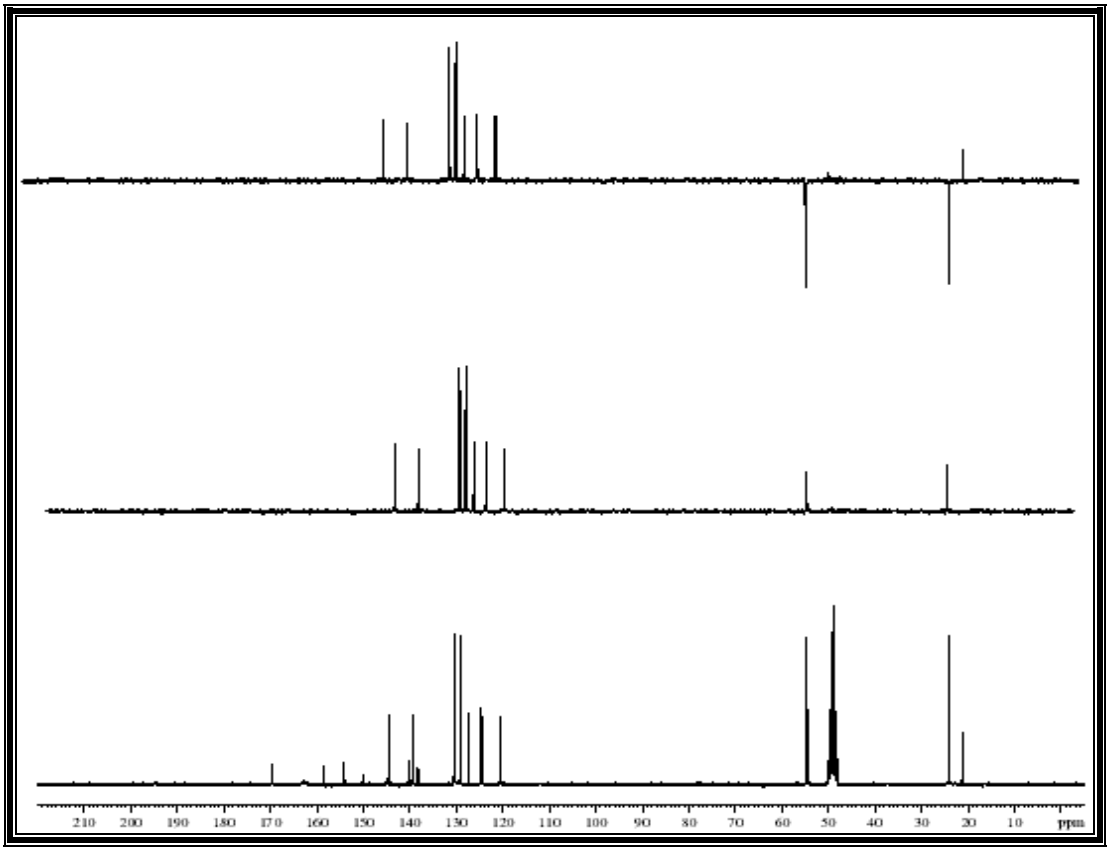


Fig. 23: ^{13}C-DEPT of Z-isomer

Being regioisomers, Acrivastine and its Z-isomers, by and large show similar ^{1}H and ^{13}C-NMR patterns. Both provide complete accountability for every proton and carbon via their δ values, types of protons, carbons, multiplicity patterns, integration and nature of carbons (methyl, methylene, methane and quaternary) via DEPT experiments. The differences are due to their geometric isomer nature, which is described below.

The characterization of geometric isomers of Acrivastine in ^{1}H-NMR, deshielding of H_{19} at 6.78 δ ppm is obvious in E-isomer, i. e. Acrivastine due to intra molecular H-bonding, whereas it is shielded at 6.25 δ ppm in Z-isomer. Similar effects of shielding (3.83 δ ppm for CH_2, H_{20}) in E-isomer and deshielding (4.00 δ ppm) of Z-isomer are noteworthy.

Form above spectroscopic data of Mass, IR and NMR (1H. 13C and DEPT) confirmed the structure of both the isomers and the values for ^{1}H-NMR, ^{13}C-NMR and Mass are in total agreement with assigned chemical structure.

5.4 Determination of the Relative Response Factor (RRF) of Z-isomer

Since, two different compounds may have different UV profile, therefore, detection of them at single wavelength will not give equal response. In order to calculate exact amount of impurity, relative response factor is required. Here, response factor of Z-isomer relative to acrivastine has been determined. The results of experiment are presented in Table 6.

Table 6: Results of Relative Response Factor

Parameters	**Acrivastine**	**Z-isomer**
Area - 1	102	57.7
Area - 2	101.4	58
Area - 3	103.6	58.3
Area - 4	103.5	58.3
Area - 5	103.1	58.6
Average Area	102.72	58.18
SD	0.87	0.31
RSD	0.85	0.53
RRF	1	0.57

Correction Factor	1	1.76

It can be seen from the Table 1 that response of the Z-isomer is less at 229nm as compare to acrivastine, which is also supported by UV spectra. The multiplication of correction factor and percentage of Z-isomer from the area normalize method will give exact amount present in the tested sample.

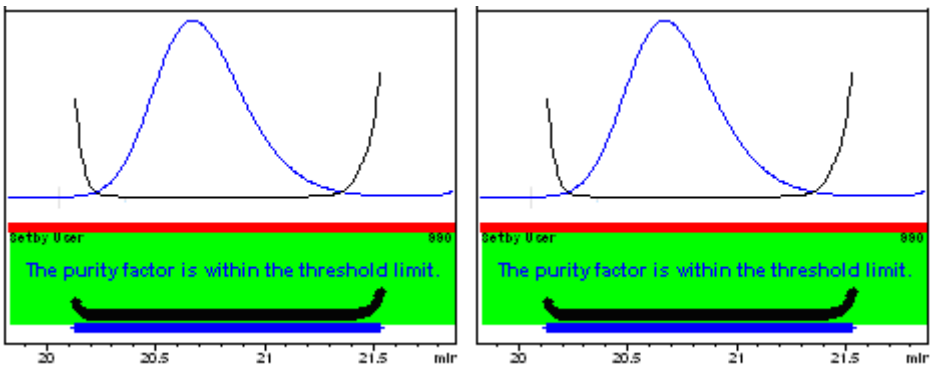
5.5 Results of Method Validation

5.5.1 Results of System Suitability and Specificity

The system suitability results are summarized in Table 7, which showed that resolution between acrivastine and its Z-isomer was not less than 2.8. Peak purity of acrivastine and its Z-isomer was not less than 0.999/990 using PDA detector. Report of peak purity is presented in Fig. 24.

Table 7: System suitability data

System suitability parameters	Z-isomer (n =3)	Acrivastine (n=3)
Retention factor (k)	2.54	2.87
Selectivity ( )		1.13
Resolution (Rs)	2.98	
Symmetry (S)	0.78	0.80
Theoretical Plates (N)	10766	12297



(a) **(b)**

Fig. 24: Peak purity report of (a) Acrivastine and (b) Z-isomer

The retention time of the peak of acrivastine and its Z-isomer was 10.8 and 9.9 minutes respectively. No interference was observed in the chromatogram of the diluent and mobile phase at the retention time of acrivastine and its Z-isomer. The retention time identification of acrivastine and its Z-isomer and chromatogram of the specificity study are presented as Fig. 25, 26 and 27.

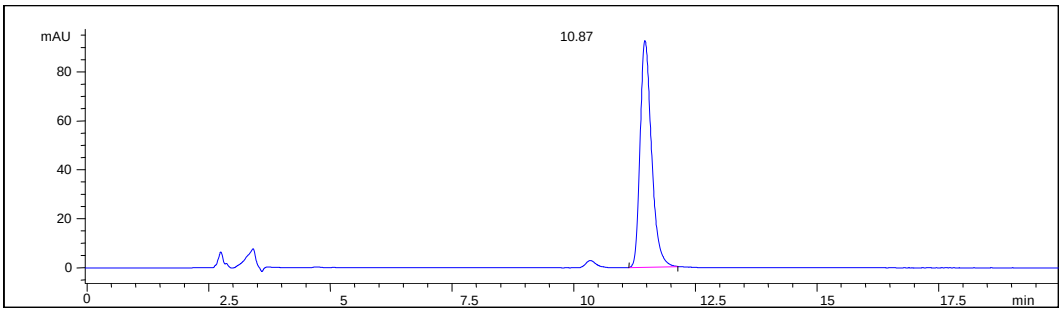


Fig. 25: Chromatogram of Acrivastine

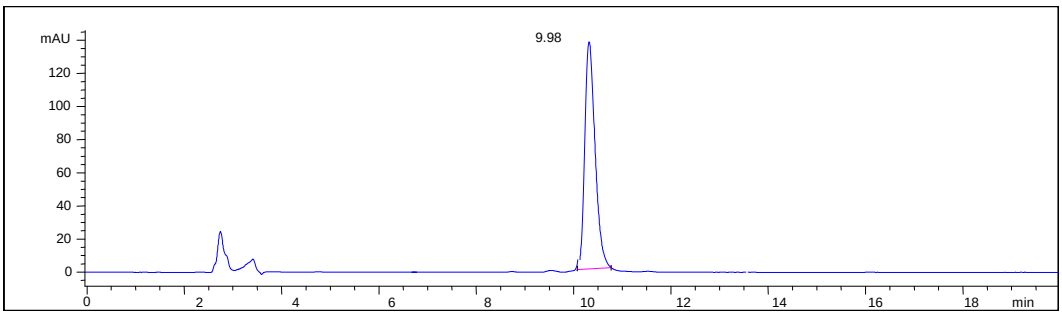


Fig. 26: Chromatogram of Z-isomer

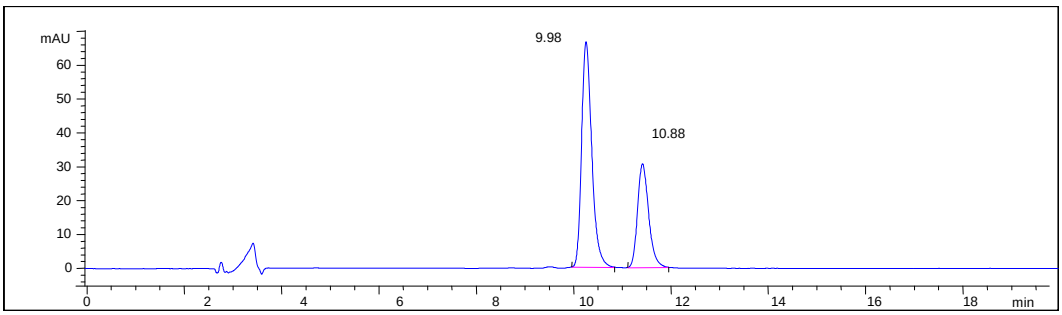


Fig. 27: Chromatogram of Acrivastine + Z-isomer

5.3.2 Result of Precision

The precision was determined for acrivastine spiked with Z-isomer at 100 % specification level. The relative standard deviation of retention time and area was calculated for six replicates injection. The area of Z-isomer was corrected with '3.5' area already present in the selected batch of acrivastine sample. The result of precision study is presented in Table 8.

Table 8: Result of Precision Study

Precision Data					
Sr. No	***Acrivastine***		***Z-isomer***		
	RT	**Area**	**RT**	**Area**	**Corrected Area**
1	10.85	11000.10	9.928	16.50	13.00
2	10.848	11040.30	9.925	16.70	13.20
3	10.838	11043.20	9.919	16.60	13.10
4	10.829	11057.70	9.912	16.60	13.10
5	10.833	11073.30	9.915	16.30	12.80
6	10.83	11104.10	9.911	16.30	12.80
Average	10.84	11053.12	9.92	16.50	13.00
SD	0.01	34.93	0.01	0.17	0.17
% RSD	**0.08**	**0.32**	**0.07**	**1.01**	**1.29**

It can be seen from the data that relative standard deviation of retention time and peak area is less than 0.1 % and 2.0 %, respectively for acrivastine and its Z-isomer. The result of precision are within general acceptance limit, therefore developed method is precise for its intended use.

5.3.3 Result of Limit of Detection and Limit of Quantitation

The LOD and LOQ concentration were estimated to be 0.05 and 0.2 ppm for Z-isomer, when the signal-to-noise ratio of 3 and 10 were used as the criteria. The method precision for Z-isomer at LOQ was 2.7 % RSD; data are presented in Table 9. The area of Z-isomer was corrected with '3.5' area already present in the selected batch of acrivastine sample. The recovery of Z-isomer at LOQ was 106.5 % in the spiked acrivastine sample. It can be said that LOQ value of the Z-isomer is quite law than the specification limit. The method is precise and accurate at LOQ level.

Table 9: Results of Precision study at LOQ level

Sr. No	Z-isomer at LOQ level		
	RT	**Area**	**Corrected Area**
1	9.844	7.30	3.80
2	9.847	7.40	3.90
3	9.847	7.20	3.70
4	9.859	7.40	3.90
5	9.863	7.30	3.80
6	9.893	7.50	4.00
Average	9.86	7.35	3.85
SD	0.02	0.10	0.11
% RSD	**0.19**	**1.43**	**2.72**

5.3.4 Result of Linearity of Z-isomer

The described method was linear in the range of 0.2-3.0 ppm for Z-isomer. Plotting the peak area versus its corresponding concentration with a correlation coefficient of 0.999 drew the calibration curve. The equation of the calibration curve for Z-isomer was $y = 12.1937x + 1.1019$. Linearity was checked for Z-isomer over the same concentration range for three consecutive days. The peak area at different concentration of Z-isomer for Day-1, Day-2 and Day-3 are presented in Table 10, 11 and 12 respectively. The linearity graph of Day-1, Day-2 and Day-3 are shown in Fig. 28, 29 and 30 respectively. The percentage relative standard deviation of the slope and Y-intercept of the calibration curve were 1.08 and 7.79, respectively. The data are summarized in Table 13. It can be concluded that method is linear within the studied range.

Table 10: Data of linearity of Z-isomer at Day-1

Concentration (ppm)	Area
0.2	3
0.5	7.6
1	13.6
1.5	19.1
2	25.9
3	37.1

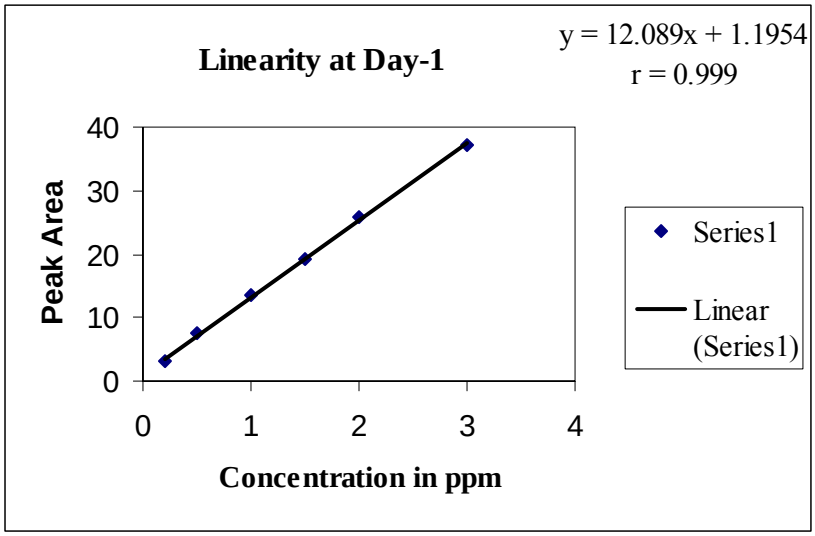


Fig. 28: Linearity plot of Z-isomer at Day-1

Table 11: Data of linearity of Z-isomer at Day-2

Concentration (ppm)	Area
0.2	3
0.5	7.6
1	13
1.5	19.2
2	25.8
3	37.2

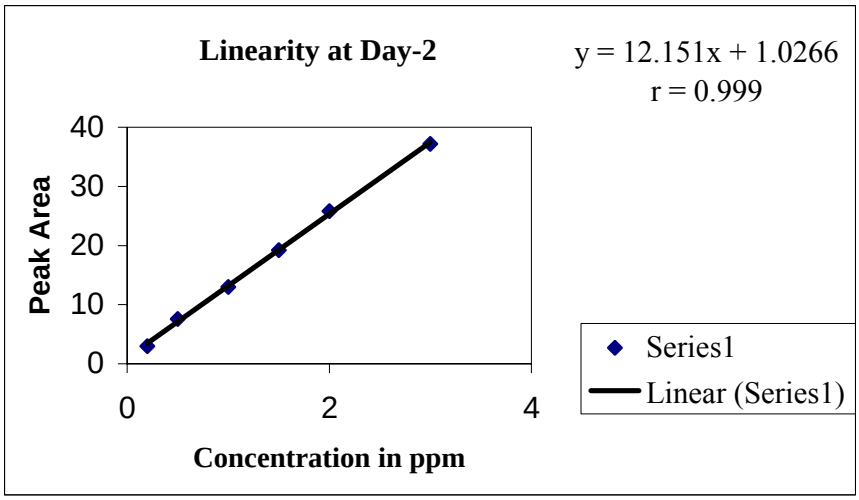


Fig. 29: Linearity plot of Z-isomer at Day-2

Table 12: Data of linearity of Z-isomer at Day-3

Concentration (ppm)	Area
0.2	3.3
0.5	7.4
1	13.2
1.5	19.9
2	26.1
3	37.8

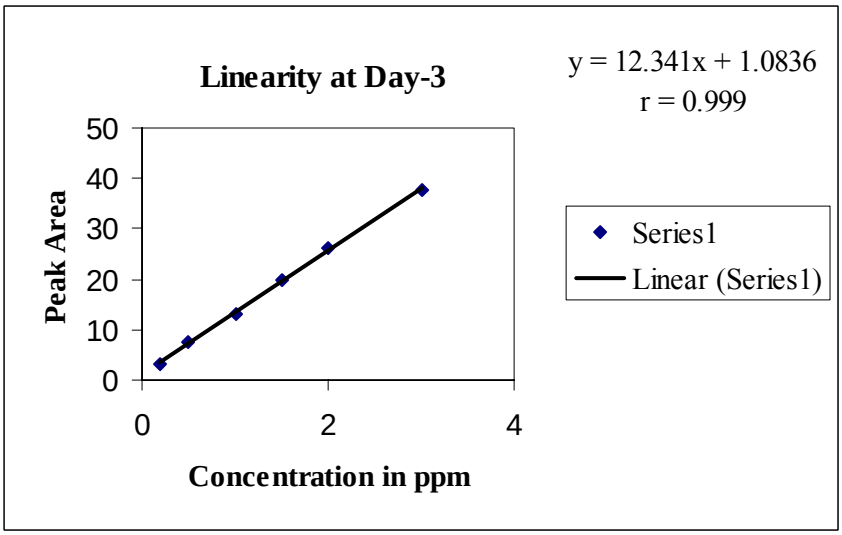


Fig. 30: Linearity plot of Z-isomer at Day-3

The general acceptance criteria for correlation coefficient are, it should not be less than 0.995. The correlation coefficient of Z-isomer was 0.999. The result showed the excellent correlation between the peak area and concentration for Z-isomer in developed method.

Table 13: Results of Linearity Study

Linearity	Equation of Calibration curve	
	Slope	**Intercept**
Day 1	12.089	1.1954
Day 2	12.151	1.0266
Day 3	12.341	1.0836
Average	12.1937	1.1019
SD	0.13	0.086
% RSD	1.08	7.79

5.3.5 Result of Accuracy/Recovery

In order to determine the accuracy of the method standard addition and recovery experiments were conducted in triplicate at 50, 100 and 150 % level of Z-isomer with respect to specification limit, i.e. 0.5, 1 and 1.5 ppm of Z-isomer. The recovery was calculated from slope and Y-intercept of the calibration curve obtained in linearity study. The acrivastine batch used for the study contains small amount of Z-isomer, therefore for the area correction, API sample was injected at same concentration. The area obtained was '3.5'.

The area obtained for Z-isomer in the spiked recovery solution was corrected by area present in the acrivastine sample solution. The recovery Z-isomer at all the tested level was calculated and mean of recovery of three different sets was also determined. The data are presented in Table 14.

Table 14: Results of accuracy/recovery of Z-isomer

Test	Accuracy at 50 % level			Accuracy at 100 % level			Accuracy at 150 % level		
	Set1	Set 2	Set3	Set1	Set 2	Set3	Set1	Set 2	Set3
Area	10.4	10.7	10.5	16.6	16.5	16.4	22.8	23.0	22.6
Corrected area	6.9	7.2	7.0	13.1	13.0	12.9	19.3	19.5	19.1
% Recovery	95.10	100.02	96.74	98.40	97.58	96.76	99.50	100.59	98.40
Mean Recovery	97.29 %			97.58 %			99.50 %		
% RSD	2.58			0.84			1.10		

It can be seen from the data that recovery of Z-isomer is between 95 to 101 % at all the tested level. The recovery experiment demonstrated excellent accuracy of the developed method for the determination of Z-isomer in acrivastine bulk drug.

5.3.6 Result of Robustness

The chromatographic resolution between acrivastine and its Z-isomer was used to evaluate the method robustness under modified conditions. The results robustness testing are summarized in Table 15.

Table 15: Results of Robustness Study

Parameter	Resolution between Acrivastine and its Z-isomer
Flow rate (mL/min)	
0.9	3.09
1.0	2.98
1.1	2.83
Mobile Phase Composition (0.05 % TFA:Acetonitrile, v/v)	
71:29	2.79
72:28	2.98
73:29	3.21

Column Temperature (° C)	
28	3.05
30	2.98
32	2.84

The resolution between acrivastine and its Z-isomer was found 2.98 in original condition. There was no major effect observed on resolution, under all the deliberately changed conditions. Therefore, it can be concluded that the developed method is robust for its intended use.

5.3.7 Result of Intermediate Precision

In order to evaluate reproducibility and ruggedness of the method, precision test was repeated using different instrument and column in second laboratory at different day. The experiment was carried out for acrivastine spiked with Z-isomer at 100 % specification level. The relative standard deviation of retention time and area was calculated for six replicates injection. The area of Z-isomer was corrected with '3.6' area of Z-isomer obtained in the selected batch of acrivastine sample on same day. The result of precision study is presented in Table 16.

The intermediate precision of the method was established by calculating percentage bias of retention time and area at two different conditions. The result of ruggedness is shown in Table. 17.

Table 16: Result of Precision Study at Different Day

Precision Data					
Sr. No	***Acrivastine***		***Z-isomer***		
	RT	**Area**	**RT**	**Area**	**Corrected Area**
1	10.825	11050.10	9.931	16.80	13.20
2	10.846	11088.30	9.926	16.70	13.10
3	10.826	11095.20	9.918	16.80	13.20
4	10.844	11064.70	9.92	16.60	13.00
5	10.824	11073.30	9.915	16.50	12.90
6	10.839	11040.10	9.935	16.90	13.30

Average	10.83	11068.62	9.92	16.72	13.12
SD	0.010	21.39	0.01	0.15	0.15
% RSD	**0.09**	**0.19**	**0.08**	**0.88**	**1.12**

Table 17: Results of Intermediate Precision Study

Compd	Average Retention Time			Average Peak Area		
	Original Conditio	Different Condition	% Bias	Original Condition	Different Condition	% Bias
Z-isomer	9.92	9.92	0.0	13.00	13.12	0.92
Acrivastine	10.84	10.83	-0.10	11053.12	11068.62	0.14

The bias between original and different conditions was found below 2.00 % for retention time peak area of acrivastine and its Z-isomer. Therefore, it can be concluded that developed method is rugged enough for its intended use.

5.3.8 Result of Solution Stability

Solution stability of acrivastine was carried out in diluent for 48 hours. The % bias of the area of Z-isomer was calculated with the initial area of fresh injection. The result of the solution stability is presented in Table 18.

Table 18: Result of Solution Stability

Sr. No.	Time (h)	Area of Z-isomer	% Bias
1	0	16.4	-
2	12	16.5	0.61
3	24	16.3	-0.61
4	48	16.2	-1.21

The bias of area of Z-isomer after 12, 24 and 48 hours was found less than 2.0 %. From data, it can be said that the sample solution is stable at room temperature for atleast 48 hours. Therefore, it can be concluded that sample solution can be use within 48 hours from the time of preparation.

6. Conclusion

A simple, rapid, robust, suitable, precise and accurate HPLC method has been developed and validated for the determination of Z-isomer in acrivastine bulk drug. The systematic method development was approached in the selection of wavelength, selection of stationary phase, selection of mobile phase components and composition. The relative response factor was also determined for Z-isomer.

The method was extended to preparative HPLC isolation of the Z-isomer from the crude reaction mixture. Successfully, Z-isomer was isolated with HPLC purity 99.02 %. The isolated pure Z-isomer was completely characterized by ^{1}H, ^{13}C-NMR and DEPT, IR, UV, Mass and HPLC. The spectroscopic difference between acrivastine and its Z-isomer was discussed.

The developed method was completely validated for the determination of the Z-isomer in acrivastine with respect to specificity, system suitability, linearity, limit of detection and quantitation, accuracy, precision, robustness, intermediate precision and solution stability. The result of validation showed satisfactory data for all the method validation parameters tested.

The developed method can be used for the determination of Z-isomer in acrivastine with limit of detection 0.05-ppm level.

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Part-B

Application of the Chromatography for the Impurity Profiling of Active Pharmaceutical Ingredient

Section-I

A UPLC Method for the Determination of Process-Related Impurities in Azathioprine

1. Introduction

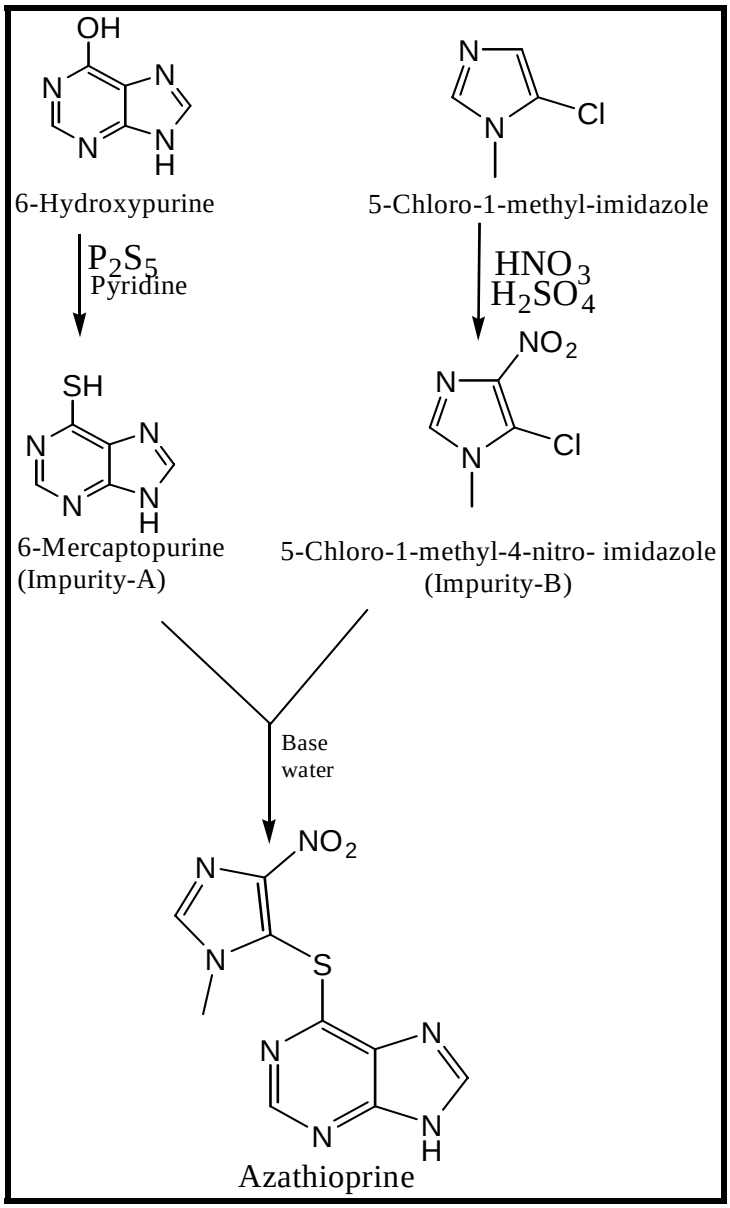
Impurities in pharmaceuticals are the unwanted chemicals thoset remain with the Active Pharmaceutical Ingredients (APIs), or develop during formulation, or upon aging of both API and formulated APIs to medicines. The presence of these unwanted chemicals even in small amounts may influence the efficacy and safety of the pharmaceutical products. Impurity profiling (i.e., the identity as well as the quantity of impurity in the pharmaceuticals), is now getting receiving important critical attention from regulatory authorities. The different pharmacopoeias, such as the British Pharmacopoeia (BP) and the United States Pharmacopoeia (USP), are slowly incorpora ting limits to allowable levels of impurities present in the APIs or formulations. Also, the International Conference on Harmonization (ICH) has published guidelines on impurities in new drug substances [1], products [2], and residual solvents [3]. In addition, Ahuja [4] and Gorog [5] have published books covering different aspects of impurities, including the governmental regulations and guidelines and the identification and monitoring of impurities found in drug products. There is a significant demand for the impurity-reference standards along with and the API reference standards for both regulatory authorities and pharmaceutical companies.

Organic impurities may arise during the manufacturing process and/or on storage of the drug substance. The major source of organic impurities is process related impurities.

Starting materials, intermediates, precursors etc. are the most common impurities found in every API unless a proper care is taken in every step involved throughout the multi-step synthesis. Sometimes, impurities of intermediate and precursor generate structurally related by-product during synthesis.

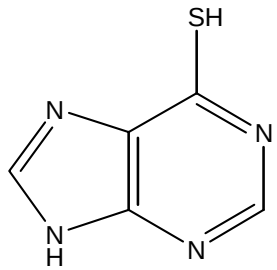
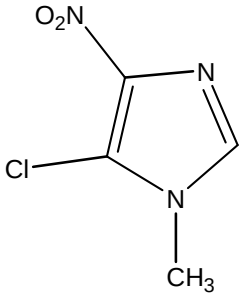
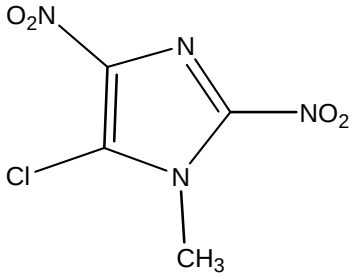
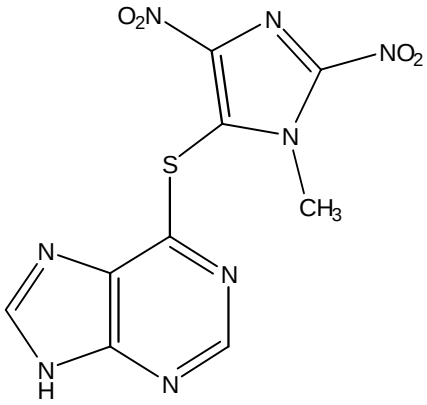
The study describes development and validation of the chromatographic method for the determination of the process related impurities. Azathioprine has been selected for the study. It is an immunosuppressive drug co-administered with cyclosporine and corticoids to prevent rejection after transplantation. Ultra Performance Liquid Chromatography (UPLC) method for the determination of four impurities (Impurity-A, Impurity-B, Impurity-C and Impurity-D) along with azathioprine has been developed.

Structural information of the impurities are presented in Table 1. Specification limit of each impurity has been decided that it should not be more than 0.2 %. The synthesis of azathioprine as described in Scheme 1[6], Impurity-A and Impurity-B may be present as an un-reacted precursor, while Impurity-C [5-Chloro-1-methyl-2,4-dinitro-1H-imidazole] is the impurity arising by over reaction during synthesis of Impurity-B and Impurity-D is the byproduct of the coupling of Impurity-A and Impurity-C as per this route of synthesis.



Scheme 1: Synthetic scheme of Azathioprine

Table 1: Structural Information About Impurities

Code of Impurity	Structure	Description
Impurity-A		**IUPAC Name:** 9H-Purine-6-thiol **Molecular Formula:** $C_5H_4N_4S$ **Molecular Weight:** 152.18 g/mol **HPLC Purity:** 99.97 %
Impurity-B		**IUPAC Name:** 5-Chloro-1-methyl-4-nitro-1H-imidazole **Molecular Formula:** $C_4H_4ClN_3O_2$ **Molecular Weight:** 161.55 g/mol **HPLC Purity:** 99.98 %
Impurity-C		**IUPAC Name:** 5-Chloro-1-methyl-2,4-dinitro-1H-imidazole **Molecular Formula:** $C_4H_3ClN_4O_4$ **Molecular Weight:** 206.54 g/mol **HPLC Purity:** 95.40 %
Impurity-D		**IUPAC Name:** 6-(3-Methyl-2,5-dinitro-3H-imidazol-4-ylsulfanyl)-9H-purine **Molecular Formula:** $C_9H_6N_8O_4S$ **Molecular Weight:** 322.26 g/mol **HPLC Purity:** 99.50 %

1.1 Drug Profile

1.1.1 Description

Azathioprine (Fig. 1) is the nitro imidazole derivative of 6-mercaptopurine. It is chemically, 6-(3-Methyl-5-nitro-3H-imidazol-4-ylsulfanyl)-9H-purine. Its molecular formula is $C_9H_7N_7O_2S$ having molecular weight 277.26 g/mole.

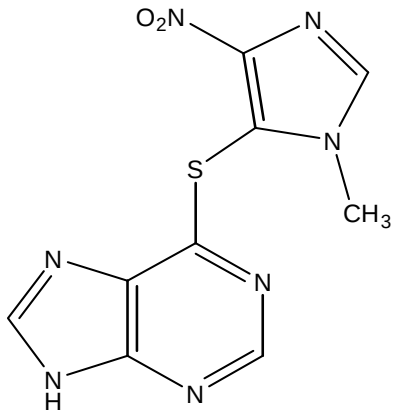


Fig. 1: Chemical Structure of Azathioprine

1.1.2 Mechanism of Action

The exact mechanism of immunosuppressive activity of azathioprine has not been determined. Azathioprine, which is an antagonist to purine metabolism, may inhibit RNA and DNA synthesis. The drug may also be incorporate into nucleic acid resulting in chromosomes break, malfunctioning of the nucleic acid, or synthesis of fraudulent proteins. The drug may also inhibit coenzyme formation and functioning, there by interfering with cellular metabolism. Mitosis may be inhibited by drug [7].

Azathioprine is also a potent inhibitor of mixed lymphocyte culture responses and can readily suppress the in vitro generation of cytotoxic T cells. These observations suggest that drugs exert preferential toxicities for murine T cells. Blymphocytes for mice appear to vary in there susceptibility for thiopurines. By contrast, the activity of human B cells can be readily suppressed with this drug whereas T helper function is completely unaltered. In addition to immunosuppressive properties, thiopurins are capable of

exerting anti-inflammatory activities, primary by inhibiting the replication of hematopoietic precursors.

1.1.3 Clinical Use

Adjunct with other agents in prevention of rejection of kidney transplants; also used in severe active rheumatoid arthritis unresponsive to other agents, other autoimmune diseases (ITP, SLE, MS, Crohn's disease).

A study has suggested that long-term treatment with Azathioprine may prevent extravation and cause reduction in neutrophile trafficking which may be beneficial for maintaining remission in IBD.

In addition immunosuppressive properties, thiopurines are capable of exerting anti-inflammatory activities by inhibiting cyclooxygenase production [7, 8].

1.1.4 Pharmacokinetics

Azathioprine is fast and extensively metabolized when administer orally in mice. Azathioprine undergoes first pass metabolism in mice indicated by the presence of thiouric acid, the end product of azathioprine metabolism in intestinal mucosa and liver. The initial concentration of 6-MP extracted from the organs was about 10 fold those found in plasma indicating rapid cellular uptake of 6-MP. The conversion of azathioprine to 6-mercaptopurine in vivo may be catalyzed largely enzymatically by glutathione S-transferase in the liver [9].

6-MP administration in rhesus monkeys, 6-MP levels were described by a two-compartment body model; mean terminal half life; plasma clearance (CLp) and volume of distribution (Vdss) were 41.6 ± 12.1 min, 48 ± 15.4 ml/min/kg and 1.76 ± 0.64 liters/kg respectively.

Distribution: Crosses placenta

Protein binding: ~30%

Metabolism: Extensively hepatic via xanthine oxidase to mercaptopurine (active); mercaptopurine requires detoxification by thiopurine methyltransferase (TPMT)

Half-life elimination: Parent drug: 12 minutes; mercaptopurine: 0.7-3 hours; End-stage
Renal disease: Slightly prolonged
Excretion: Urine (primarily as metabolites)

1.1.5 Dosage

I.V. dose is equivalent to oral dose (dosing should be based on ideal body weight):
Children and Adults: Renal transplantation: Oral, I.V.: 2-5 mg/kg/day to start, then 1-3 mg/kg/day maintenance
Adults: Rheumatoid arthritis: Oral: 1 mg/kg/day for 6-8 weeks; increase by 0.5 mg/kg every 4 weeks until response or up to 2.5 mg/kg/day

Dosing adjustment in renal impairment:
Clcr 10-50 mL/minute: Administer 75% of normal dose daily
Clcr<10 mL/minute: Administer 50% of normal dose daily
Hemodialysis: Slightly dialyzable (5% to 20%)
Administer dose posthemodialysis: CAPD effects: Unknown; CAVH effects: Unknown

1.1.6 Adverse Reactions

Central nervous system: Fever, chills
Dermatologic: Alopecia, erythematous or maculopapular rash
Gastrointestinal: Nausea, vomiting, anorexia, diarrhea, aphthous stomatitis, pancreatitis
Hematologic: Leukopenia, thrombocytopenia, anemia, pancytopenia (bone marrow suppression may be determined, in part, by genetic factors, ie, patients with TPMT deficiency are at higher risk)
Hepatic: Hepatotoxicity, jaundice, hepatic veno-occlusive disease
Neuromuscular & skeletal: Arthralgias
Ocular: Retinopathy
Miscellaneous: Rare hypersensitivity reactions which include myalgia, rigors, dyspnea, hypotension, serum sickness, rash; secondary infection may occur secondary to immunosuppression

1.1.7 Overdosage/Toxicology

Symptoms of overdose include nausea, vomiting, diarrhea, and hematologic toxicity. Following initiation of essential overdose management, symptomatic and supportive treatment should be instituted. Dialysis has been reported to remove significant amounts of the drug and its metabolites, and should be considered as a treatment option in those patients who deteriorate despite established forms of therapy[10].

1.1.8 Drug Interactions

ACE inhibitors: Concomitant therapy may induce leukopenia.

Allopurinol: May increase serum levels of azathioprine's active metabolite (mercaptopurine). Decrease azathioprine dose to 1/3 to 1/4 of normal dose.

Aminosalicylates (olsalazine, mesalamine, sulfasalazine): May inhibit TPMT, increasing toxicity/myelosuppression of azathioprine. Use caution.

Warfarin: Effect may be decreased by azathioprine[11].

1.1.9 International Brand Names

Alti-Azathioprine (CA); Apo-Azathioprine® (CA); Azafalk® (DE); Azahexal® (AU); Azamedac® (DE); Azamun® (AU, CZ, FI, NZ, RO); Azamune® (GB); Azanin® (JP); Azapress® (ZA); Aza-Q® (DE); Azarek® (CH, DE); azathiodura® (DE); Azathioprin 1A Pharma® (DE); Azathioprin AL® (DE); Azathioprin beta® (DE); Azathioprine® (GB, PL, RO, TR); Azathioprine PCH® (SG, TR); Azathioprine Pharmachemie® (TH); Azathioprin Hexal® (DE); Azathioprin-Puren® (DE); Azathioprin-ratiopharm® (DE); Azathioprin Stada® (DE); Azatioprina Asofarma® (AR); Azatioprina® (CL); Azatioprina Filaxis® (AR); Azatioprina Wellcome® (IT); Azatioprin NM Pharma® (SE); Azatrilem® (MX); Azopi® (IL); Azopine® (IE); Azoran® (IN); Colinsan® (DE); Gen-Azathioprine (CA); Immunoprin® (GB); Imuger® (IE); Imunen® (BR); Imuprin® (CY, FI, RO); Imuran® (AR, AU, BD, BE, BG, BR, CA, CL, CZ, EC, GB, HR, HU, ID, IE, IL, IN, KW, LU, MX, NL, NZ, PL, RO, RU, SG, SI, TH, TR, YU); Imuran® [inj.] (RO, TR); Imuran Paranova® (DK); Imurek® (AT, CH, DE); Imurek® [inj.] (AT, CH,

DE); Imurel® (DK, ES, FI, FR, NO, SE); Oprisine® (GB); Thioprine® (AU, NZ); Transimune® (IN); Zaprine 50® (ZA); Zytrim® (DE)

2. Literature Review

The literature survey revealed that Azathioprine was mainly determined by HPLC method, since information was not obtained on UPLC.

A through literature review, it has been observed that numerous HPLC methods have been developed for the determination of 6-MP and its metabolites in biological fluids [12-15]. In addition, a HPLC-mass spectrometry method for the detection of 6-TGNs and 6-mMPNs has been developed, recently [16]. Few of the literature input described below in detail.

US Pharmacopoeia, USP 30, 2007, 1471-72

Test for Mercaptopurine: Prepare three solutions in 6 N ammonium hydroxide containing respectively, 20 mg of Azathiopurine per mL, 2 mg of USP Azathiopurine RS per mL, and 200 µg of USP Mercaptopurine RS, on the anhydrous basis, per mL. Apply 5 µL volumes of the solution at points about 2 cm from the bottom edge of a thin-layer chromatographic plate coated with a 0.25 mm layer of microcrystalline cellulose. Allow the spot to dry, and develop the chromatogram in a suitable chamber, using butyl alcohol, previously saturated with 6 N ammonium hydroxide, as a solvent, until the solvent front has moved about 15 cm from the point of application. Remove the plate, air-dry and locate the spot by viewing under short- and long wavelength UV light: any spot in the chromatogram from Azothioprine, other than the principal spot, is not more intense than the spot in the chromatogram obtained with USP Mercaptopurine RS (1.0%) [17].

Ahmed F. Hawwa, Jeff S. Millership, Paul S. Collier, James C. McElnay Development, validation of an HPLC method for the rapid and simultaneous determination of 6-mercaptopurine and four of its metabolites in plasma and red blood cells. Journal of Pharmaceutical and Biomedical Analysis (2008), doi:10.1016/j.jpba.2008.10.045

Chromatographic separation was achieved using reversed phase chromatography with isocratic elution. An Atlantis™ dC18 column [150 mm × 3.9 mm (i.d.); particle size, 3 μm; Waters] protected with a guard cartridge [20 mm × 3.9 mm; particle size, 3 μm; Waters] was used as the stationary phase.

The mobile phase, consisting of acetonitrile, methanol and KH_2PO_4 (0.02 M; pH 2.25) (3:1:96, v/v/v), was filtered and degassed through a 0.45 μm filter and pumped at a flow rate of 1 ml/min. The column temperature was maintained at 25°C.

UV detection was employed at 322 nm (for the detection of 6-MP and the 6-mMP derivative) and 342 nm (for the detection of 6-TU, 6-TG and 6-TX) [18].

Thierry Dervieux, Roselyne Boulieu, Simultaneous determination of 6-thioguanine and methyl 6-mercaptopurine nucleotides of azathioprine in red blood cells by HPLC, Drug monitoring and toxicology, Clinical chemistry 44:3, 551-555 (1998)

6-TG and Me6-MP derivative liberated from the nucleotide moiety were analyzed by a reversed-phase HPLC method. The separation was performed on a Purospher RP 18-e column, 5-mm particle size, protected by a Purospher RP 18-e guard column (from Merck) with a linear gradient elution mode with 0.02 mol/L potassium phosphate (pH 3.5) and 0.02 mol/L potassium phosphate (pH 3.5):methanol (40:60 by vol). The concentration of methanol varied from 0 to 200 mL/L over a period of 12 min. The flow rate was 1.2 mL/min. Detection of 6-TG and Me6-MP derivative was performed at 341 nm and 304 nm respectively. Peak identity was confirmed through library matching by comparison of unknown peak to reference spectra of calibrator. All analyses were performed at ambient temperature [15].

Tatiana Tatit Fazio, Anil Kumar Singh, Erika Rosa Maria Kedor-Hackmann, Maria Ines Rocha Miritello Santoro. Quantitative determination and sampling of azathioprine residues for cleaning validation in production area. Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 1495–1498

The aim of the author for this study was to validate simple analytical method for verification of residual azathioprine in equipments used in the production area and to confirm efficiency cleaning procedure. Analytical conditions were developed through the LC system using Nova-Pak C18 (3.9mm×150 mm, 4μm) WATERS®. The mobile phase

was constituted of methanol–water–acetic acid (20:80:1, v/v/v), at a flow rate of 1.0 mL/min. UV detection was made at 280 nm. The volume of injection was fixed at 20µL. All analyses were done at room temperature 25±2 °C, approximately. The mobile phase was prepared fresh each day, vacuum-filtered through a 0.45 µm Millex® (HV) hydrophilic membrane and degassed by ultrasonication for 20 min [19].

3. Aim of Present Work

As per discussion in the literature review, a few chromatographic methods are reported for the determination of the impurities and metabolites of azathioprine. So far to our present knowledge, there is no validated UPLC method for the determination of process related impurities in azathioprine. In official compendia and in-house standard testing procedure of laboratory for old drugs, most of the methods are in HPLC. Though HPLC is a well-established reliable technique used in controlling the quality and consistency of active pharmaceutical ingredients and dosage forms, it is often a slow technique because of complexity of some of the samples, it could still be improved. In the recent trend, most of the laboratories are being equipped with fast LC. Ultra Performance Liquid Chromatography (UPLC) is a new category of separation technique based upon well-established principles of liquid chromatograph, which utilize sub-2 m particles for stationary phase. To accelerate process development and fast quality control of old drugs, chromatographic methods using advance technology are most welcomed. Therefore, our aim is to develop simple, rapid and accurate UPLC method for the determination of the process related impurities of azathioprine bulk drug, which can be used comfortably in day-to–day activities.

The present work deals with the systematic method development studies, determination of the relative response factor for each impurity and validation of the method. In the method validation, specificity, linearity, accuracy, precision and limit of quantification and detection have been evaluated for each impurity. The robustness and solution stability of the method were also established. The developed method is recommended for routine monitoring of process development and quality control analysis.

4. Experimental

Materials and Instruments

Drug and Impurities Samples

Azathioprine, Impurity-A, Impurity-B, Impurity-C and Impurity-D were in-house samples, synthesized by Process Research Department of Zydus Research Center, Cadila Healthcare Ltd., Ahmedabad.

Chemicals and Solvent Used

Methanol – s d fine-CHEM Limited, Mumbai

Acetonitrile - s d fine-CHEM Limited, Mumbai

Trifluoro acetic acid – SIGMA-ALDRICH CHEMIE GmbH, Germany

Hydrochloric acid – s d fine-CHEM Limited, Mumbai

Sodium hydroxide - s d fine-CHEM Limited, Mumbai

Hydrogen peroxide - s d fine-CHEM Limited, Mumbai

Potassium bromide - s d fine-CHEM Limited, Mumbai

Water – HPLC grade water

Instruments Used

UPLC – WATERS ACQUITY Ultra performance LC

Analytical balance – Mettler Toledo

Sonicator – Bandelin electronic Heinrichstra Be 3-4 D-12207 Berlin, Germany

Vortexer – Spinix

Preparation of 0.05 % TFA in water

Transferred accurately 500 µL of Trifluoro acetic acid into a 1000 mL volumetric flask containing approximately 700 mL of water. Dissolved and make volume up to the mark with HPLC grade water, filter through 0.45 µm filter.

4.3 Preparation of Diluent (0.1 % ammonia in methanol)

Transferred accurately 250 µL of ammonia solution into a 250 mL volumetric flask. Dissolved and make up to the volume with HPLC grade methnaol, filter through 0.45 µm filter.

4.4 Test Sample Preparation

Weighed accurately about 25 mg of Azathioprine sample into 25 mL of volumetric flask. Add 10 to 15 mL of diluent and sonicated to dissolve the solid. Diluted to make up volume up to the mark with diluent and mixed well.

4.5 Chromatographic Parameters of Finalized Method

Elution : Gradient
Column : AQUITY UPLC BEH C18, 100 x 2.1 mm, 1.7 µm
Wave length : 220 nm
Column oven temp. : 30 C
Flow rate : 0.35 mL / min.
Injection volume : 1 µL
Retention time : About 2.8 min.
Run time : 5 min
Mobile phase : 0.05 % TFA in water: Acetonitrile (Gradient)
Diluent : 0.1 % Ammonia in methanol
Sample conc. : 1.0 mg/mL
Gradient program :

Time (minutes)	0.05 % TFA in Water (%)	Acetonitrile (%)
0.0	97	03
1.0	97	03
3.5	40	60
4.0	40	60
4.1	97	03
5.0	97	03

Sequence run to determine impurities:

Sr. No.	Solution to be injected	No. of injections	Cumulative injections
1	Diluent	1	1
2	Mobile phase	1	2
3	Sample preparation	2	4

Calculation:

Calculate the amount of impurities and % purity by area normalization, calculate exact amount of impurities by multiplying the obtained value with correction factor of known individual impurities.

4.6 Relative Response Factor

Sample preparation:

Transferred an accurately weigh quantity of about 10 mg of azathioprine and each impurities into 10 mL volumetric flasks, separately. Added about 7-8 mL of diluent into volumetric flask and sonicated to dissolve. Make volume up to the mark with diluent and mixed well to prepare 1000-ppm stock solution.

100 µL of above stock solution of azathioprine and each impurities was taken in a single 20 mL volumetric flask. Make volume up to the mark with diluent and mixed well to prepare blend 5-ppm working solution.

Procedure:

Five replicate of above solution was injected in the equilibrated chromatographic system at 220 nm. Average area of azathioprine and each impurities was used to calculate relative response factor.

4.7 Method Validation for Impurities

4.7.1 Specificity and System Suitability

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

To evaluate the interference from degradants, forced degradation study was carried out by stress testing of API under the following stress conditions.

Stress Type	Condition
Acid Hydrolysis	Treated with 1 mL of 5 N HCl and heat at 60° C on a water bath for 1 hour.
Base Hydrolysis	Treated with 1 mL of 0.1 N NaOH and heat at 60° C on a water bath for 1 hour.
Peroxide Oxidation	Treated with 0.5 mL of 30 % H_2O_2 and heat at 60° C on water bath for 1 hour.

Procedure:

About 10 mg of API was accurately weighed and transferred into a 10 mL volumetric flask. 5 mL of stress solution was added to it and the solution was heated at 60° C on water bath for 1 hour. The sample solution was then allowed to cool at room temperature and approximately neutralized it. About 2 mL of diluent was added to it and the solution was sonicated for 10 minutes and then was diluted up to the mark with diluent. The solution was mixed well and then filtered through 0.22 m filters. The sample solution was then injected into equilibrated chromatographic system. Peak purity of the principle peak was checked in impurities spiked solution and stressed solution using photo diode array detector**.**

Analysis sequence:

Analysis sequence for specificity test

Sr. No.	Sample	No. of Injections
1	Diluent	1
2	Mobile phase	1
3	Standard API solution	1
4	Impurity A	1
5	Impurity B	1
6	Impurity C	1
7	Impurity D	1
8	API + Impurities	1
9	Diluent – Acid Hydrolysis	1
10	API – Acid Hydrolysis	1
11	Diluent – Base Hydrolysis	1
12	API – Base Hydrolysis	1
13	Diluent – Peroxide Oxidation	1
14	API – Peroxide Oxidation	1

The system suitability parameters were evaluated from chromatogram of blend sample solution of impurities and API.

4.7.2 Linearity

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

Preparation of Stock Solution of Each Impurity (10 ppm):

Transferred an accurately weighed quantity of about 10 mg of each impurities to 100 mL volumetric flasks, separately. Added about 50 mL of diluent to volumetric flask and sonicated to dissolve. Make volume up to the mark with diluent and mixed well to prepare 100 ppm solution. Diluted 5 mL of this solution to 50 mL with diluent to prepare 10 ppm stock solutions of each impurities.

Preparation of 150 % (at specification level) Solution:

3 mL of stock solution was taken to 10 mL volumetric flask and make up the volume up to mark with diluent and mixed well.

Preparation of 100 % (at specification level) Solution:

2 mL of stock solution was taken to 10 mL volumetric flask and make up the volume up to mark with diluent and mixed well.

Preparation of 50 % (at specification level) Solution:

5 mL of stock solution was taken to 50 mL volumetric flask and make up the volume up to mark with diluent and mixed well.

Preparation of 25 % (at specification level) Solution:

5 mL of 50 % solution was taken to 10 mL volumetric flask and make up the volume up to mark with diluent and mixed well.

Preparation of 10 % (at specification level) Solution:

5 mL of 50 % solution was taken to 25 mL volumetric flask and make up the volume up to mark with diluent and mixed well.

Preparation of 5 % (at specification level) Solution:

5 mL of 10 % solution was taken to 10 mL volumetric flask and make up the volume up to mark with diluent and mixed well.

Procedure:

1 µL solutions of 5 %, 10 %, 25 %, 50 %, 100 %, and 150 % of each impurity were injected on an equilibrated chromatographic system. Area for each concentration

was calculated and linearity was plotted for area against the concentration for each impurity. The regression coefficient was calculated for the linearity curve.

4.7.3 Limit of Detection and Quantitation

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessary quantitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest amount of an analyte in a sample which can be quantitatively determined with suitable precision and accuracy. Demonstrate the limit of detection and quantification based on the residual standard deviation ( ) and slope (S) of linearity data obtained by preparing the diluted standard solutions in the range of 150 % (3 ppm) to 5 % (0.1 ppm) of target concentration.

The linearity curve obtained in linearity study for each impurities were used to determined standard deviation ( ) and slope (S). The following formulas were used to calculate LOD and LOQ.

$$\text{Limit of Detection (LOD)} = \frac{3.3}{S}$$

$$\text{Limit of Quantification (LOQ)} = \frac{10}{S}$$

4.7.4 Accuracy and Recovery

The accuracy of an analytical method is the closeness of test results obtained by the method to the true value. Here accuracy is demonstrated at 50 %, 100 % and 150 % level with respect to the specification limit for each impurity. Recovery was determined in triplicates and reported.

Preparation of Blend Stock Solution of Impurities (10 ppm):

Transferred an accurately weighed quantity of about 10 mg of each impurities to 100 mL volumetric flasks, separately. Added about 50 mL of diluent to volumetric flask and sonicated to dissolve. Make volume up to the mark with diluent and mixed well to prepare 100 ppm solution. Took 5 mL of solution of each impurity to 50 mL volumetric flask and diluted with diluent to prepare 10 ppm blend stock solution containing each impurity.

Standard Solutions For Impurities:

3, 2 and 1 mL of 10 ppm stock solution was taken in three different 10 mL volumetric flask and make up the volume up to mark with diluent and mixed well to got the concentration of 150, 100 and 50 % respectively.

Solution A: Preparation of API Sample:

Transferred an accurately weighed quantity of about 10 mg of API into 10 mL volumetric flask. Added 7-8 mL of diluent and sonicate to dissolve. Make up the volume up to mark with diluent and mixed well.

Solution B: Preparation of API Sample with 50 % Spiked Impurities:

Transferred an accurately weighed quantity of about 10 mg of API into 10 mL volumetric flask. Added 1.0 mL of 10 ppm stock solution and sonicate to dissolve. Make up the volume up to mark with diluent and mixed well.

Solution C: Preparation of API Sample with 100 % Spiked Impurities:

Transferred an accurately weighed quantity of about 10 mg of API into 10 mL volumetric flask. Added 2.0 mL of 10 ppm stock solution and sonicate to dissolve. Make up the volume up to mark with diluent and mixed well.

Solution D: Preparation of API Sample with 150 % Spiked Impurities:

Transferred an accurately weigh quantity of about 10 mg of API into 10 mL volumetric flask. Added 3.0 mL of 10 ppm stock solution and sonicate to dissolve. Make up the volume up to mark with diluent and mixed well.

Procedure:

1 µL of each standard solution of impurities in duplicate and solution A, B, C and D were injected on equilibrated chromatographic system. Area for each impurity was determined and recovery was calculated with respect to area of standard impurity solution at the same concentration level. Same experiment was performed for three sets of recovery solutions.

4.7.5 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 prescribe conditions. The precision of an analytical procedure is usually expressed as the variance, standard deviation or co-efficient of variation of a series of measurement. Here precision is determined by spiking the impurities with API at 100 % specification level.

Preparation of Blend Stock Solution of Each Impurity (10 ppm):

Transferred an accurately weighed quantity of about 10 mg of each impurities to 100 mL volumetric flasks, separately. Added about 50 mL of diluent to volumetric flask and sonicated to dissolve. Make volume up to the mark with diluent and mixed well to prepare 100 ppm solution. Took 5 mL of solution of each impurity to 50 mL volumetric flask and diluted with diluent to prepare 10 ppm blend stock solution containing each impurity.

Preparation of API Sample with 100 % Spiked Impurities:

Transferred an accurately weigh quantity of about 10 mg of API into 10 mL volumetric flask. Added 2.0 mL of 10 ppm stock solution and sonicate to dissolve. Make up the volume up to mark with diluent and mixed well.

Procedure:

Five sample sets were prepared and 1 µL was injected from each set on an equilibrated chromatographic system. Area was determined for API and each impurity. % RSD was calculated for area and retention time.

4.7.6 Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage. The following parameters were changed and analysed the test sample against standard.

(a) Flow Rate Variation:

The flow rate of the mobile phase was changed to 0.33 mL/min and 0.37 mL/min from 0.35 mL/min. Resolution was determined between the main peak and closest impurity i.e. impurity-B.

(b) Column Oven Temperature Variation:

The temperature of the column oven was changed to 28° C and 32° C from 30° C. Resolution was determined between the main peak and closest impurity i.e. impurity-B.

(c) TFA Concentration Variation:

The concentration of the triflouro acetic acid was changed to 0.04 % and 0.06 % from 0.05 %. Resolution was determined between the main peak and closest impurity i.e. impurity-B.

4.7.7 Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions such as different laboratories, different analysts, different instrument, and different column. Ruggedness is normally expressed as the lack of the influence on the test results of operational and environmental variables of the analytical method.

Ruggedness was determined by using different laboratory, different instrument, and different column on an another day.

Sample Preparation and Procedure:

Sample preparation and procedure were same as shown in the test of precision (4.7.5). % Bias was determined between original condition and changed condition.

4.7.8 Solution Stability

Stability of the sample in diluent is required for a reasonable time to generate reproducible and reliable results. Solution stability study carried out for 24 h.

Sample Preparation:

Transferred an accurately weigh quantity of about 25 mg of API into 25 mL volumetric flask. Added 10.0 mL of diluent and sonicate to dissolve. Make up the volume up to mark with diluent and mixed well.

Procedure:

Initial, after 12 h and after 24 h % purity was determined and % bias was calculated.

5. Result and Discussion

5.1 Method Development

The present study is aimed at developing a UPLC system capable of eluting and resolving azathioprine and its impurities originated from the synthesis. Structure of impurities and azathioprine are shown in Table 1 and Fig. 1 respectively in introduction section. The blend sample solution containing about 10 ppm concentration of all the four impurities and 100 ppm azathioprine in diluent were used for the method development. To detect all the compounds in a single run, selection of wavelength become challenging task since, UV profile of all the compounds may differ. The UV spectra of all the compound was taken in diluent are presented in Fig. 3, 4, 5, 6 and 7. It can be seen that it is difficult to detect all the compounds at single wavelength with equal response. Therefore, based on UV profile of all the compounds 220 nm wavelength was selected as common wavelength for the detection. Relative response factor of all the impurities was evaluated for accurate determination.

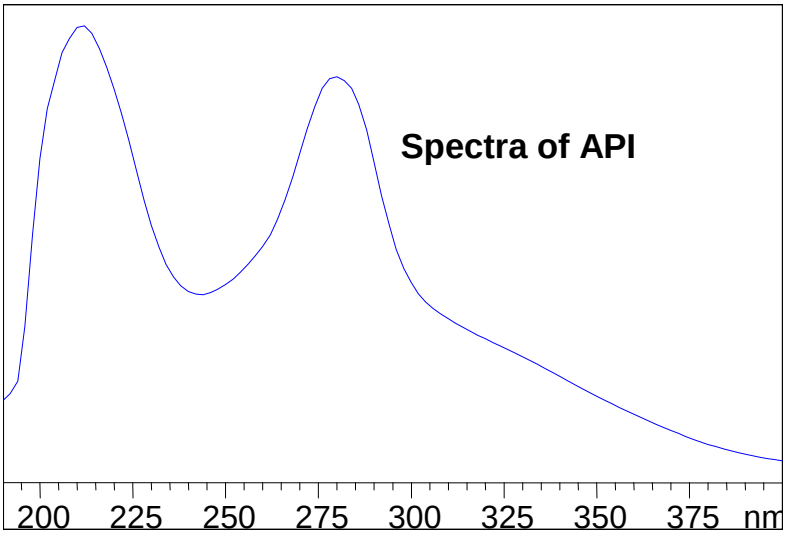


Fig. 3: UV spectra of Azathioprine

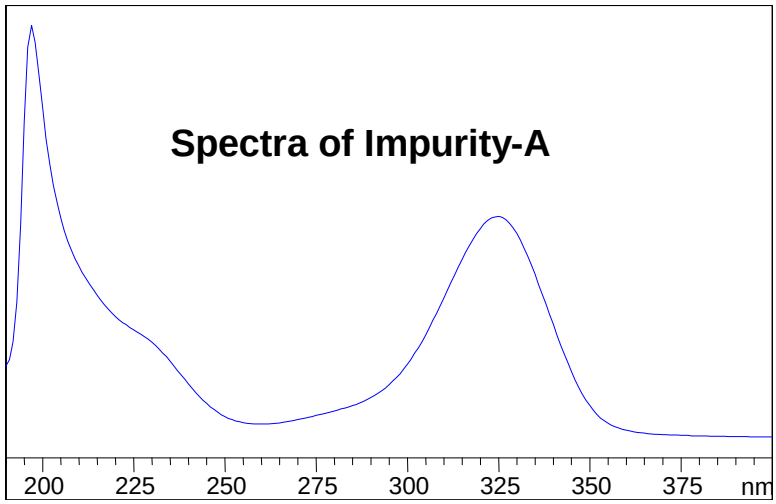


Fig. 4: UV spectra of Impurity-A

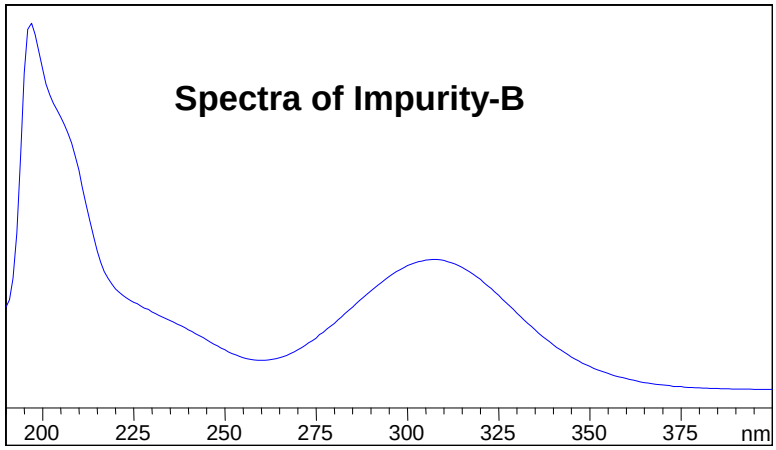


Fig. 5: UV spectra of Impurity-B

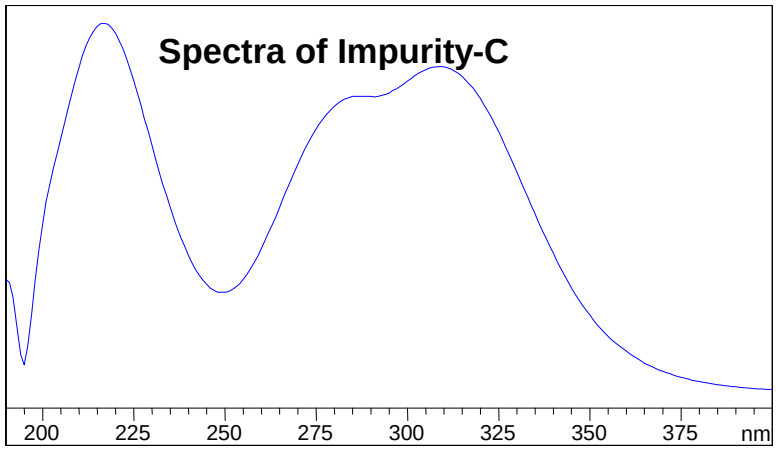


Fig. 6: UV spectra of Impurity-C

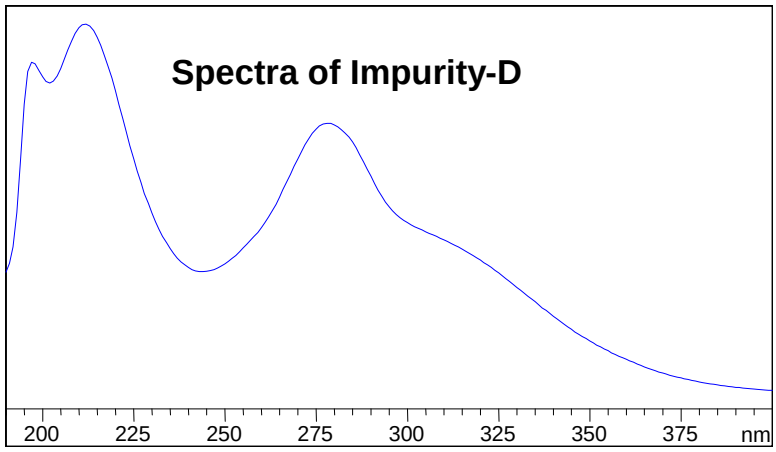


Fig. 7: UV spectra of Impurity-D

UPLC is a new technique, where sub two-micron particles are used. Because of the small particle size used in the column, chemistry of the stationary phase plays fewer roles in method development. Method development was initiated in Acquity UPLC BEH C18 (100 x 2.1 mm, 1.7 µm) column as a stationary phase. Column was kept under control temperature 30° C. Binary pump was used to flow 0.4 mL/min. TUV detector was operated at 220 nm.

As per the common development practice method development was initiated with Water: Acetonitrile as a mobile phase in isocratic mode. In due course, cross combination of the 0.05 % TFA in water and methanol was also tried as a mobile phase. 1 µL of blend sample preparation was injected in equilibrated chromatographic system using above mentioned parameters.

Trial 1: Mobile phase: Water: Acetonitrile (90:10), rest of the conditions as discussed above.

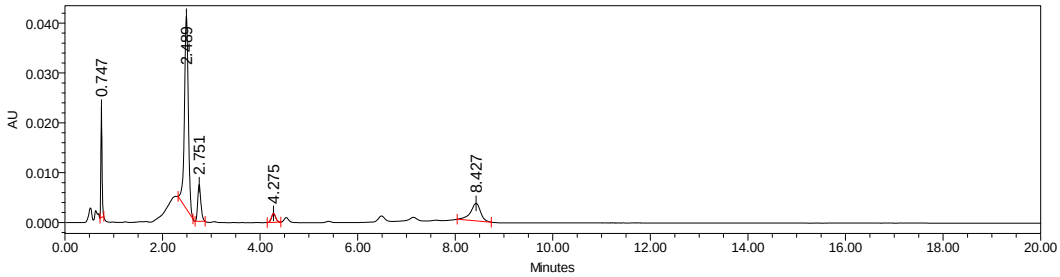


Fig. 8: UPLC chromatogram of trial 1

Trial 2: Mobile phase: Water: Methanol (90:10), rest of the conditions as discussed above.

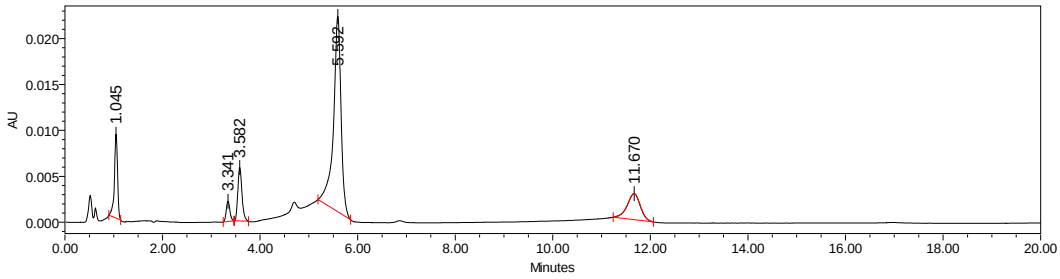


Fig. 9: UPLC chromatogram of trial 2

Observation: From the above trials 1 and 2, it was observed that impurities were not properly eluted. Last eluting peak in trial 1 (Fig. 8) was 8.4 minutes and in trial 2 (Fig. 9) was 11.6 minutes, which are too long for UPLC technique. Peak shapes were also not proper therefore, some buffer of ion pairing reagent should be tried.

Trial 3: Mobile phase: 0.05 % TFA in Water: Acetonitrile (90:10), rest of the conditions as discussed above.

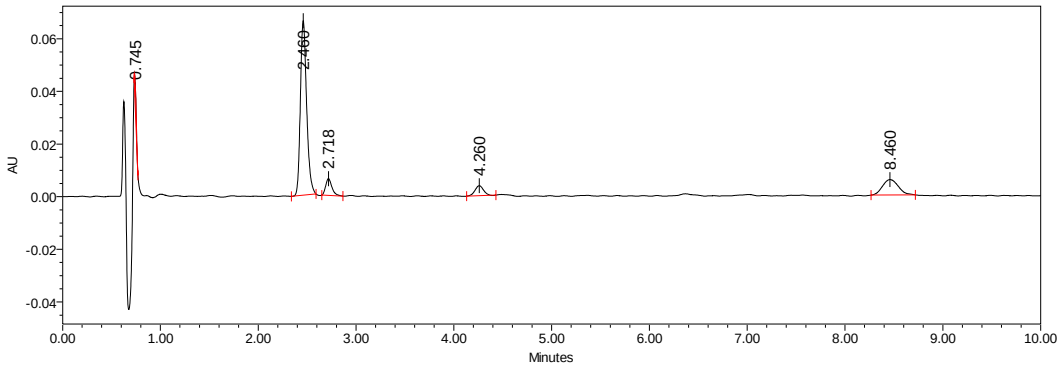


Fig. 10: UPLC chromatogram of trial 3

Trial 4: Mobile phase: 0.05 % TFA in Water: Methanol (90:10), rest of the conditions as discussed above.

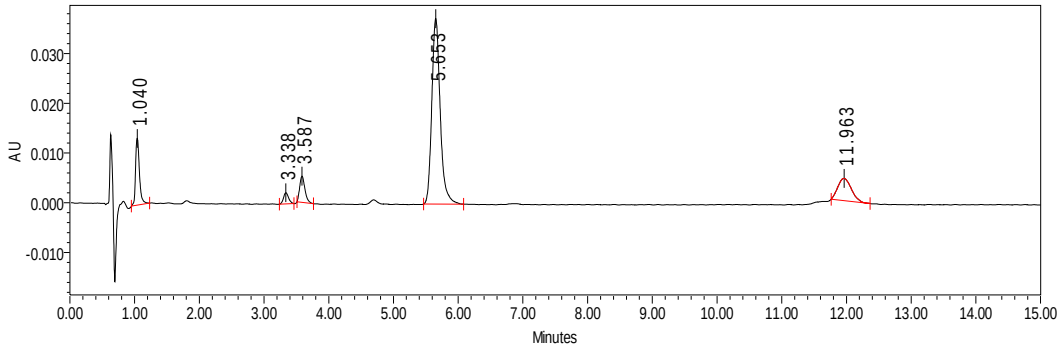


Fig. 11: UPLC chromatogram of trial 4

Observation:

From the above trials 3 (Fig. 10) and 4 (Fig. 11), it was observed that peak shapes were found to be ok. In the condition of trial 3 (Fig. 10), first eluting peak was eluted with dead volume and last eluting peak was eluted at 8.4 minutes. In the similar line, in trial 4 (Fig. 11), last eluting peak was eluted at 11.9 minutes. As, stated earlier, it is too long time for the good UPLC method. Since, all the compounds of interest have different polarity depending on its chemical structure therefore to separate and elute all the compounds within define time frame, gradient elution should be tried.

To get gradient elution pattern, small linear gradient were employed for the method development. It has been scanned for all the cross combination of water and 0.05 % TFA in water with acetonitrile and methanol to finalize mobile phase components, while column (Acquity UPLC BEH C18 (100 x 2.1 mm, 1.7 µm), flow rate (0.4ml/min), column temperature (30° C) and wavelength (220nm) were kept constant.

Gradient Program

Time (min)	0.05% TFA in water (%)	Acetonitrile (%)
1.0	95	05
2.0	10	90
3.0	10	90
3.1	95	05
4.0	95	05

Trial 5: Mobile phase: Water:Acetonitrile (Gradient)

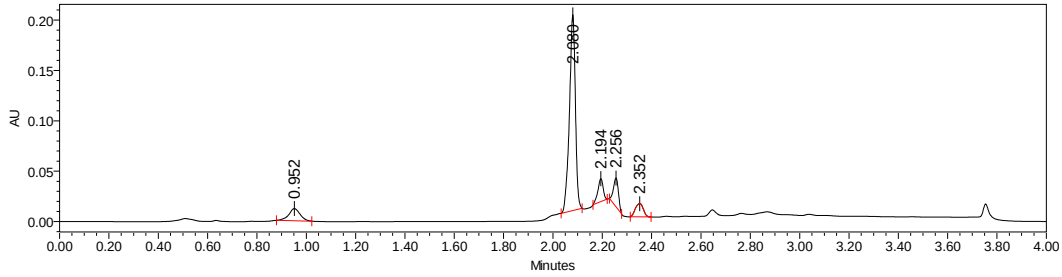


Fig. 12: UPLC chromatogram of trial 5

Trial 6: Mobile phase: Water:Methanol (Gradient)

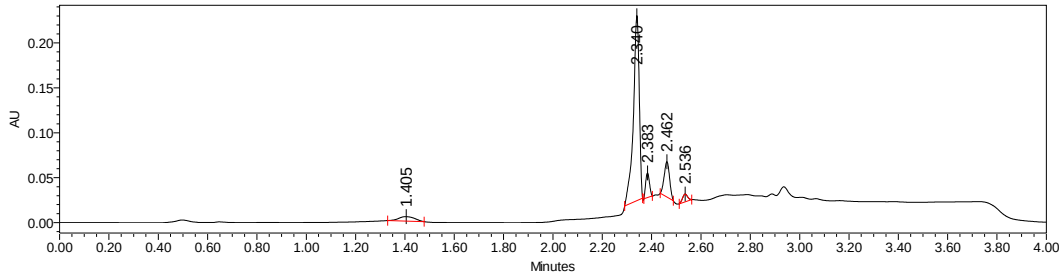


Fig. 13: UPLC chromatogram of trial 6

Trail 7: Mobile phase: 0.05 % TFA in water:Acetonitrile (Gradient)

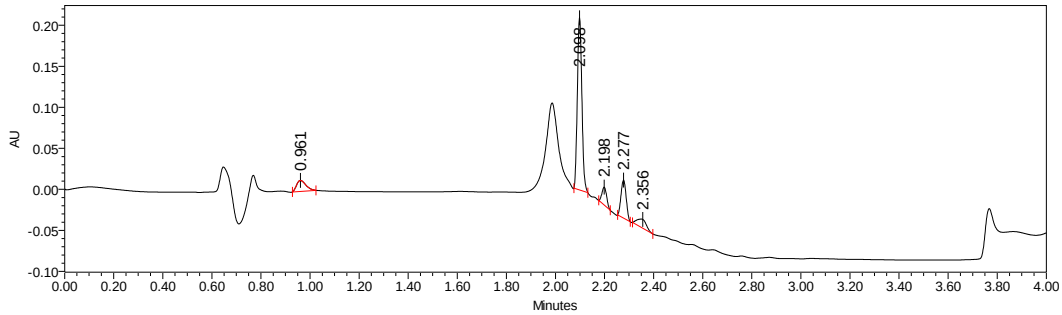


Fig. 14: UPLC chromatogram of trial 7

Trial 8: Mobile phase: 0.05 % TFA in water:Methanol (Gradient)

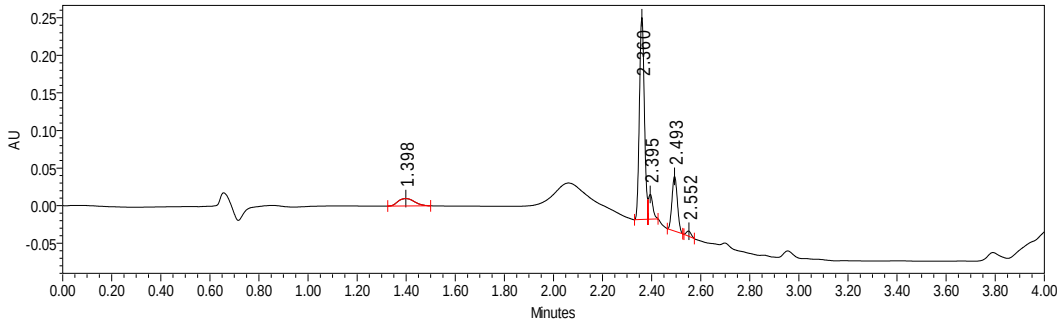


Fig. 15: UPLC chromatogram of trial 8

Observation:

From the above trials, it can be seen that gradient elution has advantage over isocratic elution. The chromatograms of screening of different mobile phase components in same gradient program are presented in Fig. 12, 13, 14 and 15. It can be observed that combination of 0.05 % TFA in water and acetonitrile (Fig. 14) gave better result than any other tested combination, which can be further improved. Therefore, now finalizing 0.05 % TFA in water and acetonitrile as a mobile phase components, gradient program should be optimized to get desire retention and resolution.

After above experimental input, further attempt was made to optimize the gradient program. Couple of slow gradient programs were tried to increase resolution between impurities maintaining peak shape and retention and also played with flow rate to achieve separation goal. Finally, flow rate 0.35 mL/min and following gradient program were found to be optimal for determination of process related impurities in azathioprine. Chromatogram of the finalize condition is presented in Fig. 16.

Gradient Program

Time (minutes)	0.05 % TFA in Water (%)	Acetonitrile (%)
1.0	97	03
3.5	40	60
4.0	40	60
4.1	97	03
5.0	97	03

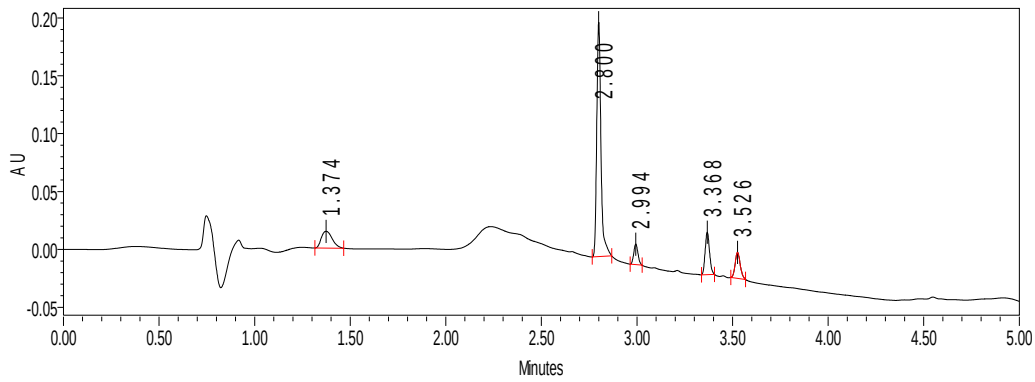


Fig. 16: Chromatogram of finalize condition

It can be seen from the Fig. 16 that all the impurities and azathioprine are well separated within 5 minutes run time. The method was found to be simple and rapid for its routine use. The developed method was subjected for validation. Just to check effect of different column chemistry, Acquity UPLC HSS T3 1.8 µm (100 x 2.1mm) column was tried in optimized chromatographic parameters. The chromatogram is presented in Fig. 17.

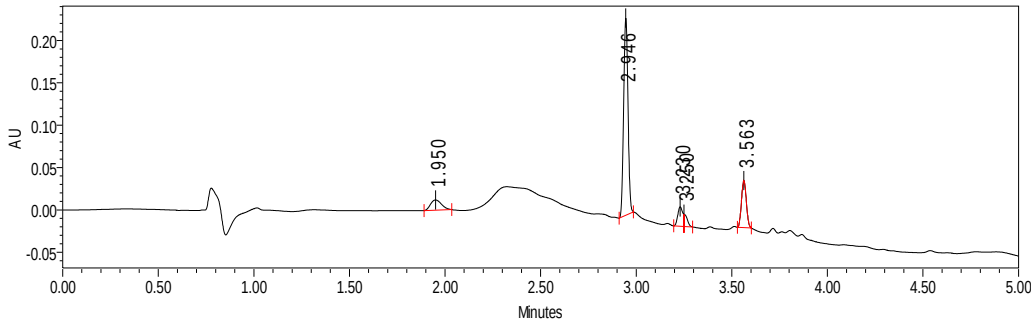


Fig. 17: Chromatogram on Acquity UPLC HSS T3 1.8 µm (100 x 2.1mm) Column

It can be seen from Fig. 17 that two impurities were merged in column Acquity UPLC HSS T3 1.8 µm (100 x 2.1mm). Therefore, earlier column, AQUITY UPLC BEH C18 (100 x 2.1 mm, 1.7 µm) was found to be right choice.

From the entire method development discussion the final optimized parameters of the UPLC method for the determination of the process related impurities in azathioprine are as below.

Elution : Gradient
Column : Acquity UPLC BEH C18, 100 x 2.1 mm, 1.7 µm
Wave length : 220 nm
Column oven temp. : 30 C
Flow rate : 0.35 mL / min.
Injection volume : 1 µL
Run time : 5 min
Mobile phase : 0.05 % TFA in water: Acetonitrile (Gradient)
Diluent : 0.1 % Ammonia in methanol
Sample conc. : 1.0 mg/mL
Gradient program :

Time (minutes)	**0.05 % TFA in Water (%)**	**Acetonitrile (%)**
1.0	97	03
3.5	40	60
4.0	40	60
4.1	97	03
5.0	97	03

5.2 Determination of the Relative Response Factor (RRF)

Since, all the impurities have different UV profile, therefore, detection of them at single wavelength will not give equal response. In order to calculate exact amount of impurity, relative response factor is required. Here, response factor of all the four impurities relative to azathioprine have been determined. Results of experiment are presented in Table 2.

Table 2: Result of Relative Response Factor Determination Study

Parameters	API	Imp-A	Imp-B	Imp-C	Imp-D
Area - 1	27432	25666	12360	20886	25578
Area - 2	25792	24852	12479	19763	24554
Area - 3	26571	25983	12584	20224	25055
Area - 4	27083	25681	12875	20292	25235
Area - 5	26942	25536	12851	19911	25015
Average Area	26764.00	25543.60	12629.80	20215	25087.40
SD	624.84	419.91	227.31	434	371.91
RSD	2.33	1.64	1.80	2.15	1.48
RRF	1	0.94	0.47	0.80	0.94
Correction Factor	1	1.06	2.12	1.25	1.06

It can be seen from the Table 2 that response of the Impurity-B is very less at 220nm as compare to other impurities, which is also supported by UV spectra of Impurity-B (Fig. 5). The response of Impurity-A and Impurity-D is almost equivalent to azathioprine. The multiplication of correction factor and percentage of impurity from the area normalize method will give exact amount of that impurity.

5.3 Result of Method Validation

5.3.1 Result of Specificity and System Suitability

The retention time of the peak of interest in the sample solution was 2.8 minutes. Impurities were well resolved from each other as well as from the main peak. No interference was observed in the chromatogram of the diluent and mobile phase at the retention time of the sample as well as impurities. Retention time and compound are described in Table 3. The retention time identification of the impurities and chromatogram of the specificity study after subtraction with diluent are presented as Fig. 18, 19, 20, 21, 22 and 23.

Table 3: Retention time for API and its Impurities

Name of the Sample	Retention time of the main peak (minutes)
Diluent	No peak
Mobile phase	No peak
Impurity-A	1.4 min
Impurity-B	3.0 min
Impurity-C	3.5 min
Impurity-D	3.4 min
Azathioprine (API)	2.8 min

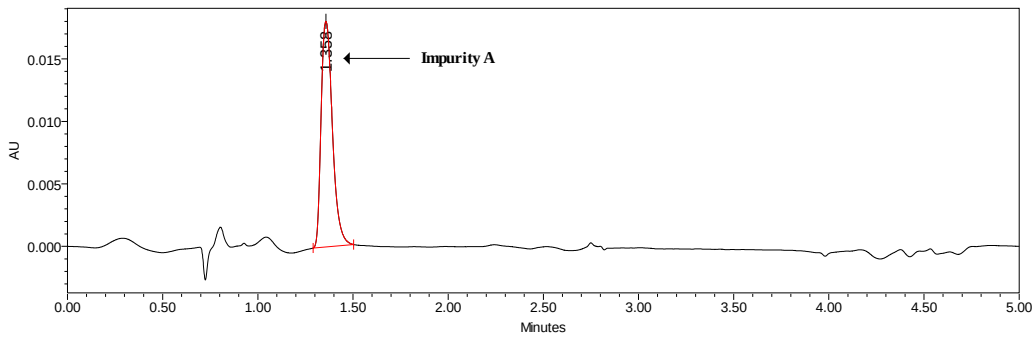


Fig. 18: Chromatogram of Impurity-A

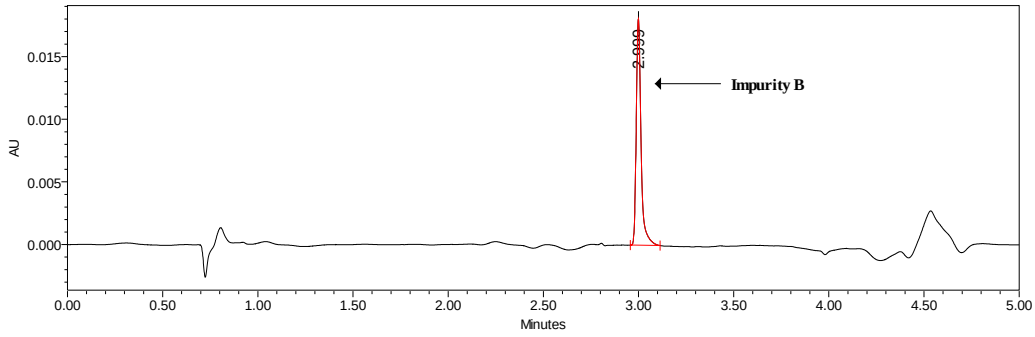


Fig. 19: Chromatogram of Impurity-B

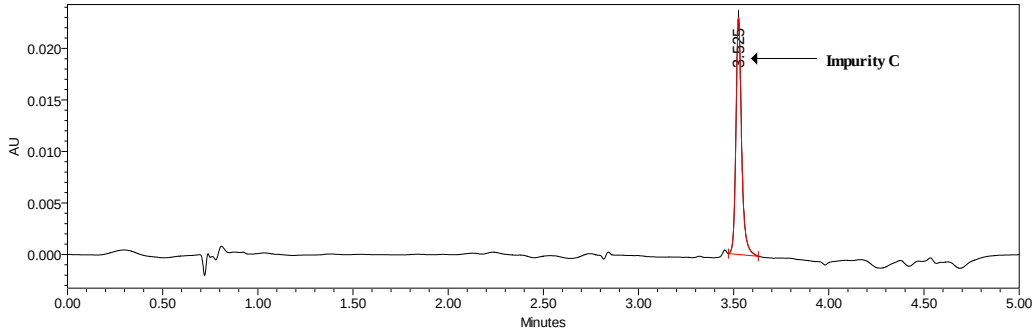


Fig. 20: Chromatogram of Impurity-C

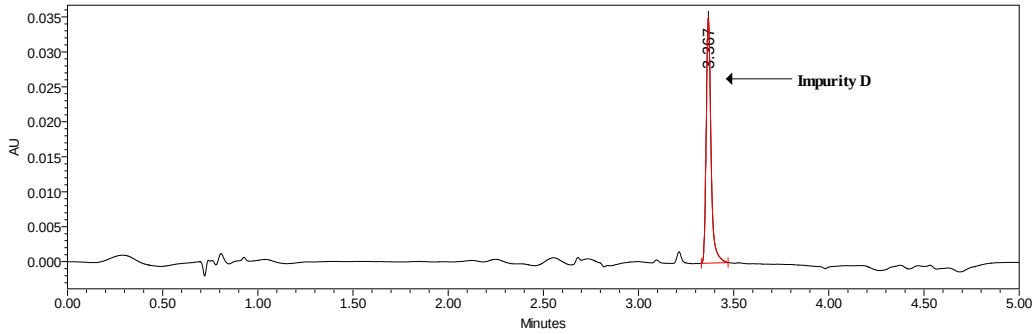


Fig. 21: Chromatogram of Impurity-D

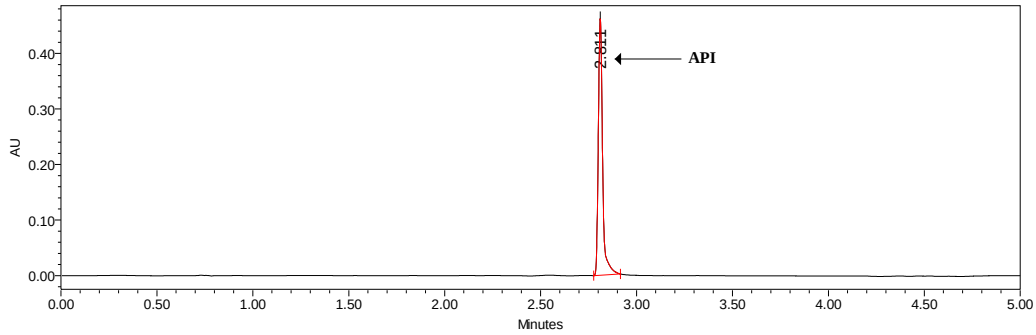


Fig. 22: Chromatogram of Azathioprine (API)

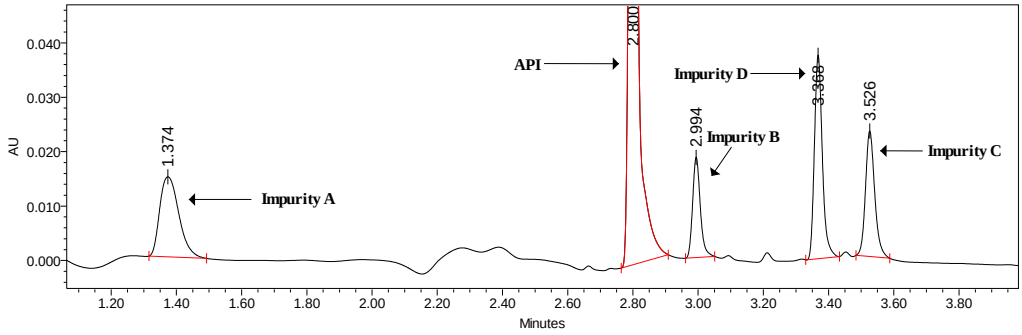


Fig. 23: Chromatogram of blend sample containing API and all the impurities

After specificity establishment, system suitability parameters were evaluated for the all compounds. The system suitability results are shown in Table 4. The system suitability chromatogram after subtraction with diluent is presented in Fig. 24 and without diluent subtraction is presented in Fig. 25.

Table 4: Results of System Suitability

Name	Retention Time	Selectivity	Resolution	USP Resolution	USP Tailing	USP Plate Count
Impurity-A	1.374				1.29	2901.07
API	2.800	2.63	19.6	20.1	1.42	84984.33
Impurity-B	2.994	1.08	4.88	4.78	1.29	83346.89
Impurity-D	3.368	1.15	8.93	8.75	1.27	100817.7
Impurity-C	3.526	1.06	3.45	3.36	1.38	80375.01

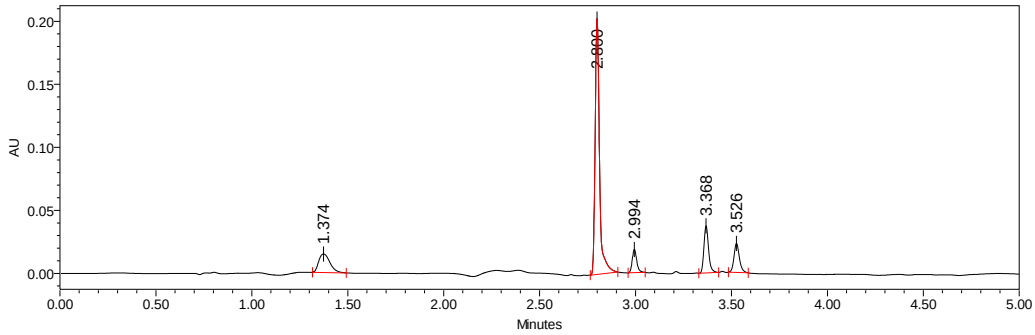


Fig. 24: Chromatogram of System suitability after subtraction with diluent

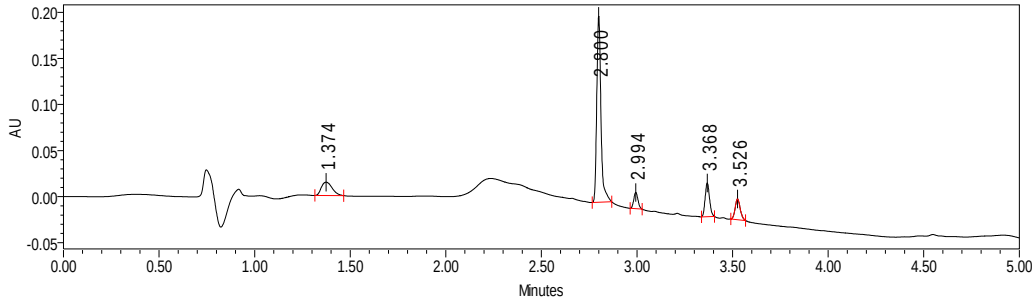


Fig. 25: Chromatogram of System suitability without subtraction of diluent

The forced degradation of the azathioprine under all the stress condition was carried out as a part of specificity study. The result and observation of the forced degradation study are discussed below.

Acid Hydrolysis:

Minor degradation was observed after acid treatment. A degradation product of 0.67 % was observed in the sample chromatogram, which was 0.03 % present initially. The relative retention time of degradant is 1.11 with respect to main peak. All degradant products were well resolved from the peak of azathioprine. The % purity of sample was found 99.33 %, which was initially 99.86 %. The Chromatogram of acid hydrolysis is presented in Fig. 26.

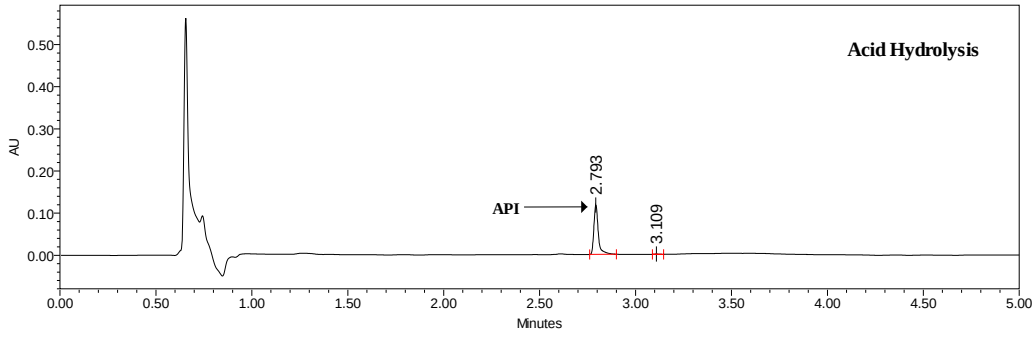


Fig. 26: Chromatogram of Acid hydrolysis

Base Hydrolysis:

Degradation was observed after base treatment. A degradation product of 2.07 % was observed in the sample chromatogram, which was 0.02 % present initially. The relative retention time of degradant is 0.50 with respect to main peak. All degradant products were well resolved from the peak of azathioprine. The % purity of sample was found 97.93 %, which was initially 99.86 %. The Chromatogram of base hydrolysis is presented in Fig. 27. It can be seen form the chromatogram that degradation product observed at 1.4 minutes. The retention time was same as Impurity-A. Therefore, Impurity-A may be base degradation product also. It is also supported by literature that 6-mercaptopurine is active metabolite of the azathioprine.

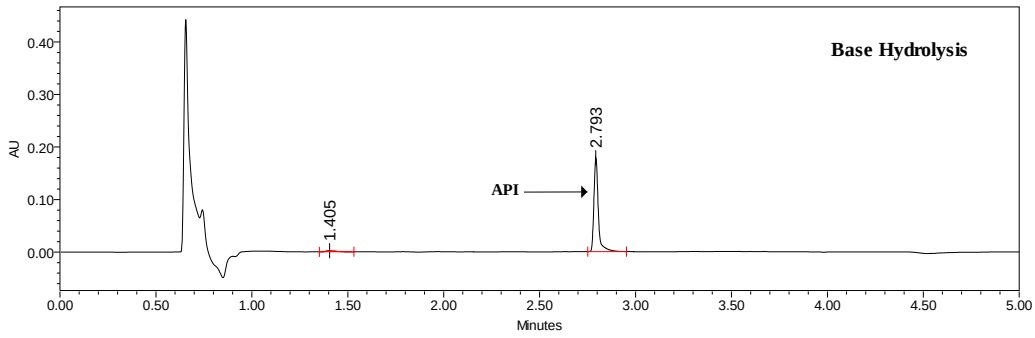


Fig. 27: Chromatogram of Acid hydrolysis

Peroxide Oxidation:

Degradation was not observed after peroxide treatment. The % purity of sample was found 99.85 %, which was initially 99.86 %. The Chromatogram of peroxide oxidation is presented in Fig. 28.

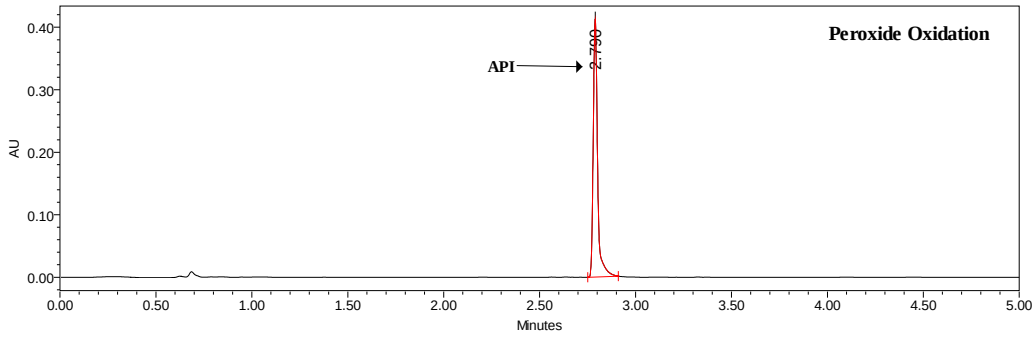


Fig. 28: Chromatogram of Acid hydrolysis

Peak Purity:

Peak purity of the principle peak was checked in impurities spiked solution and stressed solution using photo diode array detector. Peak purity was passed for all the solution at the threshold level 990. Representative peak purity report is shown in Fig. 29.

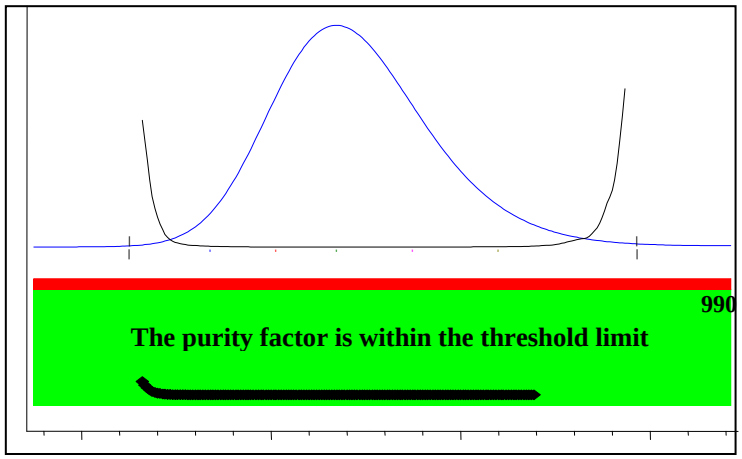


Fig. 29: Peak purity chromatogram of principle peak

Conclusion: Method is capable to resolve degradants and that conformed stability indicating power of the UPLC method.

5.3.2 Result of Linearity of Impurities

The linearity of all the impurities was evaluated from the 5 % to 150 % of the specification limit. The described method was linear in the range of 0.1 - 3 ppm for all the impurities. The calibration curve was drawn by plotting the concentration verses its corresponding peak area with a correlation coefficient greater than 0.995. The equation of the calibration curve was also calculated for all the impurities. The data of slope, Y-intercept and correlation coefficient for all the impurities are presented in Table 9.

Impurity-A:

The peak area at different concentration for Impurity-A is presented in Table 5 and linearity plot is shown in Fig. 30.

Table 5: Data of linearity of Impurity-A

Concentration		Area
Percentage of specification limit	**ppm**	
150	3	19992
100	2	13970
50	1	7335
25	0.5	3557
10	0.2	1354
5	0.1	941

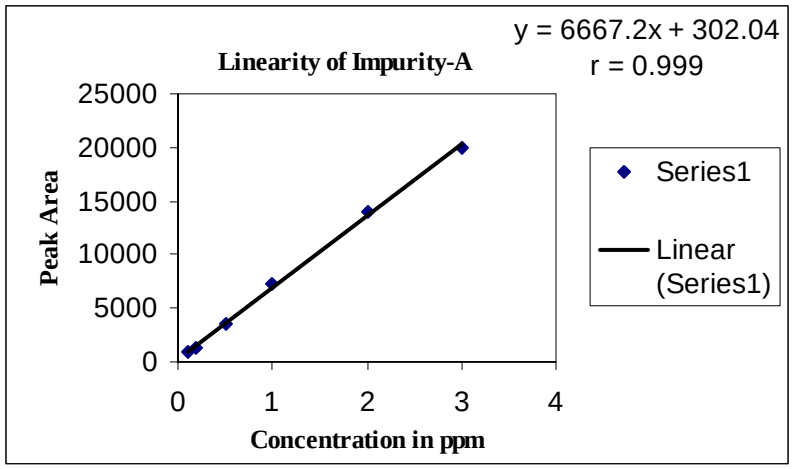


Fig. 30: Linearity plot of Impurity-A

Acceptance Criteria: Correlation coefficient should not be less than 0.995.

Conclusion:

The correlation coefficient of Impurity-A was 0.999. The result showed the excellent correlation between the peak area and concentration for Impurity-A in developed method.

Impurity B:

The peak area at different concentration for Impurity-B is presented in Table 6 and linearity plot is shown in Fig. 31.

Table 6: Data of linearity of Impurity-B

Concentration		Area
Percentage of specification limit	**ppm**	
150	3	9784
100	2	5963
50	1	3020
25	0.5	1032
10	0.2	380
5	0.1	139

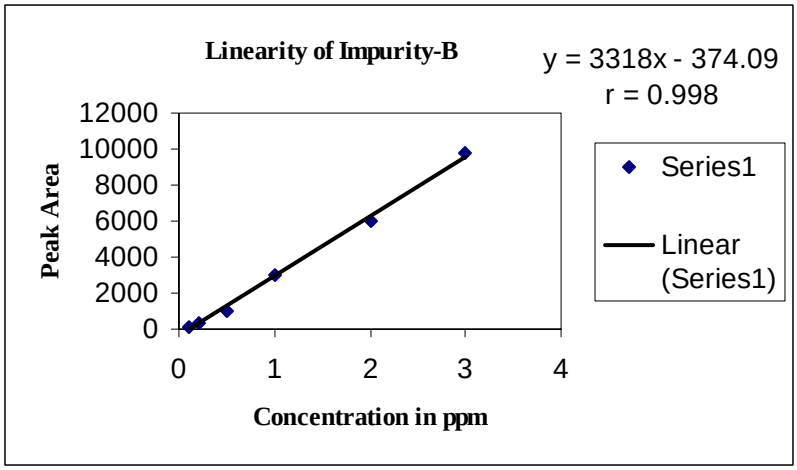


Fig. 31: Linearity plot of Impurity-B

Acceptance Criteria: Correlation coefficient should not be less than 0.995.

Conclusion:

The correlation coefficient of Impurity-B was 0.998. The result showed the excellent correlation between the peak area and concentration for Impurity-B in developed method.

Impurity-C:

The peak area at different concentration for Impurity-C is presented in Table 7 and linearity plot is shown in Fig. 32.

Table 7: Data of linearity of Impurity-C

Concentration		Area
Percentage of specification limit	**ppm**	
150	3	14198
100	2	9365
50	1	5065
25	0.5	2004
10	0.2	706
5	0.1	333

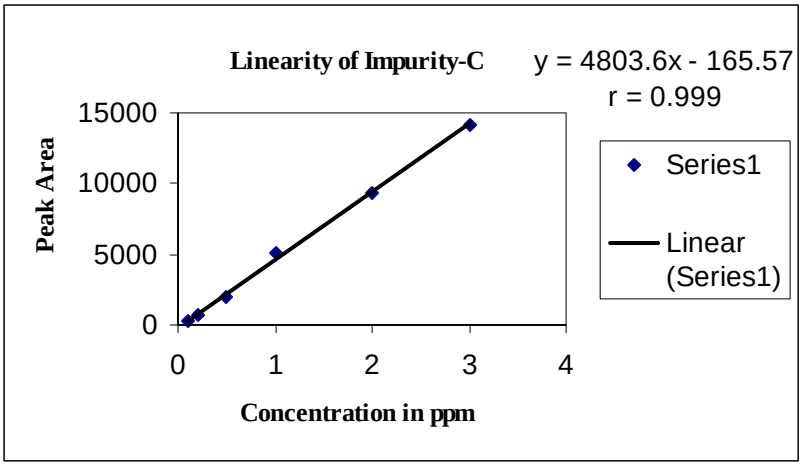


Fig. 32: Linearity plot of Impurity-C

Acceptance Criteria: Correlation coefficient should not be less than 0.995.

Conclusion:

The correlation coefficient of Impurity-C was 0.999. The result showed the excellent correlation between the peak area and concentration for Impurity-C in developed method.

Impurity-D:

The peak area at different concentration for Impurity-D is presented in Table 8 and linearity plot is shown in Fig. 33.

Table 8: Data of linearity of Impurity-D

Concentration		Area
Percentage of specification limit	ppm	
150	3	17324
100	2	11435
50	1	6192
25	0.5	2479
10	0.2	1020
5	0.1	580

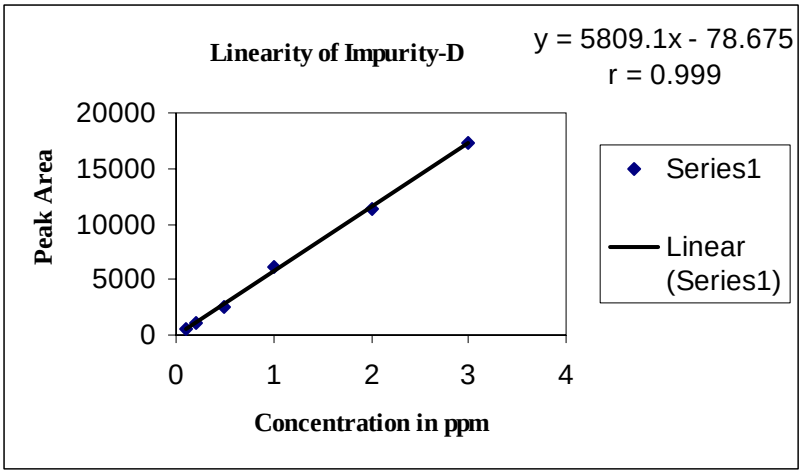


Fig. 33: Linearity plot of Impurity-D

Acceptance Criteria: Correlation coefficient should not be less than 0.995.

Conclusion:

The correlation coefficient of Impurity-D was 0.999. The result showed the excellent correlation between the peak area and concentration for Impurity-D in developed method.

Table 9: Results of Linearity Study

Parameter	Result			
	Impurity A	**Impurity B**	**Impurity C**	**Impurity D**
Correlation coefficient	0.999	0.998	0.999	0.999
Slope	6667.2	3318	4803.6	5809.1
Y-Intercept	302.04	- 374.09	- 165.57	- 78.68

5.3.3 Result of Limit of Detection and Quantitation

The six point linearity plot with range from 0.1 to 3.0 ppm concentration , obtained in linearity study *(Section 5.3.2)* for all the impurities were used to determine limit of detection and limit of quantitation by calibration curve method. The result obtained in limit of detection and quantitation are presented in Table 10.

Table 10: Result for LOD and LOQ test

Parameters	Impurity A	Impurity B	Impurity C	Impurity D
Residual Standard Deviation	327.24	245.87	251.30	297.88
Slope	6667.2	3318	4803.6	5809.1
LOD	0.16	0.24	0.17	0.17
LOQ	0.49	0.74	0.52	0.51

It can be seen from the Table 10 that limit of detection and quantitation of Impurity-A, Impurity-C and Impurity-D are almost same. The value of limit of detection and quantitation of Impurity-B is higher compare to other impurities, which is also supported by RRF data. It can concluded that limit of quatitation of all the impurities are far below than the specification limit.

5.3.4 Result of Accuracy and Recovery

In order to determine the accuracy of the method standard addition and recovery experiments were conducted in triplicate at 50, 100 and 150 % level of each impurity with respect to specification limit, i.e. 1, 2 and 3 ppm of each impurity. The recovery of the each impurity spiked with API was calculated from the area of impurity standard solution at that particular level of concentration of accuracy study. The PI batch used for the study contains small amount of Impurity-A and Impurity-D, therefore for the correction of these impurities, API sample was injected at same concentration. The area obtained in the API for these impurities are shown in Table 11.

Table 11: Area of impurities in API sample solution

Impurity	Impurity A	Impurity B	Impurity C	Impurity D
Area	1914	ND	ND	1604

Impurity A:

The area obtained from the standard solution of the impurity-A at 50, 100 and 150 % level, i.e. 1, 2 and 3 ppm are presented in Table 12.

Table 12: Area of standard sample solution of Impurity-A

Injection No.	Standard at 50 % level	Standard at 100 % level	Standard at 150 % level
1	4470	10440	16730
2	4465	10528	16796
3	4459	10495	16768
Average area	4464.67	10487.67	16764.67

The area obtained for Impurity-A in the spiked recovery solution was corrected by area present in the API sample solution. The recovery of Impurity-A at all the tested level was calculated and mean of recovery of three different sets was also determined. The data are presented in Table 13.

Table 13: Results of accuracy and recovery of Impurity-A

Test	Accuracy at 50 % level			Accuracy at 100 % level			Accuracy at 150 % level		
	Set1	**Set 2**	**Set3**	**Set1**	**Set 2**	**Set3**	**Set1**	**Set 2**	**Set3**
Area	6352	6398	6379	12401	12360	12398	18689	18726	18769
Corrected area	4438	4484	4465	10487	10446	10484	16775	16812	16855
% Recovery	99.40	100.43	100.01	99.99	99.60	99.97	100.06	100.28	100.54
Mean	99.95 %			99.85 %			100.29 %		

Conclusion:

It can be seen from the data that recovery of impurity-A is between 99 to 101 % at all the tested level. The recovery experiment demonstrated excellent accuracy of the developed method for the determination of Impurity-A in azathioprine bulk drug.

Impurity B:

The area obtained from the standard solution of the impurity-B at 50, 100 and 150 % level, i.e. 1, 2 and 3 ppm are presented in Table 14.

Table 14: Area of standard sample solution of Impurity-B

Injection No.	Standard at 50 % level	Standard at 100 % level	Standard at 150 % level
1	2042	4126	6205
2	2050	4165	6183
3	2058	4136	6195
Average area	2050	4142.33	6194.33

The recovery of Impurity-B at all the tested level was calculated and mean of recovery of three different sets was also determined. The results and data are presented in Table 15.

Table 15: Results of accuracy and recovery of Impurity-B

Test	Accuracy at 50 % level			Accuracy at 100 % level			Accuracy at 150 % level		
	Set1	**Set 2**	**Set3**	**Set1**	**Set 2**	**Set3**	**Set1**	**Set 2**	**Set3**
Area	1998	2046	2010	4075	4085	4123	6215	6096	6108
% Recovery	97.46	98.80	98.05	98.37	98.62	99.53	100.33	98.41	98.61
Mean	98.44 %			98.84 %			99.12 %		

Conclusion:

It can be seen from the data that recovery of impurity-B is between 98 to 100 % at all the tested level. The recovery experiment demonstrated excellent accuracy of the developed method for the determination of Impurity-B in azathioprine bulk drug.

Impurity C:

The area obtained from the standard solution of the impurity-C at 50, 100 and 150 % level, i.e. 1, 2 and 3 ppm are presented in Table 16.

Table 16: Area of standard sample solution of Impurity-C

Injection No.	Standard at 50 % level	Standard at 100 % level	Standard at 150 % level
1	2382	4856	7035
2	2372	4869	6991
3	2361	4859	7015
Average area	2371.67	4861.33	7013.67

The recovery of Impurity-C at all the tested level was calculated and mean of recovery of three different sets was also determined. The results and data are presented in Table 17.

Table 17: Results of accuracy and recovery of Impurity-C

Test	Accuracy at 50 % level			Accuracy at 100 % level			Accuracy at 150 % level		
	Set1	Set 2	Set3	Set1	Set 2	Set3	Set1	Set 2	Set3
Area	2403	2376	2397	4866	4823	4896	6985	6934	7023
% Recovery	101.32	100.18	101.07	100.10	99.21	100.71	99.59	98.86	100.13
Mean	100.19 %			100.01 %			99.53 %		

Conclusion:

It can be seen from the data that recovery of impurity-C is between 99 to 101 % at all the tested level. The recovery experiment demonstrated excellent accuracy of the developed method for the determination of Impurity-C in azathioprine bulk drug.

Impurity D:

The area obtained from the standard solution of the impurity-D at 50, 100 and 150 % level, i.e. 1, 2 and 3 ppm are presented in Table 18.

Table 18: Area of standard sample solution of Impurity-D

Injection No.	Standard at 50 % level	Standard at 100 % level	Standard at 150 % level
1	4706	11010	17593
2	4715	10994	17629
3	4728	10999	17609
Average area	4716.33	11001	17610.33

The area obtained for Impurity-D in the spiked recovery solution was corrected by area present in the API sample solution. The recovery of Impurity-D at all the tested level was calculated and mean of recovery of three different sets was also determined The data are presented in Table 19.

Table 19: Results of accuracy and recovery of Impurity-D

Test	Accuracy at 50 % level			Accuracy at 100 % level			Accuracy at 150 % level		
	Set1	Set 2	Set3	Set1	Set 2	Set3	Set1	Set 2	Set3
Area	6386	6350	6302	12563	12605	12598	19205	19298	19268
Corrected area	4782	4746	4698	10959	11001	10994	17601	17694	17664
% Recovery	101.39	100.63	99.61	99.62	100.0	99.94	99.95	100.48	100.30
Mean	100.21 %			99.85 %			100.24 %		

Conclusion:

It can be seen from the data that recovery of impurity-D is between 99 to 101 % at all the tested level. The recovery experiment demonstrated excellent accuracy of the developed method for the determination of Impurity-D in azathioprine bulk drug.

5.3.5 Result of Precision

The precision was determined for all the impurities at 100 % specification level spiked in API sample. The relative standard deviation of retention time and area was calculated for five replicates injection. The result of precision of retention time and area is presented in Table 20 and Table 21, respectively.

Table 20: Results of Precision of Retention Time

Injection No.	Impurity-A	Impurity-B	Impurity-C	Impurity-D	API
1	1.362	3.012	3.528	3.376	2.813
2	1.364	3.013	3.529	3.377	2.815
3	1.361	3.012	3.528	3.376	2.813
4	1.361	3.012	3.528	3.376	2.813
5	1.359	3.011	3.527	3.375	2.812
Average	1.36	3.01	3.53	3.38	2.81
SD	0.001	0.001	0.001	0.001	0.001
% RSD	0.13	0.02	0.02	0.02	0.04

Table 21: Results of Precision of Peak Area

Injection No.	Impurity-A	Impurity-B	Impurity-C	Impurity-D	API
1	11908	3893	4963	11532	5695412
2	12162	4015	5026	11922	5784108
3	11955	3977	4958	11567	5685863
4	11999	3987	4913	11969	5799792
5	11899	3956	4911	11875	5756444
Average	11984.6	3965.6	4954	11773.0	5744323.8
SD	106.95	45.80	47	207.09	51518.04
% RSD	0.89	0.02	0.95	1.76	0.90

Conclusion:

It can be seen from the data that relative standard deviation of retention time and peak area is less than 0.2 % and 2.0 %, respectively for all the impurities and API. The result of precision are within general acceptance limit, therefore developed method is precise for its intended use.

5.3.6 Result of Robustness

The chromatographic resolution between API and closest eluting impurity, i. e. Impurity-B was used to evaluate the method robustness under modified conditions. The results robustness testing are summarized in Table 22.

Table 22: Results of Robustness Study

Parameter	Resolution between API and Impurity-B
Flow rate (mL/min)	
0.33	5.08
0.35	4.85
0.37	4.65
Column Temperature (° C)	
28	4.94
30	4.85
32	4.76
Concentration of Triflouro acetic acid (%)	
0.04	4.83
0.05	4.85
0.06	4.84

Conclusion:

The resolution between API and closest eluting impurity, i.e. Impurity-B was found 4.85 in original condition. There was no major effect observed on resolution, under all the deliberately changed conditions. Therefore, it can be concluded that the developed method is robust for its intended use.

5.3.7 Result of Ruggedness

In order to evaluate reproducibility and ruggedness of the method, precision test was repeated using different instrument and column in second laboratory at different day. The experiment was carried out for all the impurities at 100 % specification level spiked in API sample. The relative standard deviation of retention time and area was calculated for five replicates injection. The result of precision of retention time and area is presented in Table 23 and Table 24, respectively. The ruggedness of the method was established by calculating percentage bias of retention time and area at two different conditions. The result of ruggedness is shown in Table. 25.

Table 23: Results of Precision of Retention Time at Different Set of Conditions

Injection No.	Impurity-A	Impurity-B	Impurity-C	Impurity-D	API
1	1.361	3.011	3.529	3.386	2.814
2	1.363	3.021	3.527	3.384	2.818
3	1.362	3.018	3.529	3.389	2.813
4	1.363	3.013	3.528	3.392	2.815
5	1.36	3.024	3.527	3.387	2.82
Average	1.36	3.02	3.53	3.39	2.82
SD	0.001	0.01	0.001	0.001	0.001
% RSD	0.10	0.18	0.03	0.09	0.10

Table 24: Results of Precision of Peak Area at Different Set of Conditions

Injection No.	Impurity-A	Impurity-B	Impurity-C	Impurity-D	API
1	11970	3991	4955	11666	5701234
2	12110	3952	4965	11824	5699524
3	11980	3967	4977	11867	5723546
4	12110	4012	4945	11905	5699584
5	11954	3911	4966	11766	5712683
Average	12024.8	3966.6	4962	11805.6	5707314.2
SD	78.33	38.60	12	93.60	10603.15
% RSD	0.65	0.97	0.24	0.79	0.19

Table 25: Results of Ruggedness study

Compd	Average Retention Time			Average Peak Area		
	Original Condition	Different Condition	% Bias	Original Condition	Different Condition	% Bias
Impurity A	1.36	1.36	0.0	11984.6	12024.8	0.34
Impurity B	3.01	3.02	0.33	3965.6	3966.6	0.03
Impurity C	3.53	3.53	0.0	4954	4962	0.16
Impurity D	3.38	3.39	0.30	11773.0	11805.6	0.28
API	2.81	2.82	0.36	5744323.8	5707314.2	-0.64

Conclusion:

The bias between original and different conditions was found below 2.00 % for retention time and peak area of all the impurities and API. Therefore, it can be concluded that developed method is rugged enough for its intended use.

5.3.8 Result of Solution Stability

Solution stability of azathioprine (API) was carried out in diluent for 24 hours. The % bias of purity was calculated with the purity of fresh injection. The result of the solution stability is presented in Table 26.

Table 26: Result of Solution Stability

Sr. No.	Time (hrs)	% Purity	% Bias
1	0	99.86	-
2	12	99.87	0.01
3	24	99.85	-0.01

Conclusion:

The bias of % purity of Azathioprine after 12 and 24 hours was found 0.01 %. The data it can be said that the sample solution is stable at room temperature for atleast 24 hours. Therefore, it can be concluded that sample solution can be use within 24 hours from the time of preparation.

6. Conclusion

A simple, suitable, precise and accurate UPLC method has been developed and validated for the determination of process related impurities in azathioprine bulk drug. The systematic method development was approached in the selection of wavelength, mode of elution and mobile phase components. The forced degradation was also performed to demonstrate specificity of the method.

The developed method was completely validated for all the four impurities with respect to specificity, system suitability, linearity, limit of detection and quantitation, accuracy, precision, robustness, ruggedness and solution stability. The result of validation showed satisfactory data for all the method validation parameters tested.

The developed method can be used for the determination of process related impurities (Impurity-A, Impurity-B, Impurity-C and Impurity-D) and azathioprine in the bulk drug substance.

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Part-B

Application of the Chromatography for the Impurity Profiling of Active Pharmaceutical Ingredient

Section-II

Isolation and Characterization of Degradation Products of Pioglitazone Hydrochloride

1. Introduction

Pioglitazone hydrochloride (Actos) was previously under joint development by Takeda Chemical Industries (Osaka, Japan) and Upjohn (Kalamazoo, MI) as an oral treatment for non-insulin dependent diabetes mellitus (NIDDM), i.e. type-2 diabetes and was undergoing clinical testing for safety and efficacy. It has been shown to affect abnormal glucose and lipid metabolism associated with insulin resistance by enhancing insulin action on peripheral tissues in animal models [1-3].

Type 2 diabetes (formerly called non-insulin-dependent diabetes mellitus (NIDDM), or adult-onset diabetes) is a disorder that is characterized by high blood glucose in the context of insulin resistance and relative insulin deficiency [4]. While it is often initially managed by increasing exercise and dietary modification, medications are typically needed as the disease progresses. There are an estimated 23.6 million people in the U.S. (7.8% of the population) with diabetes with 17.9 million being diagnosed [5], 90% of whom are type 2 [6]. With prevalence rates doubling between 1990 and 2005, CDC has characterized the increase as an epidemic [7].

Traditionally considered a disease of adults, type 2 diabetes is increasingly diagnosed in children in parallel to rising obesity rates [8] due to alterations in dietary patterns as well as in life styles during childhood [9].

According to CDC, about 23.613 million people in the United States, or 8% of the population, have diabetes. The total prevalence of diabetes increased to 13.5% from 2005-2007. It is thought that only 24% of diabetes is now undiagnosed, down from an estimated 30% in 2005 and from the previously estimated 50% in ca 1995.

About 90–95% of all North American cases of diabetes are type 2 [10], and about 20% of the population over the age of 65 has diabetes mellitus type 2. The fraction of type 2 diabetics in other parts of the world varies substantially, almost certainly for environmental and lifestyle reasons, though these are not known in detail. Diabetes affects over 150 million people worldwide and this number is expected to double by 2025 [10]. In india also about 20% population are suffering from diabetes and it is fast grawing. In coming future india may be known as Diabetes capital of the world, looking total popultation. There is also a strong inheritable genetic connection in type 2 diabetes: having relatives (especially first degree) with

type 2 increases risks of developing type 2 diabetes very substantially. In addition, there is also a mutation to the Islet Amyloid Polypeptide gene that results in an earlier onset, more severe, form of diabetes [11, 12]. About 55 percent of type 2 are obese [13]-chronic obesity leads to increased insulin resistance that can develop into diabetes, most likely because adipose tissue (especially that in the abdomen around internal organs) is a (recently identified) source of several chemical signals to other tissues (hormones and cytokines). Other research shows that type 2 diabetes causes obesity as an effect of the changes in metabolism and other deranged cell behavior attendant on insulin resistance [14] However, genetics play a relatively small role in the widespread occurrence of type 2 diabetes. This can be logically deduced from the huge increase in the occurrence of type 2 diabetes which has correlated with the significant change in western lifestyle [15].

Drug Profile

1.1.1 Description

Pioglitazone hydrochloride is chemically ( )-5-{[[4-2-(5-ethyl-2-pyridinyl)ethoxy]phenyl]methyl}-2,4-thiaz-olidinedione monohydrochloride (Fig. 1), Its molecular formula is $C_9H_6N_7O_2S \cdot HCl$ and molecular weight is 406.93 g/mole.

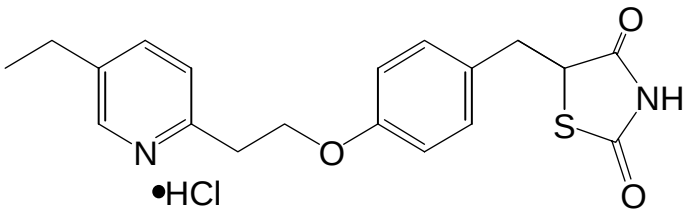


Fig. 1: Chemical Structure of Pioglitazone Hydrochloride

1.1.2 Mechanism of Action

Pioglitazone is a drug that reduces the amount of glucose (sugar) in the blood. It is in a class of anti-diabetic drugs called "thiazolidinediones" that are used in the treatment of type II diabetes. The other member in this class is rosiglitazone (Avandia). (Another member of this class, troglitazone or Rezulin, was removed from the market because of liver toxicity.) Patients with type II diabetes cannot make enough insulin, and the cells of their body do not respond normally to the reduced

amounts of insulin that are present. (Insulin is the hormone produced by the pancreas that stimulates cells to remove glucose from the blood.) Pioglitazone often is referred to as an "insulin sensitizer" because it attaches to the insulin receptors on cells throughout the body and causes the cells to become more sensitive (more responsive) to insulin. As a result, more glucose is removed from the blood. At least some insulin must be produced by the pancreas in order for pioglitazone to work [16].

Pioglitazone also lowers the level of glucose in the blood by reducing the production and secretion of glucose into the blood by the liver. In addition, pioglitazone may alter the blood concentrations of lipids (fats) in the blood. Specifically, it decreases triglycerides and increases the "good" (HDL) cholesterol.

1.1.3 Clinical Use

Pioglitazone is an anti-diabetic drug (thiazolidinedione-type, also called "glitazones") used along with a proper diet and exercise program to control high blood sugar in patients with type 2 diabetes (non-insulin-dependent diabetes). It works by helping restore body's proper response to insulin, thereby lowering blood sugar. Effectively controlling high blood sugar helps prevent heart disease, strokes, kidney disease, blindness, and circulation problems, as well as sexual function problems (impotence). Since it requires naturally-secreted insulin to be effective, pioglitazone is not recommended in type I diabetes where the amount of insulin is very low or absent. Pioglitazone is used either alone or in combination with other anti-diabetic medications (e.g., metformin, sulfonylureas, insulin) [17].

1.1.4 Pharmacodynamics/Kinetics [18]

Onset of action: Delayed

Peak effect: Glucose control: Several weeks

Distribution: Vss (apparent): 0.63 L/kg

Protein binding: 99.8%

Metabolism: Hepatic (99%) via CYP2C8/9 and 3A4 to both active and inactive metabolites

Half-life elimination: Parent drug: 3-7 hours; Total: 16-24 hours

Time to peak: ~2 hours

Excretion: Urine (15% to 30%) and feces as metabolites

1.1.5 Dosage

Oral dose for Adults:

Monotherapy: Initial: 15-30 mg once daily; if response is inadequate, the dosage may be increased in increments up to 45 mg once daily; maximum recommended dose: 45 mg once daily

1.1.6 Side Effects

Some common side effects associated with pioglitazone use are listed below:

Cough or cold

Headache

Inflammation of the sinuses or throat

Muscle pain

Swelling or fluid retention

Weight gain

Decreased triglycerides and increased HDL (good cholesterol)

1.1.7 Drug Interaction

Pioglitazone may reduce the effectiveness of oral contraceptives. In some cases, other forms of birth control are needed.

High blood sugar is more likely to occur if pioglitazone is taken with drugs that elevate blood sugar, such as the following:

Isoniazid

Diuretics

Steroids

Phenothiazines (Compazine and others)

Thyroid medicine

Oral contraceptives and other hormones

Seizure medicines

Diet pills or medicines to treat asthma, colds or allergies.

Low blood sugar is more likely to occur if pioglitazone is given with drugs that lower blood sugar, such as the following:

Nonsteroidal anti-inflammatory drugs (NSAIDs)

Aspirin or other salicylates (including Pepto-Bismol)

Sulfa drugs

Monoamine oxidase inhibitors (MAOI)

Beta-blockers

Probenecid

The following drugs can interact with pioglitazone:

Midazolam (Versed)

Gemfibrozil (Lopid)

Rifampin (Rifadin)

Furosemide (Lasix)

Nifedipine (Adalat, Procardia)

1.1.8 Research

Recent research findings have shed light on some of pioglitazone's long-term effects. In particular, pioglitazone therapy is associated with a decrease in adverse cardiac outcomes, including recurrent heart attack, in high-risk subjects [19]. A separate review of several different clinical trials concluded that pioglitazone is associated with a significantly lower risk of death, heart attack, or stroke among a diverse population of patients with diabetes [20].

1.1.9 International Brand Names

Actos® (AR, AT, BE, BR, CA, CH, CO, CR, CZ, DE, DK, ES, FI, FR, GB, GT, HN, IT, JP, NO, NZ, PA, RO, SE, SI, SV, TH); Cereluc® (AR); Glizone® (IN); G-Tase® (IN); Opam® (IN)

1.2 Forced Degradation

Forced degradation is the process of subjecting drug compounds to extreme chemical and environmental conditions to determine product breakdown levels and preliminary degradation kinetics, and to identify degradant species. It is particularly useful when little information is available about potential degradation products.

These studies also provide information about the degradation pathways and degradation products that could be formed during storage. The results of degradation studies facilitate stability indicating method (SIM) development, the design of formulations, the choice of storage conditions and packaging, an understanding of the

chemistry of the drug molecule, and stability problem solving, in which knowledge of chemical behaviour can be used to improve a drug product.

Forced degradation studies are designed to generate product-related variants and develop analytical methods to determine the degradation products formed during accelerated pharmaceutical studies and long-term stability studies. Any significant degradation product should be evaluated for potential hazard and therefore there is a need for characterization and quantitation.

2. Literature Review

A thorough literature review, it has been observed that few articles were published on analytical method of pioglitazone hydrochloride and its metabolites for its biological studies [21-29]. Few papers are also published on the analytical methods for the bulk drug and formulation of pioglitazone hydrochloride and its combination with Metformine and Glimepiride [30-34]. A stability indicating RP-HPLC methods were also reported for pioglitazone hydrochloride bulk drug and tablets [35, 36]. T. Radhakrishna et al. reported HPLC and MECK methods for the determination of the pioglitazone hydrochloride in bulk and pharmaceutical formulation [37]. The structural characterization of the impurities in pioglitazone was also published by Y. R. Kumar et. al. [38].

The literature survey revealed that there are articles regarding HPLC determination of the pioglitazone in bulk drug and formulations. There is also report regarding impurities characterization of pioglitazone. However, there is no report available regarding isolation and characterization of degradation products of pioglitazone hydrochloride. To get literature insight, few of the articles are discussed below in detail.

T. Radhakrishna, D. Sreenivas Rao, G. Om Reddy. Determination of pioglitazone hydrochloride in bulk and pharmaceutical formulations by HPLC and MEKC methods. Journal of Pharmaceutical and Biomedical Analysis, 29 (4) (2002) 593-607.

In this study, author has developed High Performance Liquid Chromatographic (HPLC) and Micellar Electrokinetic Chromatographic (MEKC) methods for the determination of pioglitazone hydrochloride. Pioglitazone and its unsaturated impurity were separated by MEKC in less than 7 min using a 43 cm x 50 m i.d. uncoated fused-silica capillary with extended light path for better sensitivity (25 kV at 30 °C) and a background electrolyte (BGE) consisting of 20% acetonitrile (v/v) in 20 mM sodium borate buffer pH 9.3 containing 50 mM sodium dodecyl sulphate (SDS). The MEKC method was compared with HPLC method using a 5 m symmetry C18 column (250 x 4.6 mm i.d.) eluted with a mobile phase consisting of a

mixture of 50% (v/v) acetonitrile and 10 mM potassium dihydrogen phosphate buffer, adjusting the pH to 6.0 with 0.1 M KOH. was used for the separation. The flow rate of mobile phase was 1.0 mL/min and the column was operated at ambient temperature (~ 22 °C). The sample injection volume was 10 l. The photodiode array detector was set at a wavelength of 225 nm. [37].

Y. R. Kumar, A. R. Reddy, S. Eswaraiah, K. Mukkanti, M. S. Reddy, M. V. Suryanarayana. Structural characterization of impurities in pioglitazone. Pharmazie. 59 (11) (2004) 836-839.

In this article, author described characterization of three prominent impurities of pioglitazone bulk drug, which were detected up to concentrations of 0.1% (ranging from 0.05-0.1%) by reversed phase HPLC. These impurities were isolated from enriched mother liquor samples and characterized as 5-(4-hydroxybenzyl)-1,3-thiazolidine-2,4-dione (I) 5-(4-fluorobenzyl)-1,3-thiazolidine-2,4-dione (II), 2-[2-(4-bromophenoxy) ethyl-5-ethyl pyridine (III) based on their ^{1}H, and ^{13}C NMR, DEPT, Mass and IR spectral data [38].

T. Thilak kumar, B. M. Rao, A. Jamal Abdul Nasser, A. Nallasivam, K. Balakumaran. A validated specific stability-indicating RP-HPLC method for pioglitazone hydrochloride. Analytical Chemistry An Indian Journal. 7(4) (2008) 255-262.

The author have reported HPLC method employing linear gradient elution in Zorabax Bonus RP18 column with water :trifluoroacetic acid in the ratio of 100:0.05(v/v) as a mobile phase-A and acetonitrile:trifluoroacetic acid in the ratio of 100:0.05(v/v) as a mobile phase - B. The gradient program: time/% of MP-B is 0/10, 12/65,16/65, 17/10 with post run 5 min. The flow rate of the mobile phase was 1.0mL/min. The column was maintained at 30° C and the wavelength was monitored at a wavelength of 225nm. The injection volume was 10 L [36].

3. Aim of Present Work

As discussed in the literature review, few HPLC methods have been developed and reported for the determination of pioglitazone hydrochloride in bulk drug, formulation and biological metrics. In 2002, T. Radhakrishna et. al. [37] performed stress testing of the pioglitazone as a part of specificity study, where they observed complete degradation in 0.1N NaOH. They have also referred about degradation in oxidative condition but degradation products were not characterized. Six years later, in 2008 T. Thilak kumar et. al. [36] have also performed forced degradation of pioglitazone to evaluate stability indicating power of the method, where they have observed degradation in 0.5N NaOH but degradants were not characterized.

The International Conference on Harmonization (ICH) guideline entitled 'Stability Testing of New Drug Substances and Products' (Q1A(R2)) requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance [39]. It suggests that the degradation products that are formed under a variety of conditions should be identified and degradation pathways established. The characterization of the degradation products formed during stress testing is also very important to understand the impurity profile of stability batches. Literature search reveal that Pioglitazone hydrochloride is very prompt to degrade in basic condition. To the best of our knowledge, there are no reports available regarding isolation and characterization of the major degradation products of pioglitazone hydrochloride. Therefore, it is necessary to isolate and characterize major degradation products of pioglitazone hydrochloride under basic condition to contribute some information in the world of pharmaceuticals.

Since, research and development is a continuous process, in line of this we have extended the research done by T. Thilak kumar et. al. [36] further. They have reported significant degradation under base hydrolysis. The major degradant formed at RRT of 1.05 in the range of 10 % a/a. Apart from that 4 more degradants formed in the level of 1-3 % a/a. They have claimed their method as a stability indicating, it means that all the degradation products forms during stress testing are well-separated form the peak of pioglitazone. Therefore, we have adopted the same method for our study and repeated forced degradation of pioglitazone under variety of stress conditions. We have attempted scale of degradation process and isolated the

degradation products by preparative HPLC system. The isolated pure material was characterized by spectroscopic techniques, such as, LC-MS, FT-IR and NMR (^{1}H, ^{13}C, DEPT) for structure elucidation. Mechanistic arguments have been advanced to support structure elucidation. Additionally, chemical transformations have been utilized to prove the structure of both degradation products.

4. Experimental

Materials and Instruments

Drug Sample

Process research department of Zydus Research Centre, Cadila Healthcare Ltd., Ahmedabad, kindly provided the sample of pioglitazone hydrochloride drug substance with purity of 99.86 %.

Chemicals and Solvent Used

Acetonitrile - s d fine-CHEM Limited, Mumbai

Methanol - Merck, India

Trifluoro acetic acid – SIGMA-ALDRICH CHEMIE GmbH, Germany

Hydrochloric acid – s d fine-CHEM Limited, Mumbai

Sodium hydroxide - s d fine-CHEM Limited, Mumbai

Hydrogen peroxide - s d fine-CHEM Limited, Mumbai

Chloroform - s d fine-CHEM Limited, Mumbai

Deuteriated DMSO – 99.9 atom % D

Water – HPLC grade water

Instruments Used

HPLC – A Agilent 1100 series (Germany) HPLC system equipped with degasser auto sampler, auto injector, thermostatic compartment and photo diode array detector was utilized for method development and validation. The out put signal was monitored and processed using Agilent Chemstation software.

Preparative HPLC – SHIMADZU LC-8A, with class-VP software

FT-IR - SHIMADZU 8400S FT-IR, Shimadzu Make, Japan

Mass Spectrometry - Waters Quatromicro MSMS, Waters Make.

NMR – Bruker 300MHz

Freeze dryer - Sharp freeze -110, aapptec make

Rotavapour – BUCHI MAKE, Buchi rotavapor-R205,
Buchi vaccume controller V-805

Analytical balance – Mettler Toledo, AX 105, Delta Range

Sonicator – Bandelin electronic Heinrichstra Be 3-4 D-12207 Berlin, Germany

Vortexer – Spinix

Preparation of Mobile Phase-A (0.05 % TFA in water)

Transferred accurately 500 µL of Trifluoro acetic acid into a 1000 mL volumetric flask containing 900 mL of water. Dissolved and make up to the volume with HPLC grade water, filter through 0.45 µm filter.

Preparation of Mobile Phase-B (0.05 % TFA in acetonitrile)

Transferred accurately 500 µL of Trifluoro acetic acid into a 1000 mL volumetric flask containing 900 mL of acetonitrile. Dissolved and make up to the volume with acetonitrile, filter through 0.45 µm filter.

4.4 Preparation of Diluent [Water:Acetonitrile (60:40, v/v)]

Mixed accurately 300 mL of water and 200 mL of acetonitrile in 500 mL volumetric flask. After proper mixing, filter through 0.45 µm filter.

4.5 Sample preparation (0.25 mg/mL)

Weighed accurately about 25 mg of pioglitazone hydrochloride sample into 100 mL of volumetric flask. Add 50 to 60 mL of diluent and sonicated to dissolve the solid. Make up the volume with diluent and mixed well.

4.6 Chromatographic parameters for analytical HPLC

Elution	: Gradient
Column	: Zorbax eclipsed C18 (150 x 4.6 mm, 5 µm)
Wave length	: 225 nm
Column temperature	: 30 C
Flow rate	: 1.0 mL / min.
Injection volume	: 10 µL
Run time	: 22 min
Mobile phase-A	: 0.05 % TFA in water
Mobile phase-B	: 0.05 % TFA in acetonitrile
Diluent	: Water:Acetonitrile (60:40)
Sample conc.	: 0.25 mg/mL

Gradient program :

Time (minutes)	Mobile Phase-A (%)	Mobile Phase-B (%)
0.0	90	10
12.0	35	65
16.0	35	65
17.0	90	10
22.0	90	10

Purity of the sample was calculated as per area normalization method.

4.7 Forced Degradation of Pioglitazone Hydrochloride

4.7.1 Preparation of 5N HCl

44.14 mL of concentrate HCl (11.3N) added in 100 mL volumetric flask containing approximately 40 mL water and make up to the mark with water and mixed well.

4.7.2 Acid Degradation

About 25 mg of pioglitazone hydrochloride was accurately weighed and transferred into a 10 mL volumetric flask. 5 mL of 5N HCl was added to it and the solution was heated at 70° C on water bath for 1 hour. The sample solution was then allowed to cool at room temperature and approximately neutralized it using 5N NaOH. The solution was sonicated for 10 minutes and diluted up to the mark with diluent. The solution was mixed well and then filtered through 0.45 m filters. Took 1 mL of this solution in 10 mL volumetric flask and diluted upto the mark with diluent and mixed well. The sample solution (10 L) was injected on an equilibrated chromatographic system.

4.7.3 Preparation of 5N NaOH

Accurately, weighed 20 g of NaOH in beaker and dissolved it in minimum amount of water, sonicated to dissolve. Then, transferred it into 100 mL volumetric flask, washed beaker twice with water and transferred into the same volumetric flask. Make up volume upto the mark with water and mixed well.

4.7.4 Base Degradation

About 25 mg of pioglitazone hydrochloride was accurately weighed and transferred into a 10 mL volumetric flask. 5 mL of 5N NaOH was added to it and the solution was heated at 70° C on water bath for 1 hour. The sample solution was then allowed to cool at room temperature and approximately neutralized it using 5N HCl. The solution was sonicated for 10 minutes and diluted up to the mark with diluent. The solution was mixed well and then filtered through 0.45 m filters. Took 1 mL of this solution in 10 mL volumetric flask and diluted upto the mark with diluent and mixed well. The sample solution (10 L) was injected on an equilibrated chromatographic system.

4.7.5 Preparation of 30 % H_2O_2

16.6 mL of H_2O_2 (Assay 30.0 %) added in 100 mL volumetric flask containing approximately 40 mL water and make up to the mark with water and mixed well.

4.7.6 Peroxide Degradation

About 25 mg of pioglitazone hydrochloride was accurately weighed and transferred into a 10 mL volumetric flask. 5 mL of 5 % H_2O_2 was added to it and the solution was heated at 70° C on water bath for 1 hour. The sample solution was then allowed to cool at room temperature. The solution was hessy, small amount of methanol was added and sonicated for 10 minutes to dissolve and diluted up to the mark with diluent. The solution was mixed well and then filtered through 0.45 m filters. Took 1 mL of this solution in 10 mL volumetric flask and diluted upto the mark with diluent and mixed well. The sample solution (10 L) was injected on an equilibrated chromatographic system.

4.7.7 Degradation Without Degrading Agent

About 25 mg of pioglitazone hydrochloride was accurately weighed and transferred into a 10 mL volumetric flask. 5 mL of diluent was added to it and the solution was heated at 70° C on water bath for 1 hour. The sample solution was then allowed to cool at room temperature. The solution was sonicated to dissolve and diluted up to the mark with diluent. The solution was mixed well and then filtered through 0.45 m filters. Took 1 mL of this solution in 10 mL volumetric flask and

diluted upto the mark with diluent and mixed well. The sample solution (10 L) was injected on an equilibrated chromatographic system.

4.7.8 Thermal Degradation

About 100 mg of pioglitazone hydrochloride spreaded in the pettri dish and exposed it, in the oven at 105°C for 52 hrs. Took, accurately weighed 25 mg sample after thermal exposure in 10 mL volumetric flask. 5 to 6 mL diluent added and sonicated to dissolve the solid. Make up the volume with diluent and mixed well. Took 1 mL of this solution in 10 mL volumetric flask and diluted upto the mark with diluent and mixed well. The sample solution (10 L) was injected on an equilibrated chromatographic system.

4.7.9 UV-light Degradation

About 100 mg of pioglitazone hydrochloride spreaded in the pettri dish and exposed it, under UV-light for 52 hrs. Took, accurately weighed 25 mg sample after UV-light exposure in 10 mL volumetric flask. 5 to 6 mL diluent added and sonicated to dissolve the solid. Make up the volume with diluent and mixed well. Took 1 mL of this solution in 10 mL volumetric flask and diluted upto the mark with diluent and mixed well. The sample solution (10 L) was injected on an equilibrated chromatographic system.

4.8 Scale-up of Degradation of Pioglitazone Hydrochloride

4.8.1 Experiment - 1

Accurately, weighed 10g of NaOH in beaker and dissolved it in 25 mL of water, sonicated to dissolve. Took 1.5 g, pioglitazone hydrochloride in 150 mL RBF and then, transferred solution of NaOH from beaker to RBF and mixed well. The RBF was heated at 100° C and 50 rpm for 2 hours. The reaction solution was allowed to cool at room temperature and approximately neutralized with HCl. To get clear solution, it was sonicated and mixed well using vortex. The reaction solution was subjected to HPLC analysis to check extend of degradation and HPLC profile.

4.8.2 Experiment - 2

About 750 mg of pioglitazone hydrochloride was weighed and transferred into a 150 mL RBF. 25 mL of 5N NaOH was added to it and the solution was heated at 75° C and 50 rpm on water bath for 2 hours. The reaction solution was allowed to cool at room temperature and approximately neutralized it using 5N HCl. To get clear solution, it was sonicated and mixed well using vortex. The reaction solution was subjected to HPLC analysis to check extend of degradation and HPLC profile.

4.9 Isolation of Degradation Products

4.9.1 Preparative HPLC Method Parameters

Elution : Gradient

Column : Modcol, (250X50mm) Self packed column, Vydac Make

Media used : ODS-AQ, 10 m, 120A, YMC, Make

Wave length : 225 nm

Column temp. : Ambient Temperature

Flow rate : 70.0 mL / min.

Injection volume : 5 mL

Mobile phase : A : 0.05 % TFA in water

B: Acetonitrile

Gradient Program :

Time (minutes)	0.05 % TFA in Water	Acetonitrile
0	78	22
4	78	22
30	50	50
35	10	90
40	10	90
41	78	22
45	78	22

4.9.2 Procedure

The reaction mixture obtained from scale-up experiments of base degradation was filtered through 0.45 m filter and 5 mL of this solution was injected manually in equilibrated preparation HPLC system. The run was monitored using Class-VP software and desired peak was collected manually in different fractions for both the

degradation product. The collected fractions were subjected to HPLC analysis. The procedure was repeated to purify remaining reaction mixture to isolate both degradation products.

The HPLC analysis of the collected fractions was used to segregate fractions as per its purity. The fractions of less than 98 % purity was mixed and re-injected through pump in preparative HPLC system to get desire purity level. Again, collected fractions were monitored in analytical HPLC system. Finally, all the fractions with more than 98.0 % purity were mixed and concentrated using rotavapor. Concentrated fractions were subjected to freeze drying, to get purified material of both the degradation products.

4.10 Characterization of Degradation Products

The isolated materials using preparative HPLC were characterized by spectroscopic techniques such as, Mass, IR, UV and NMR (^{1}H, ^{13}C, DEPT).

4.10.1 Mass Spectrometry

The isolated compound was dissolved (about 100 ppm) in methanol and injected (2 L) through liquid chromatography flowed flow with methanol-water (90:10, v/v) at a flow rate 0.3 mL/min. The mass was operated in positive spary ionization (ESI+) mode with capillary voltage 3.5 KV, cone voltage 20V, source temperature 120°C, desolvation temperature 400°C and desolvation gas flow 800L/hr.

4.10.2 Infra-red Spectrocopy

The IR spectra were recorded in chloroform in the region 4000 cm^{-1} to 400 cm^{-1}. The operated parameters are; Numbers of scan:20, Resolution:4, Beam: internal, Mirror speed: 2.8 and Gain: auto.

4.10.3 Nuclear Magnetic Resonance

^{1}H (300 MHz) and ^{13}C (300 MHz) NMR spectra of isolated degradation products were recorded on an Avance DPX- 300MHz spectrometer Bruker (Germany). The probe was a 5 mm BBO z-gradient probe, optimized for inverse detection. Spectra were recorded in deuteriated DMSO (5-mm tubes) at 300 K. Sample concentration was 10 mg in 0.5 ml. The residual protonated resonance of the

solvent (DMSO-*d*6) was used as an internal chemical shift standard, which was related to tetramethylsilane with chemical shifts of 2.5 and 39.5 ppm, respectively, for ^{1}H and ^{13}C. Processing of the raw data was performed using Bruker XWin 3.5 Nmr software. The pulse conditions were 9.8μs (attenuation 0db) for ^{1}H and 7.10μs (attenuation -1.0db) for ^{13}C.

5. Result and Discussion

As, we have discussed in preceding sections that forced degradation of the pioglitazone hydrochloride was attempted as a part of specificity study or to evaluate stability indicating power of the analytical method in literature. Here, our goal of the study was to isolate and characterize the degradation products of the pioglitazone hydrochloride under basic conditions. Therefore, we have adopted recently published HPLC method [36] for the study and the forced degradation of the pioglitazone hydrochloride was carried out at analytical scale for five different stress conditions. The stress condition and level of stress was increased, than the reported in the literature for better understanding of the degradation in different conditions.

The degradation procedure was scaled up for the isolation of degradants. The major degradation product was isolated using preparative HPLC and characterized by spectroscopic techniques.

5.1 Result of Forced Degradation

The sample of pioglitazone hydrochloride was prepared at analyte concentration in diluent and analyzed using HPLC method before exposure. The chromatogram of untreated sample is shown in Fig. 1. The purity of the sample was found 99.74 %.

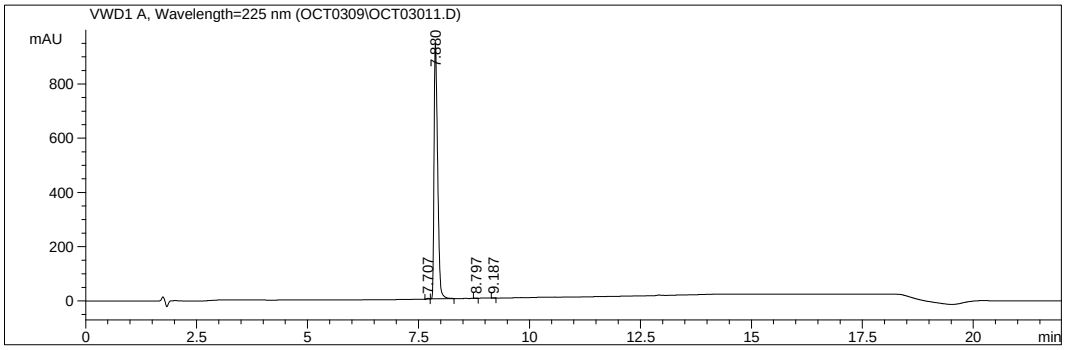


Fig. 1: Chromatogram of pioglitazone hydrochloride sample (untreated)

The pioglitazone hydrochloride was forced to degrade by acid and base hydrolysis, peroxide oxidation, heat exposure and UV-light exposure. The analysis of the degradation samples was done by HPLC to compare chromatographic profile with untreated sample and to understand degradation.

5.1.1 Acid Degradation

The pioglitazone hydrochloride was treated with 5N HCl, as described in 'experimental section' and subjected to HPLC analysis. The chromatogram of the sample treated by acid is shown in Fig. 2. The purity of the sample after acid degradation was found 99.76 %, which was equal to the untreated sample and there was no new peak observed in the chromatogram. Therefore, it can be concluded that pioglitazone hydrochloride is not degraded under acidic stress condition.

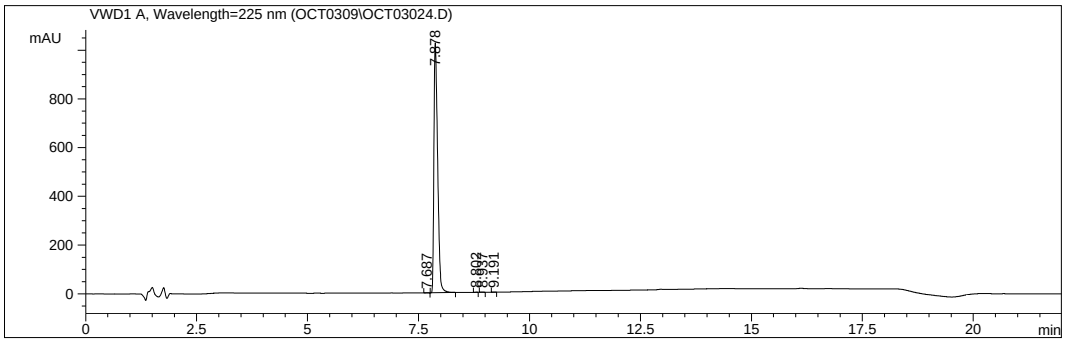


Fig. 2: Chromatogram of acid degradation

5.1.2 Base Degradation

The pioglitazone hydrochloride was treated with 5N NaOH, as described in 'experimental section' and subjected to HPLC analysis. The chromatogram of the sample treated by base is shown in Fig. 3.

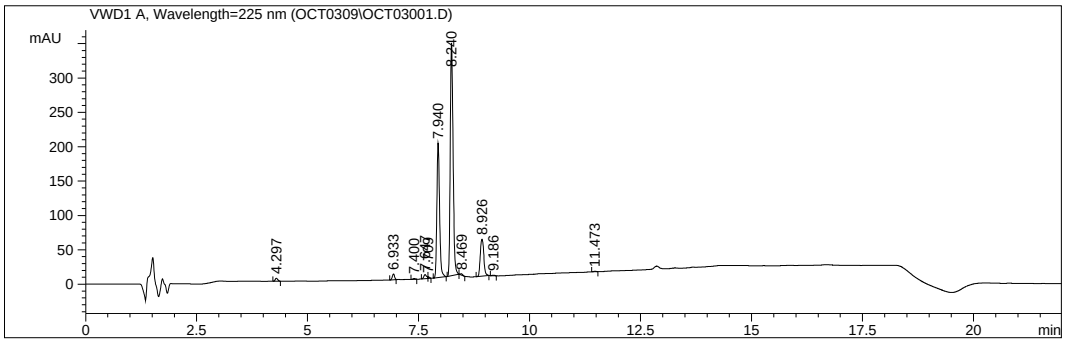


Fig. 3: Chromatogram of base degradation

It can be seen from the Fig. 3 that many new impurities are formed by base degradation of pioglitazone hydrochloride. There was significant degradation under base treatment. The purity of the sample was found only 32.1 %. The two major degradation products were observed, at retention time 8.24 (RRT:1.04) and 8.93

(RRT:1.12) minutes with 55% and 11% a/a contribution respectively. It has also been observed that if sample was treated under exhaustive base degradation condition, i. e. 100°C for 2 hours then only first degradation product (RRT: 1.04) was formed (Chromatogram: Fig. 4).

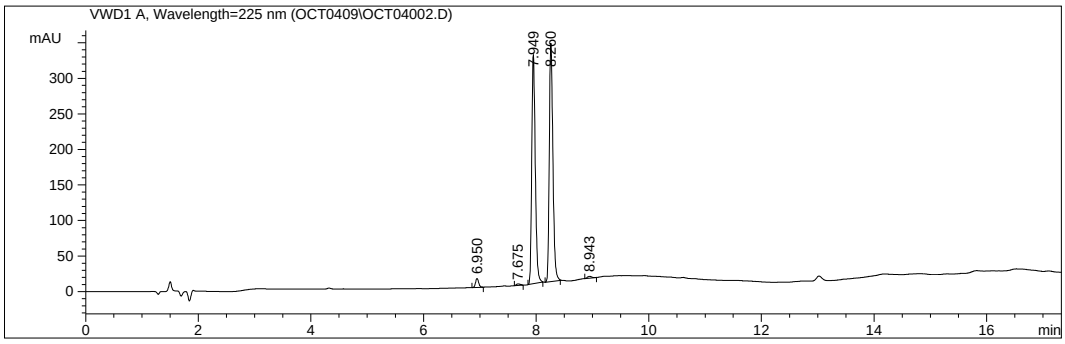


Fig. 4: Chromatogram of base degradation with harder condition

5.1.3 Peroxide Degradation

The pioglitazone hydrochloride was treated with 5% H_2O_2 (Hydrogen Peroxide) in water, as described in 'experimental section' and subjected to HPLC analysis. The chromatogram of the sample treated by hydrogen peroxide is shown in Fig. 5.

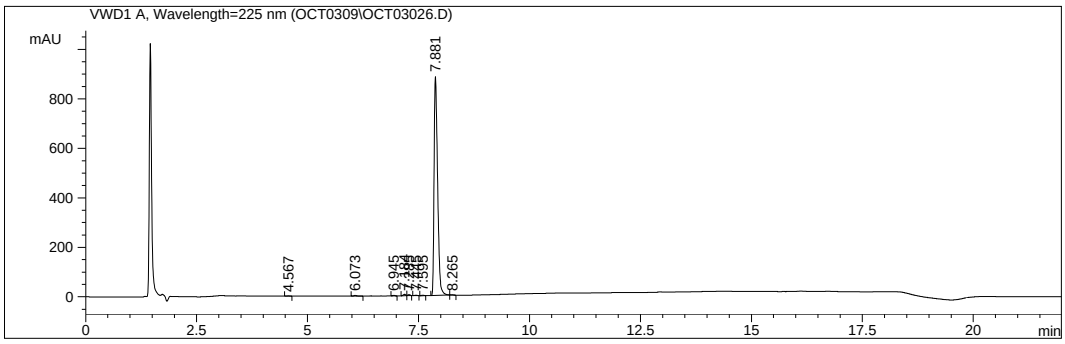


Fig. 5: Chromatogram of peroxide degradation

It can be seen from Fig. 5 that very mild degradation is observed under perxode treatment. The new impurities formed are in the range of less than 0.5 % a/a by area-normalized method. The purity of the sample after peroxide degradation was found 98.58 %. The major peak observed at retention time 1.54 minutes was found in the diluent containing same concentration of hydrogen peroxide. Therefore, it has not been integrated in the chromatogram.

5.1.4 Thermal Degradation

The pioglitazone hydrochloride was exposed at 105° C for 52 hours, as described in 'experimental section' and subjected to HPLC analysis. The chromatogram of the sample treated by heat for thermal decomposition is shown in Fig. 6. The purity of the sample after thermal degradation was found 99.75 %, which was equal to the untreated sample and there was no new peak observed in the chromatogram. Therefore, it can be concluded that pioglitazone hydrochloride is not degraded under thermal stress condition.

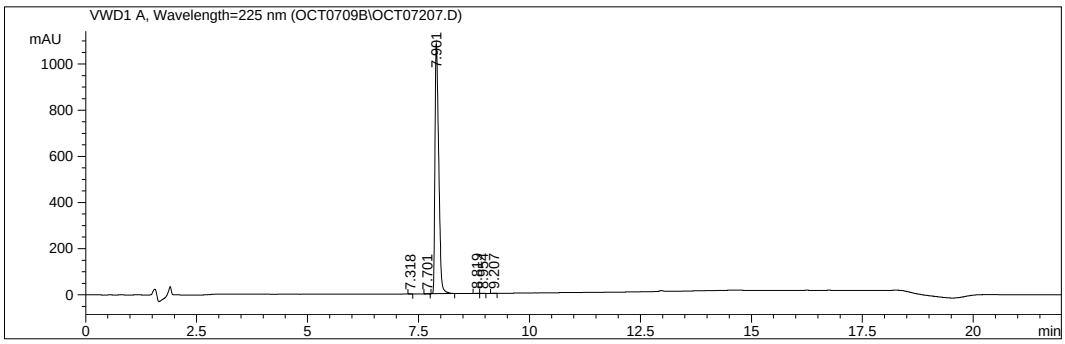


Fig. 6: Chromatogram of thermal degradation

5.1.5 UV-light Degradation

The pioglitazone hydrochloride was exposed under UV-light for 52 hours, as described in 'experimental section' and subjected to HPLC analysis. The chromatogram of the sample treated by UV-light is shown in Fig. 7. The purity of the sample after UV-light degradation was found 99.73 %, which was equal to the untreated sample and there was no new peak observed in the chromatogram. Therefore, it can be concluded that pioglitazone hydrochloride is not degraded under UV-light stress condition.

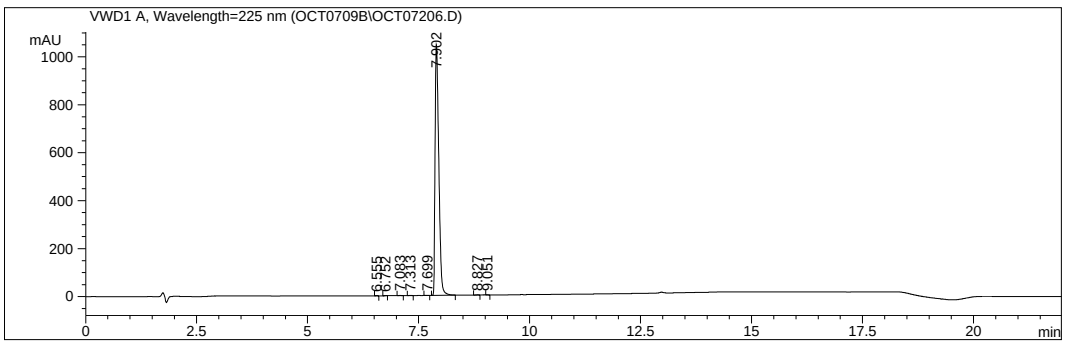


Fig. 7: Chromatogram of UV-light degradation

It can be seen from above degradation studies that the major degradation was observed only in base treatment while all other tested stress conditions were not shown major degradation of pioglitazone hydrochloride. The peroxide oxidation showed very mild degradation.

To study the effect of physical forces and to confirm the role of degrading agent, the experiment was conducted using same experimental procedure and conditions in absence of degrading agent, i.e. HCl, NaOH, H_2O_2. The chromatogram is presented in Fig. 8. The purity of the sample was found 99.78 %, which was equal to the untreated sample and there was no new peak observed in the chromatogram. Therefore, it can be concluded that only physical forces do not degrade pioglitazone hydrochloride in absence of degrading agent.

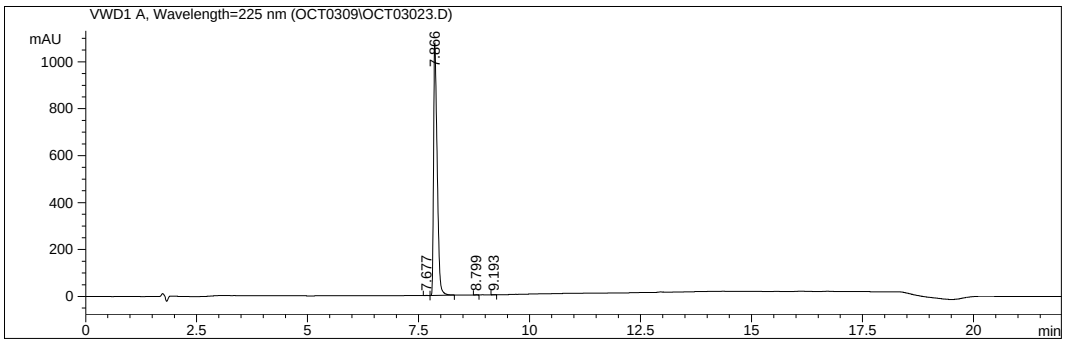


Fig. 8: Chromatogram of degradation without degrading agent

The major degradation product, observed in base degradation study was at RRT 1.04, which is same as reported by T. Thilak kumar et al. [36]. The author has not referred anything about second degradation product at RRT 1.12. He has also not characterized degradation product. Since, the pioglitazone hydrochloride mainly degrades under base treatment and forms two major degradation products. Therefore, we have focused only in base degradation and targeted to isolate and characterize two major degradation products observed at RRT 1.04 and 1.12.

5.2 Scale-up of Base Degradation

In order to isolate degradation product by preparative HPLC, the base degradation was scaled up from milligram to gram level. The scale-up experiment-1, as described in 'experimental section' showed that pioglitazone hydrochloride was almost degraded and converted in the 95 % degradation product-1 (RRT:1.04). The producr-2 was not formed. The chromatogram of experiment-1 is shown in Fig. 9.

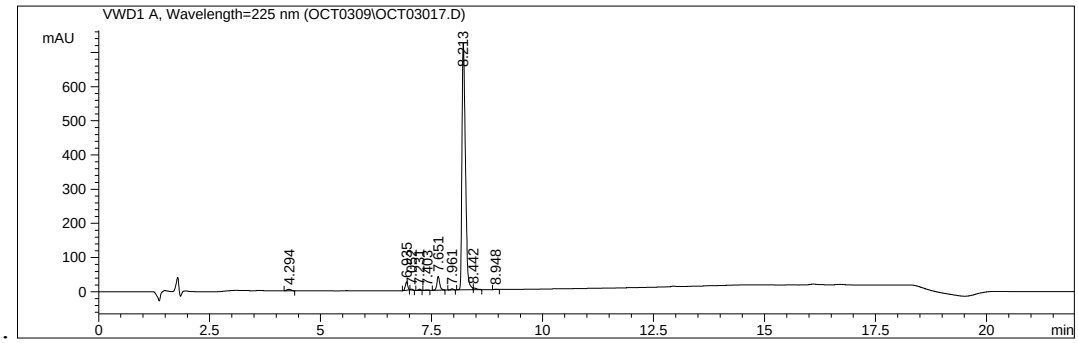


Fig. 9: Chromatogram of scale-up experiment-1 for base degradation.

To get degradation product-2, scale-up experiment-2 was planned as described in 'experimental section' using mild condition than the experiment-1. The HPLC chromatogram of the experiment-2 of scale reaction is shown in Fig. 10.

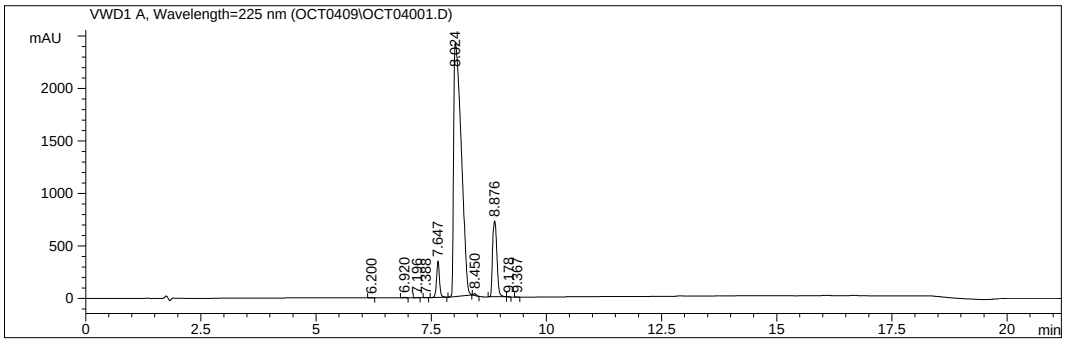


Fig. 10: Chromatogram of scale-up experiment-2 for base degradation

It can be seen from the Fig. 10 that both the desired degradation products were formed. The area contribution of the degradation product-1 and degradation product-2 in the chromatogram was 81.7% and 13.5 % respectively while un-reacted pioglitazone remained 4.5 %. The both reaction mixture was subjected to preparative isolation.

5.3 Preparative HPLC Isolation

The degradation products present in reaction mixture obtained from scale-up experiments was isolated by preparative HPLC. The fractions collected manually at five different points of the peak and purity was checked by analytical HPLC. It has been observed that fraction collected at the apex of peak was pure more than 98 %, for degradation product-1 and 99 % for degradation product-2 rest all fractions were below 98 % purity. The typical chromatogram of preparative HPLC separation of degradation products is shown in Fig. 11.

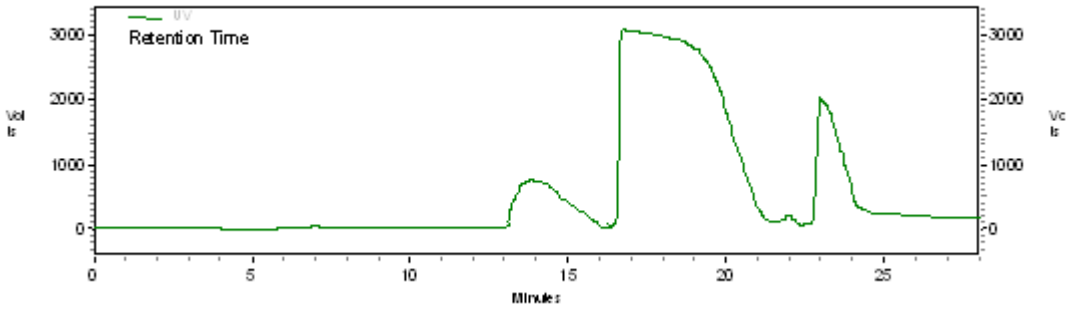


Fig. 11: Chromatogram of preparative HPLC separation

All the fractions below 98 % purity were mixed and re-injected for the further purification. The peak was collected in three parts and its purity was checked by analytical HPLC. The purity of all the three factions was found more than 98 % for degradation product-1 and 99 % for degradation product-2. To get purified material, fractions were concentrated and then lyophilized using lyophilizer. After three days drying cycle, sticky semi solid material was observed for both the degradation products. The purity of the purified materials was check by analytical HPLC. The HPLC chromatograms of isolated degradation product-1 and 2 are shown in Fig. 12 and 13, respectively. The purity of degradation product-1 was 98.1 % and degradation product-2 was 99.2 %. These materials were subjected to complete characterization by spectroscopic techniques.

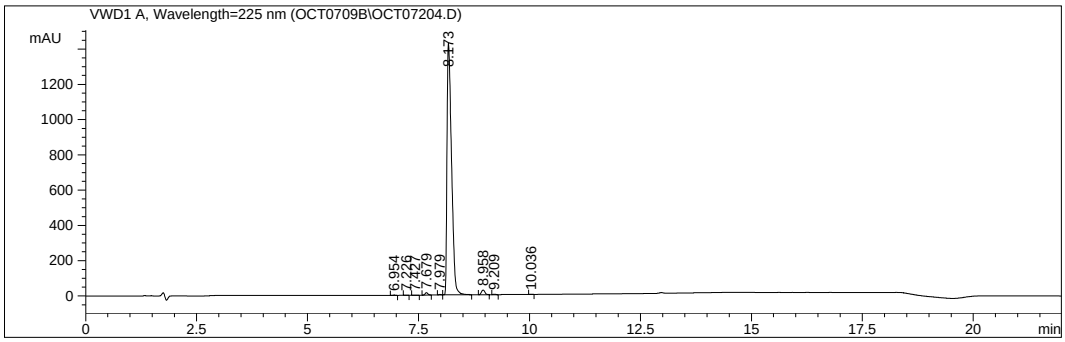


Fig. 12: HPLC Chromatogram of isolated degradation product-1 (98.1 %)

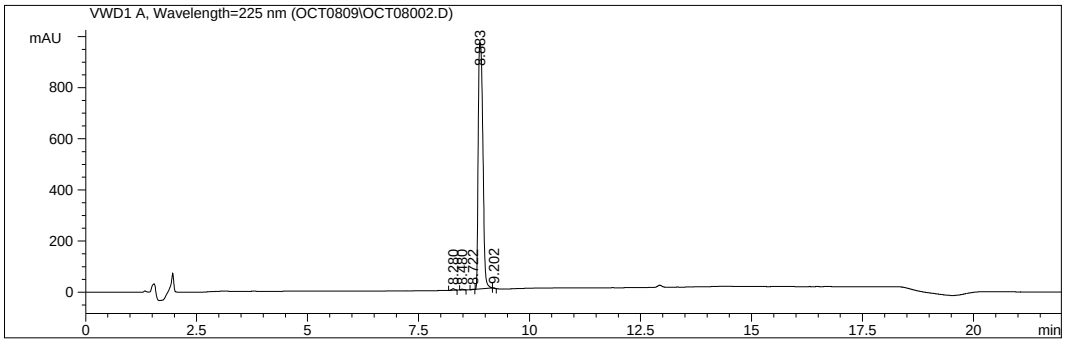


Fig. 13: HPLC Chromatogram of isolated degradation product-2 (99.2 %)

5.3 Characterization of Degradation Products

The isolated degradation products and pioglitazone were subjected to complete identification and characterization. The retention time and UV spectrum obtained in the PDA detection during HPLC analysis was matched with degradation products intended to isolate for characterization.

The mass spectra obtained for Pioglitazone, Degradation Product-1 and Degradation Product-2 are shown in Fig. 14, 15 and 16 respectively. The mass spectrum, in positive ESI mode was confirmed molecular weight.

The presence of the different functional group was confirmed by IR spectroscopy. The IR bands observed at different wave number are presented in Table 1. The IR spectra obtained for Pioglitazone, Degradation Product-1 and Degradation Product-2 are shown in Fig. 17, 18 and 19 respectively.

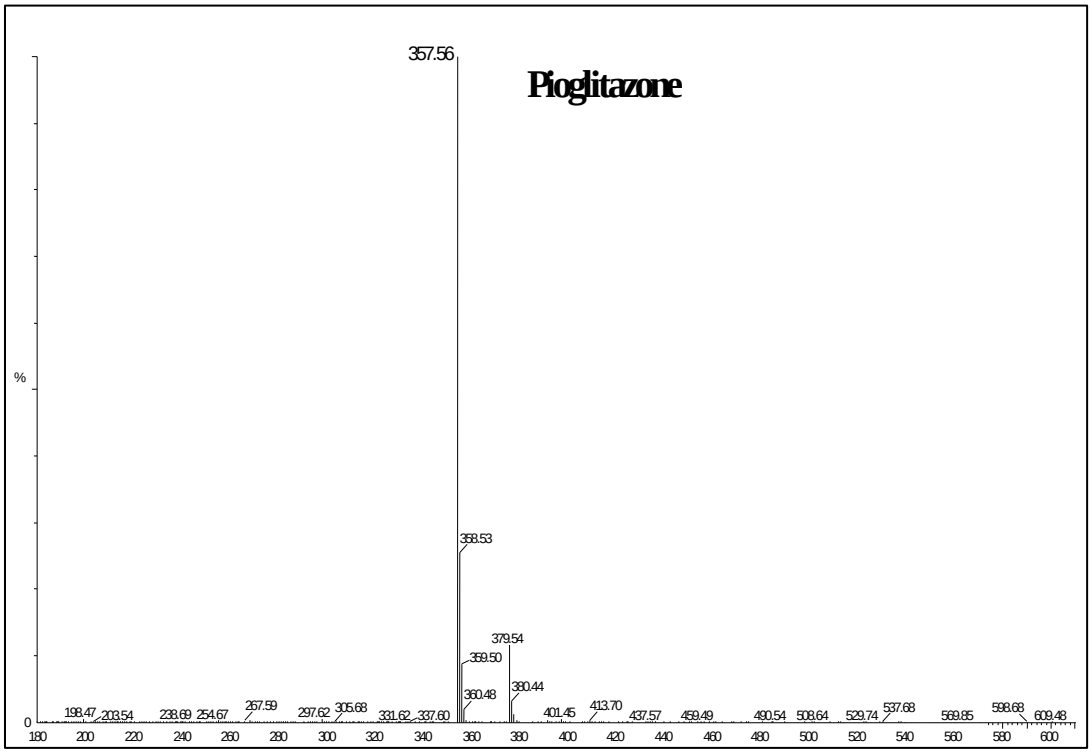


Fig. 14: Mass spectrum of Pioglitazone

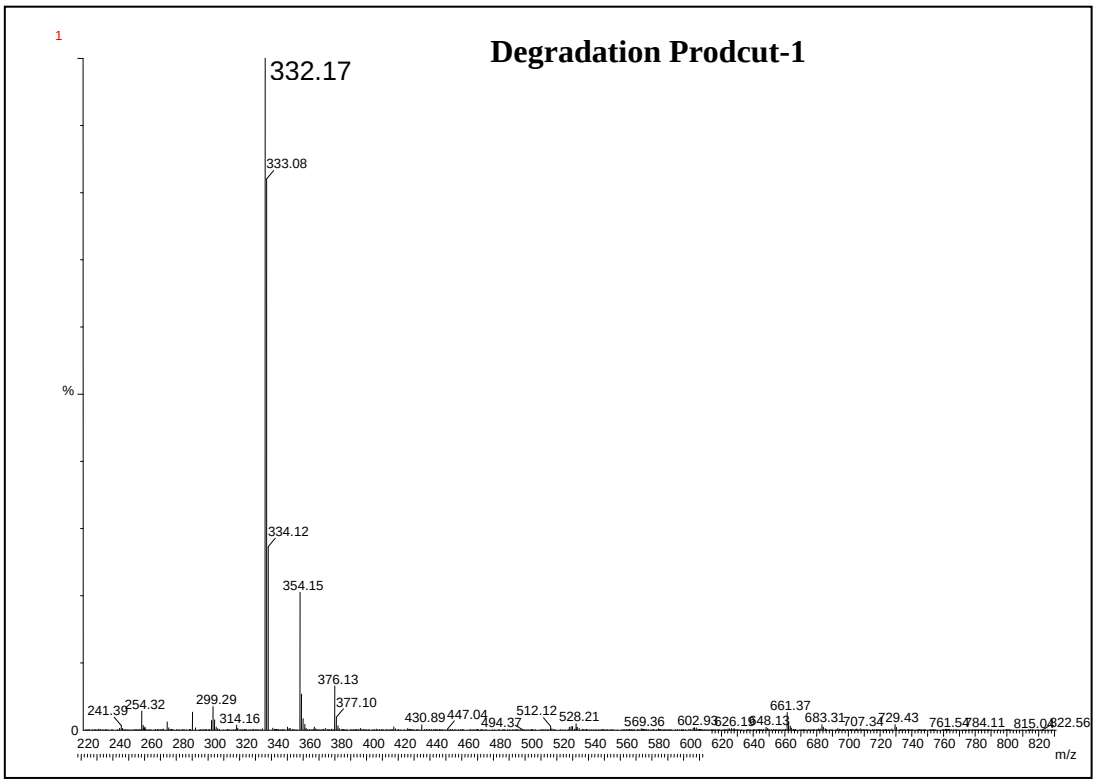


Fig. 15: Mass spectrum of Degradation Product-1

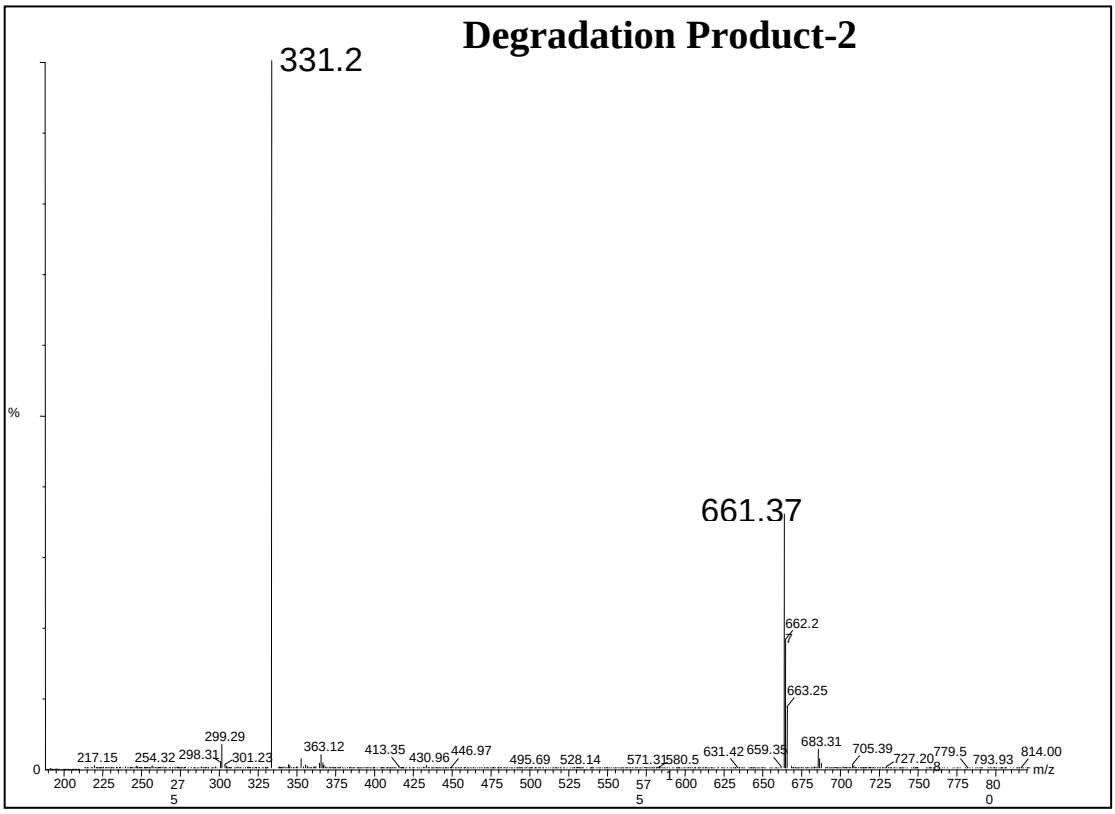


Fig. 16: Mass spectrum of Degradation Product-2

Table 1: IR bands of at different wave number

Wave number (cm^{-1})		
Pioglitazone	**Degradation Product-1**	**Degradation Product-2**
3084, 2928, 1744, 1685, 1508, 1460, 1313, 1244, 1150	3414, 3019, 1678, 1521, 1433, 1384, 1215, 1141	3416, 3021, 1686, 1512, 1385, 1215

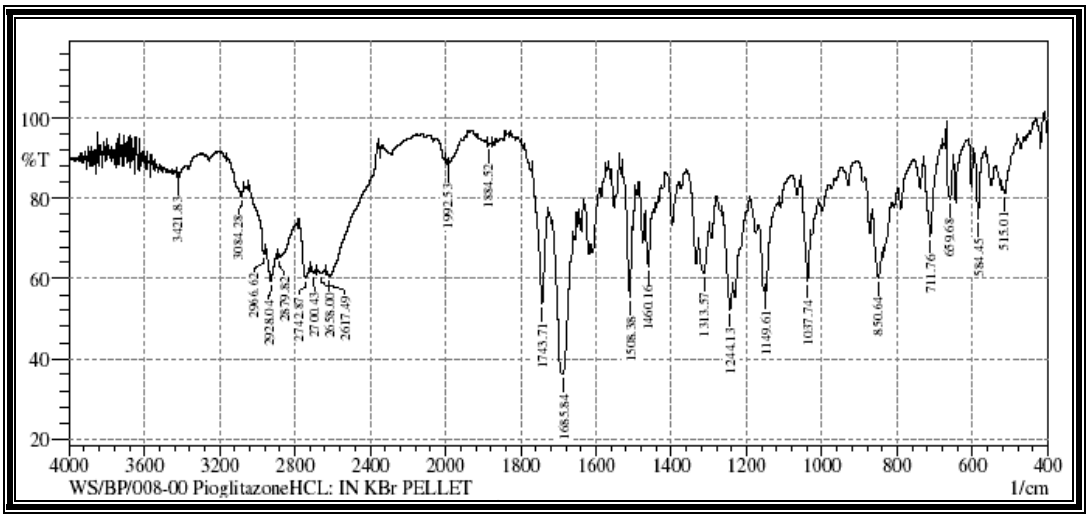


Fig. 17: IR spectrum of Pioglitazone

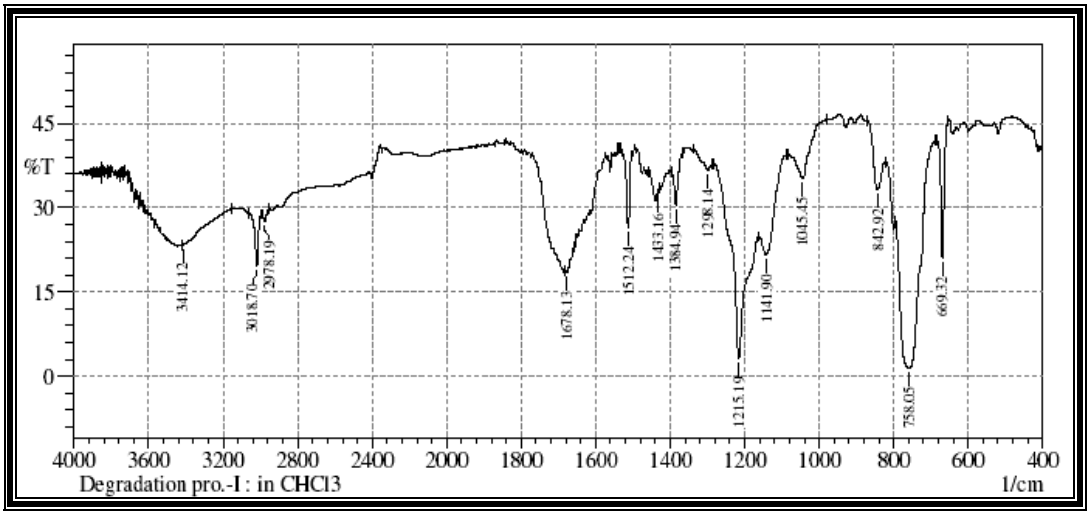


Fig. 18: Mass spectrum of Degradation Product-1

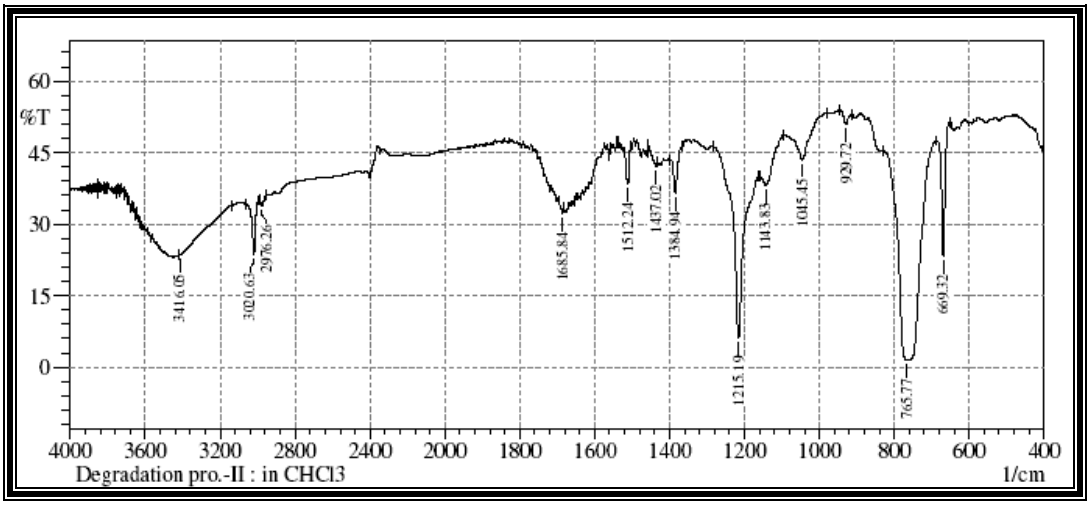


Fig. 19: Mass spectrum of Degradation Product-2

The widely used and acceptable technique for the structure elucidation and confirmation is NMR. The NMR spectra was obtained using 300 MHz instrument for ^{1}H, ^{13}C and DEPT in deuteriated dimethyl sulphoxide. The peak observed in ^{1}H and ^{13}C NMR are complied in Table 2 and 3 for Pioglitazone, Table 4 and 5 for Degradation Product-1 and Table 6 and 7 for Degradation Product-2 respectively.

1H-NMR spectra of Degradation Product-1, Degradation Product-2 and Pioglitazone are almost comparable for proton position no. 3, 4, 6, 7, 8, 9, 10, 12, 13, 15, 16 and 17 while, proton at position 18 (-CH) showed multiple signal at 4.87-4.83 δ ppm in Pioglitazone. The same proton (18) shielded at 3.72-3.51 δ ppm (m) in Degradation Product-1 and at 3.72 δ ppm (t) in Degradation Product-2. Therefore, the structure of degradation products should be very similar to pioglitazone without thiazolidinedione ring.

^{13}C-NMR spectra of degradation products clearly revealed that there is one quaternary carbon less than Pioglitazone, based on preceding discussion on ^{1}H-NMR, quaternary carbon might be lost from thiazolidinedione ring i. e. carbonyl group. It is also noticed that methylene carbon at position -17 is deshielded to the extend of ~ 4.0 δ ppm in Degradation Product-1 while methine carbon at position-18 is shielded to the extend of ~13 δ ppm, which indicates that electronegativity of the neighboring atoms are differed than the pioglitazone, the similar effect is not observed in Degradation Product-2. The mass spectrum of Degradation Product-2 showed molecular ion peak at almost double m/z value than the base peak, as well as molecular ion peak of Degradation Product-1. Therefore, mass result and ^{13}C NMR observation revealed that Degradation product-2 may be dimer of Degradation Product-1. It is also supported by chemical evidences.

The NMR spectra of Pioglitazone for ^{1}H, ^{13}C and DEPT are shown in Fig. 20, 21 and 22 respectively. The NMR spectra of Degradation Product-1 for ^{1}H, ^{13}C, DEPT and HMQC are shown in 23, 24, 25 and 26 respectively. The NMR spectra of Degradation Product-2 for ^{1}H, ^{13}C, DEPT and HMQC are shown in 27, 28, 29 and 30 respectively.

Pioglitazone

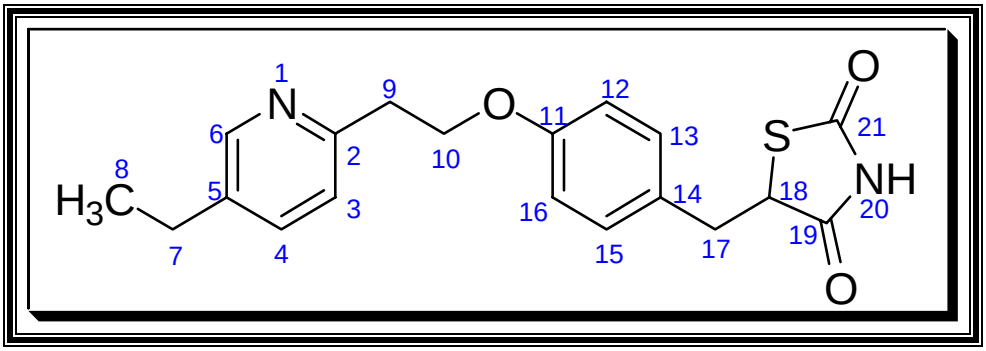


Table 2: ^{1}H-NMR of Pioglitazone

Chemical Shift (ppm)	Proton No.
12.06 (bs, 1H, NH)	20
8.70 (d, J=1.23 Hz, 1H, ArH)	6
8.41 (dd, J=1.65 & 8.28 Hz, 1H, ArH)	4
7.98 (d, J=8.25 Hz, 1H, ArH)	3
7.39(d, J=7.92 Hz, 2H, ArH)	12, 16
7.13(d, J=8.55 Hz, 2H, ArH)	13, 15
4.87 – 4.83 (m, 1H, CH)	18
4.40 (t, 2H, CH_2)	10
3.52 (t, 2H, CH_2)	9
3.31 – 3.00 (q, 2H, CH_2)	17
2.77 (q, 2H, CH_2)	7
1.21 (t, 3H, CH_3)	8

Table 3: ^{13}C-NMR of Pioglitazone

Chemical Shift (ppm)	Nature of Carbon	Assignment
175.61, 171.58	Quaternary	19, 21
156.97, 151.05		2, 11
141.31		5
129.48		14
145.22	Tertiary	6
139.90		4
130.36		12, 16
127.15		3
114.41		13, 15
52.90		18
65.42	Secondary	10
36.19, 32.12		17, 9
24.55		7
14.53	Primary	8

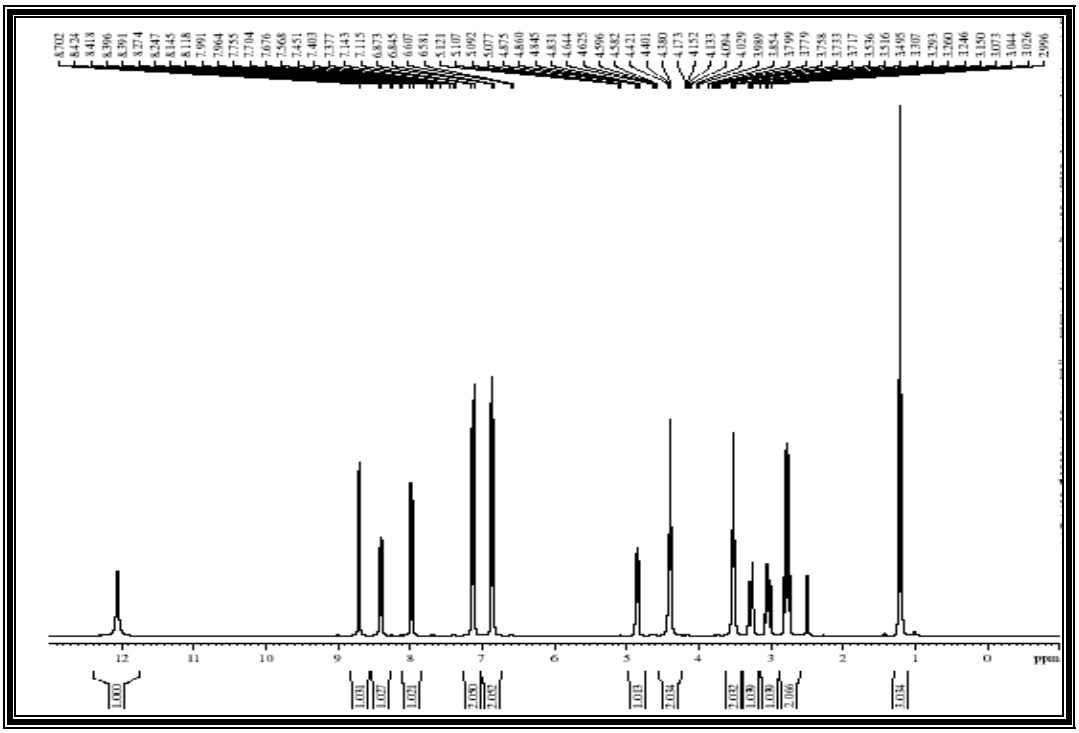


Fig. 20: ^{1}H-NMR spectrum of Pioglitazone

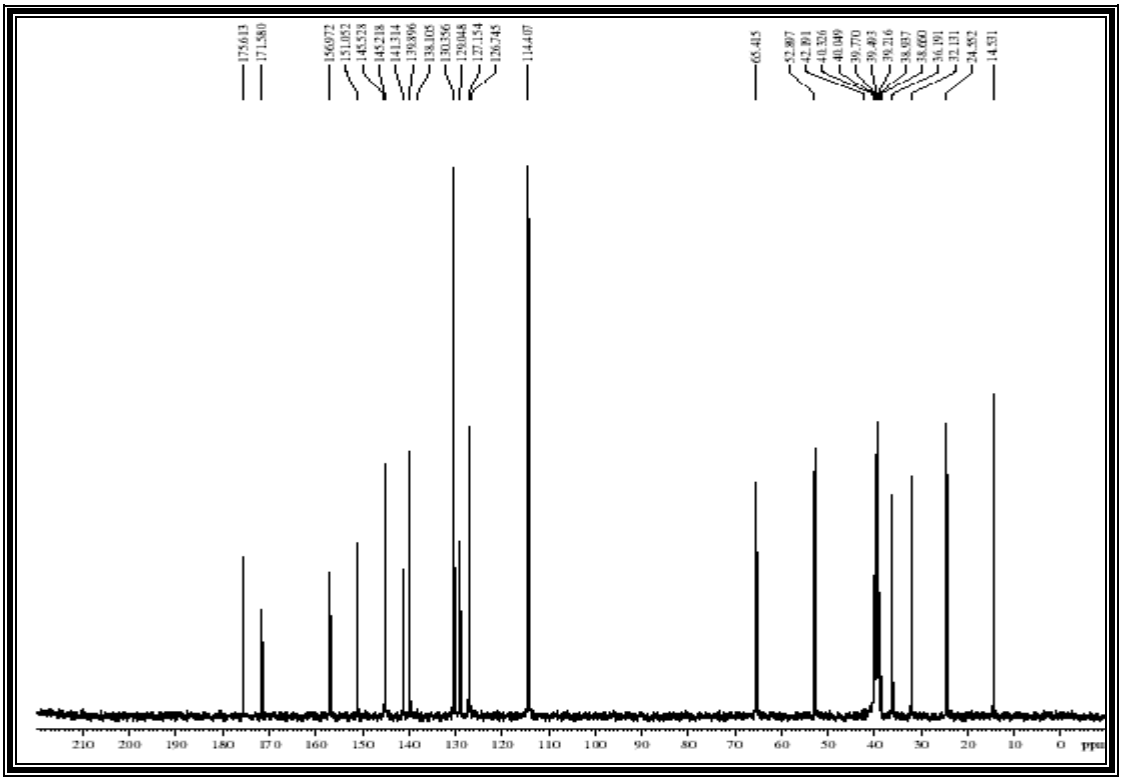


Fig. 21: ^{13}C-NMR spectrum of Pioglitazone

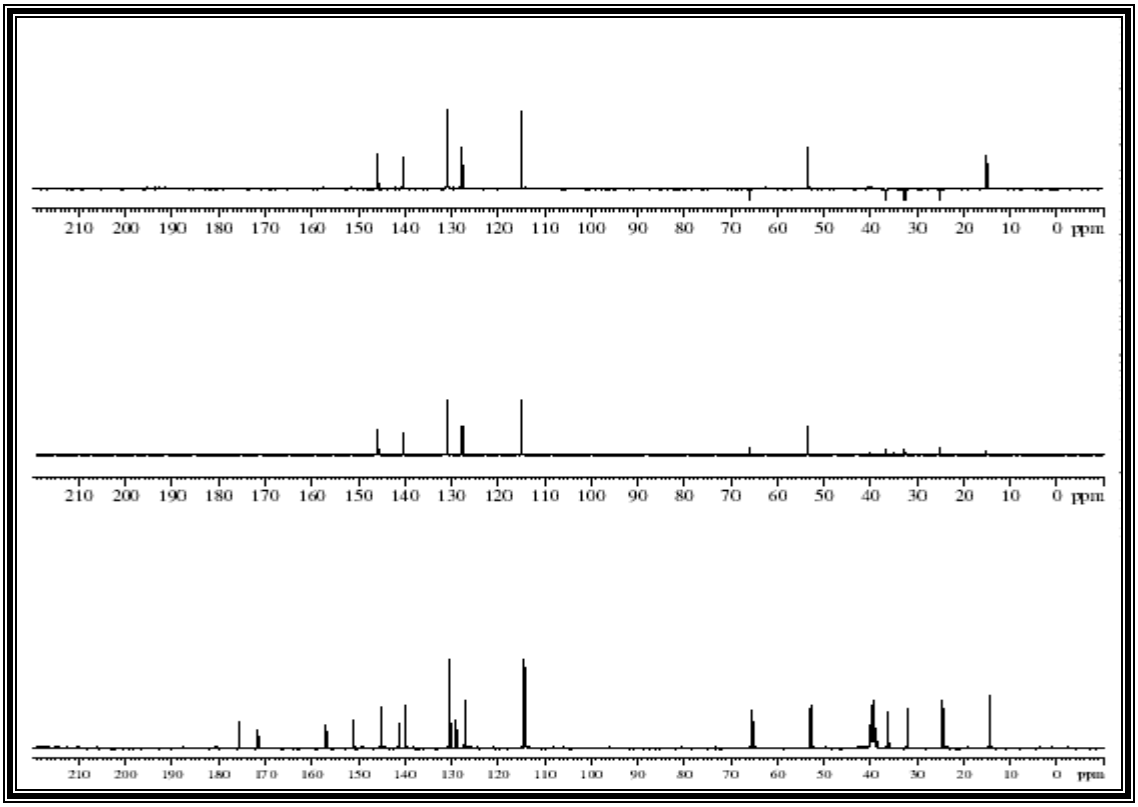


Fig. 22: ^{13}C-DEPT spectrum of Pioglitazone

Degradation Product-1

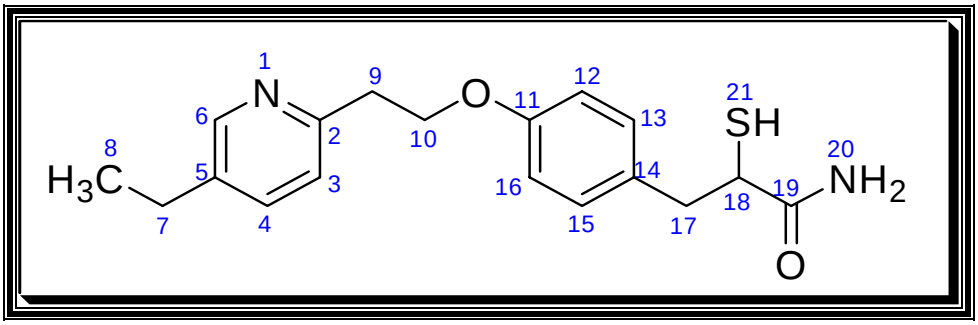


Table 3: ^{1}H-NMR of Degradation Product-1

Chemical Shift (ppm)	Proton No.
8.63 (s, 1H, ArH)	6
8.17 (d, J=8.04 Hz, 1H, ArH)	4
7.78 (d, J=8.1 Hz, 1H, ArH)	3
7.10 (d, J=8.5 Hz, 2H, ArH)	12, 16
6.80 (d, J=8.5 Hz, 2H, ArH)	13, 15
4.31 (t, 2H, CH_2)	10
3.72 – 3.51 (m, 1H, CH)	18
3.35 (t, 2H, CH_2)	9
3.06 – 2.94 (m, 2H, CH_2)	17
2.70 (q, 2H, CH_2)	7
1.18 (t, 3H, CH_3)	8

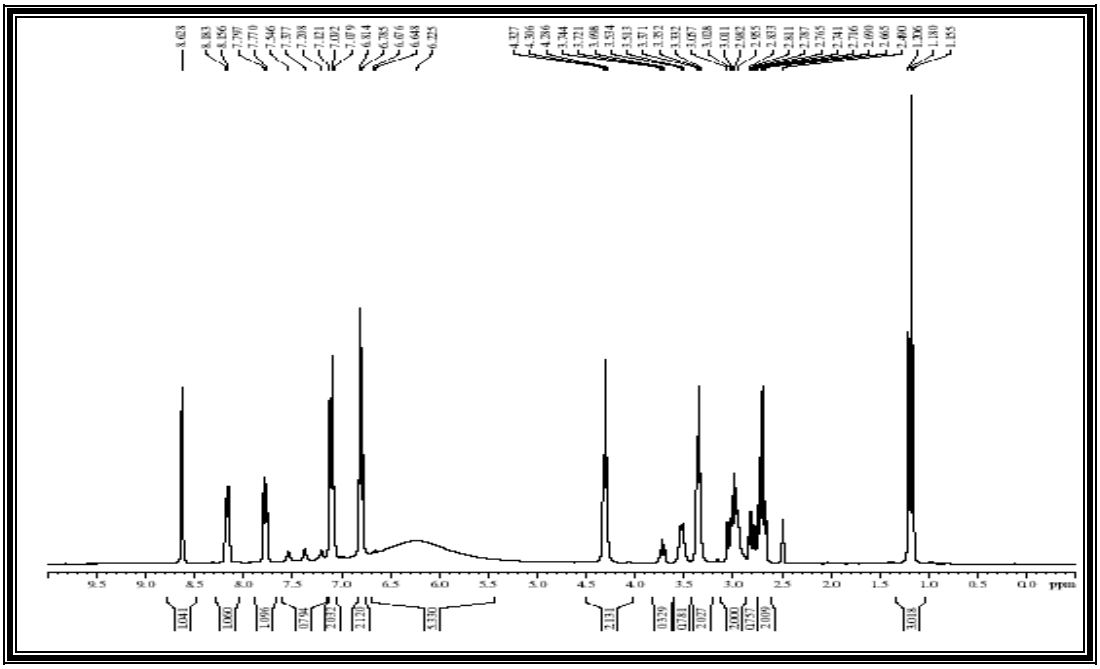


Fig. 23: ^{1}H-NMR spectrum of Degradation Product-1

Table 4: ^{13}C-NMR of Degradation Product-1

Chemical Shift (ppm)	Nature of Carbon	Assignment
173.90	Quaternary	19
157.00, 152.72		2, 11
140.26		5
130.90		14
142.96, 142.67	Tertiary	6, 4
130.30, 126.29		13, 15, 3
114.46		12, 16
42.00		**18**
65.94	Secondary	10
40.41		**17**
33.80		9
24.88		7
14.83	Primary	8

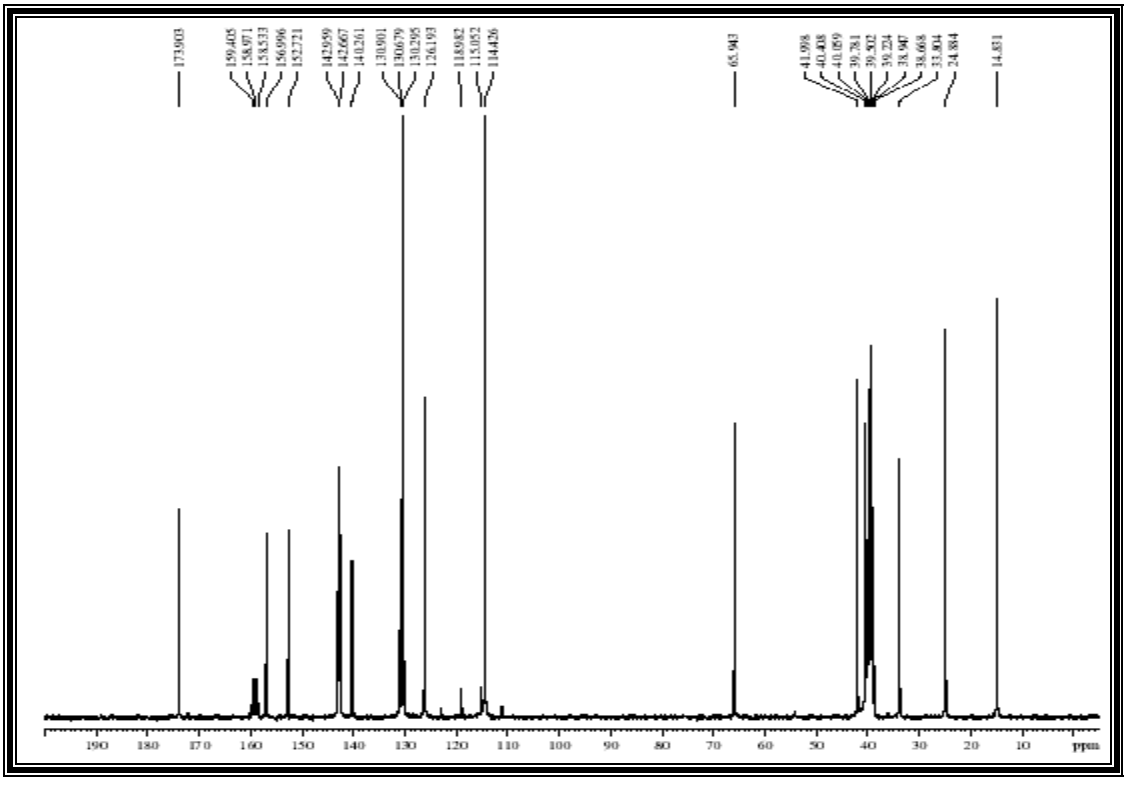


Fig. 24: ^{13}C-NMR spectrum of Degradation Product-1

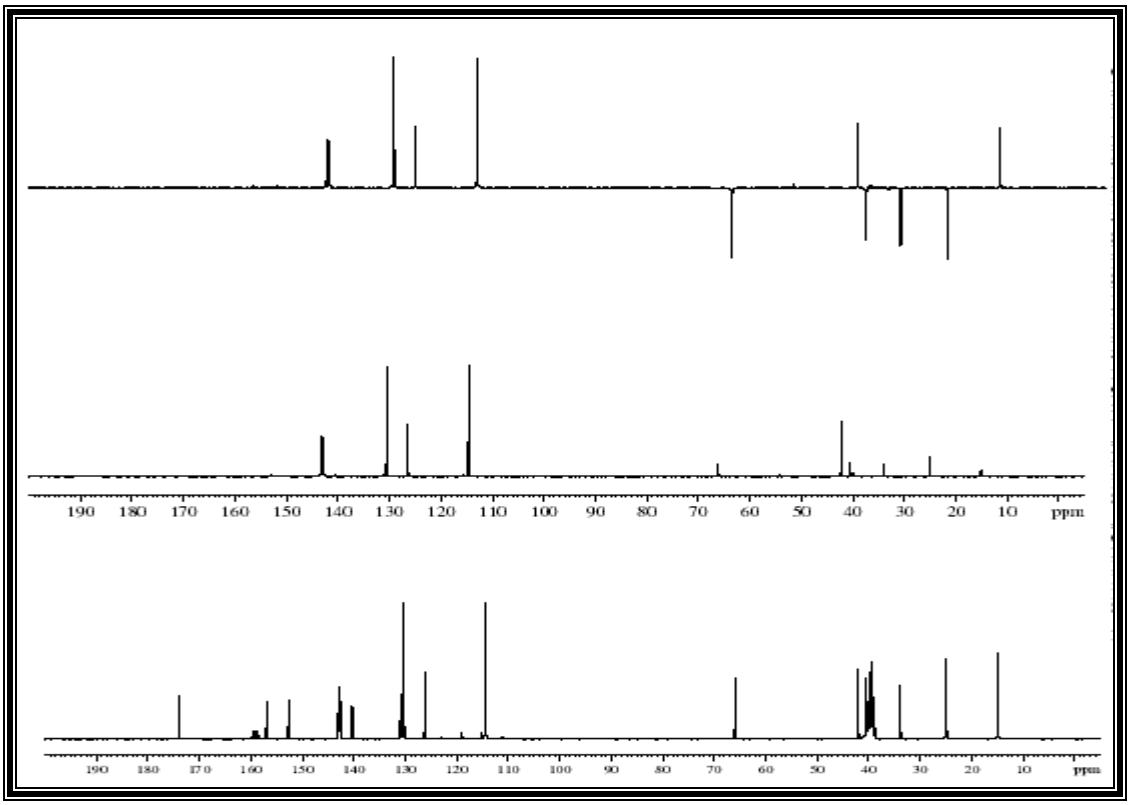


Fig. 25: ^{13}C-DEPT spectrum of Degradation Product-1

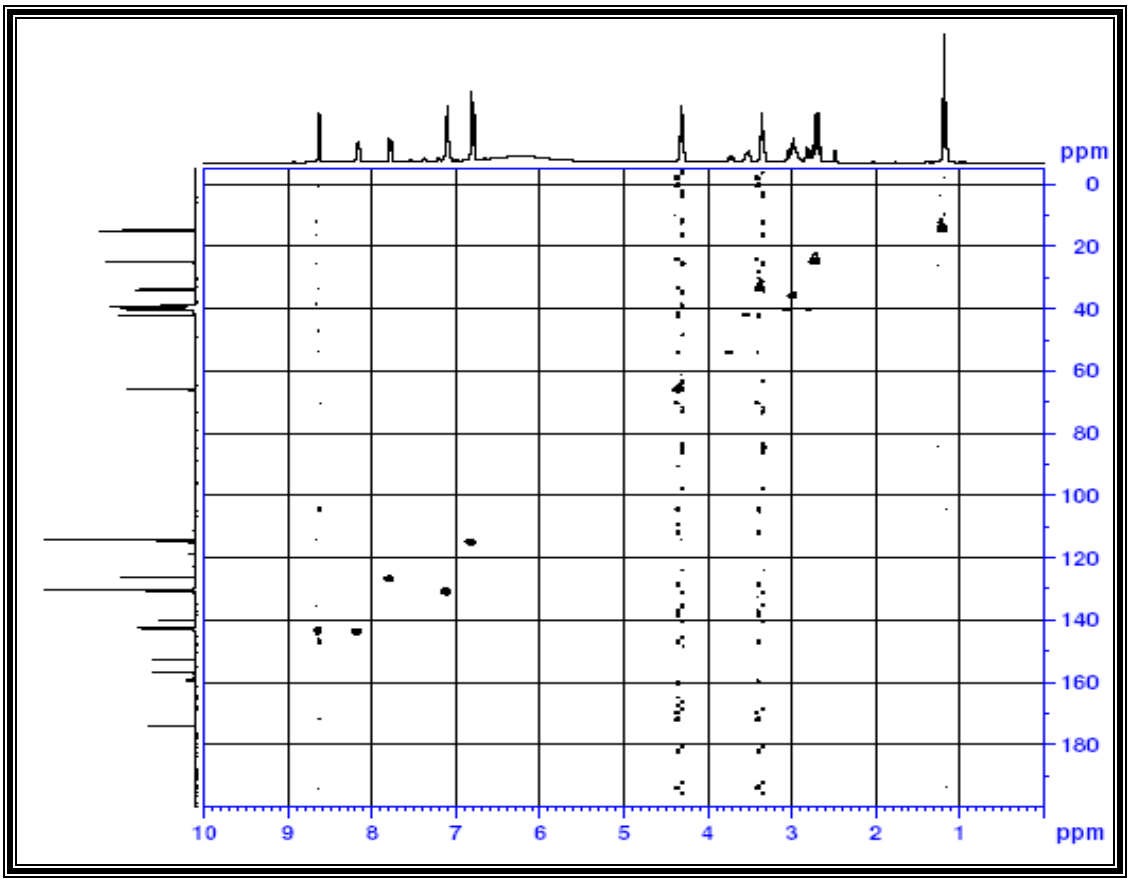


Fig. 26: HMQC spectrum of Degradation Product-1

Degradation Product-2

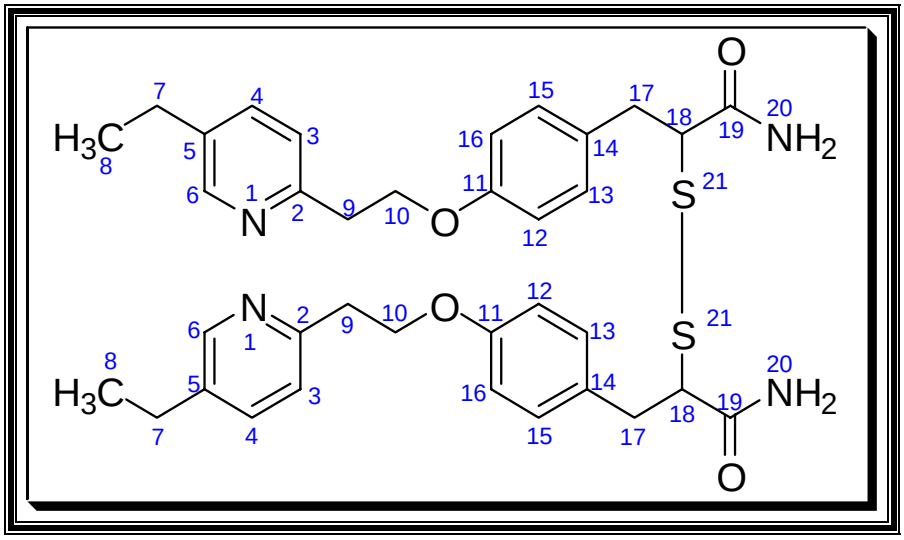


Table 5: ^{1}H-NMR of Degradation Product-2

Chemical Shift (ppm)	Proton No.
8.63 (s, 1H, ArH)	6
8.19 (d, J=6.9 Hz, 1H, ArH)	4
7.79 (d, J=8.2 Hz, 1H, ArH)	3
7.09 (d, J=7.9 Hz, 2H, ArH)	12, 16
6.81 (d, J=8.3 Hz, 2H, ArH)	13, 15
4.32 (t, 2H, CH_2)	10
3.72 (t, 1H, CH)	18
3.35 (t, 2H, CH_2)	9
3.04 – 2.95 (m, 2H, CH_2)	17
2.72 (q, 2H, CH_2)	7
1.20 (t, 3H, CH_3)	8

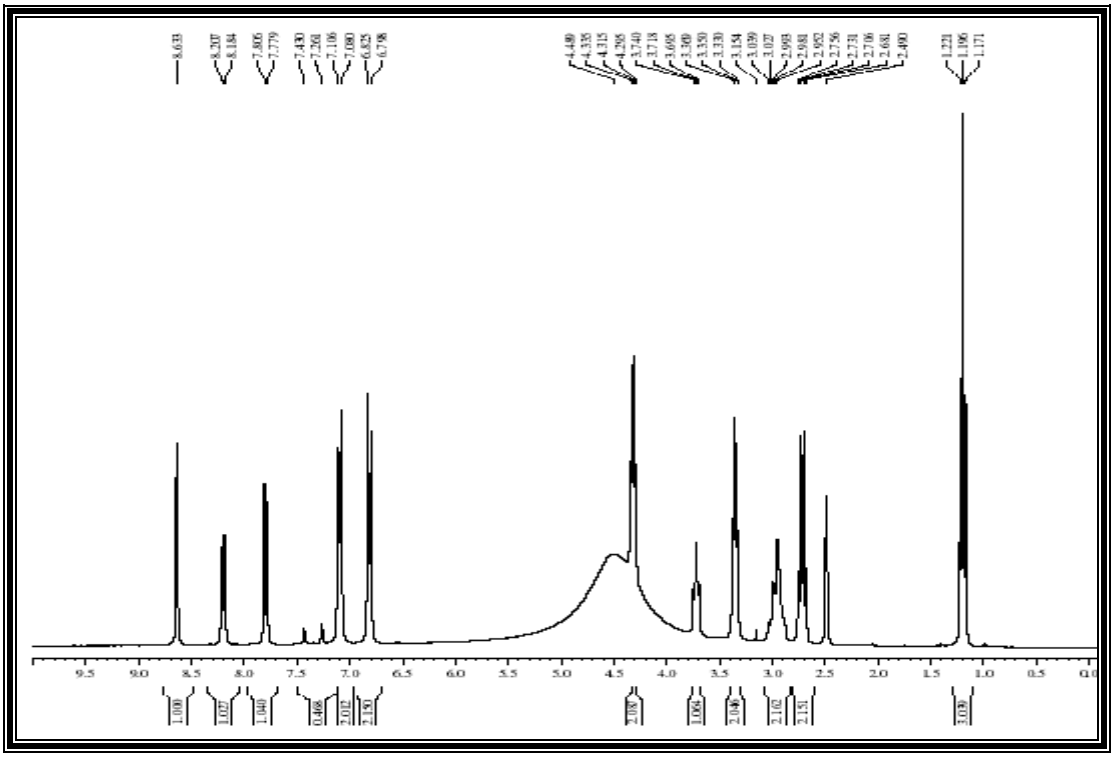


Fig. 27: ^{1}H-NMR spectrum of Degradation Product-2

Table 6: ^{13}C-NMR of Degradation Product-2

Chemical Shift (ppm)	Nature of Carbon	Assignment
172.04, 171.96	Quaternary	19
156.92, 152.54		2, 11
140.23		5
130.02		14
143.07, 142.48	Tertiary	6, 4
130.22		13, 15
126.13		3
114.41		12, 16
53.84		**18**
65.82	Secondary	10
35.96		**17**
33.66		9
24.78		7
14.82	Primary	8

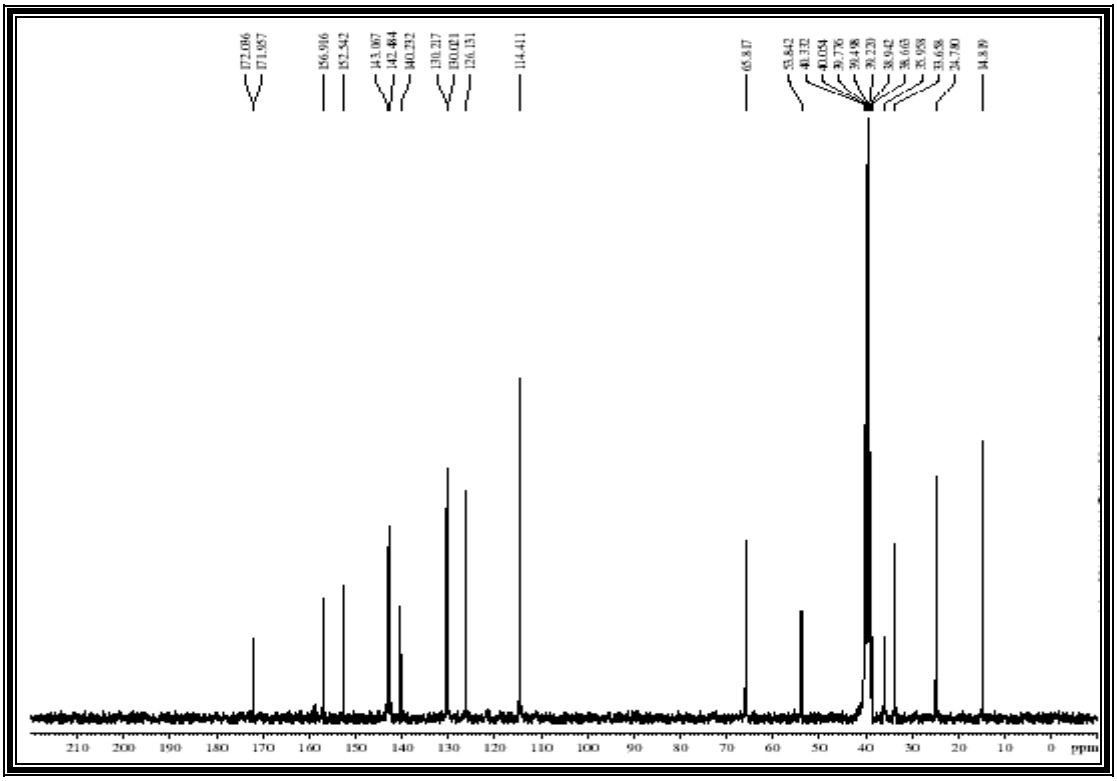


Fig. 28: ^{13}C-NMR spectrum of Degradation Product-2

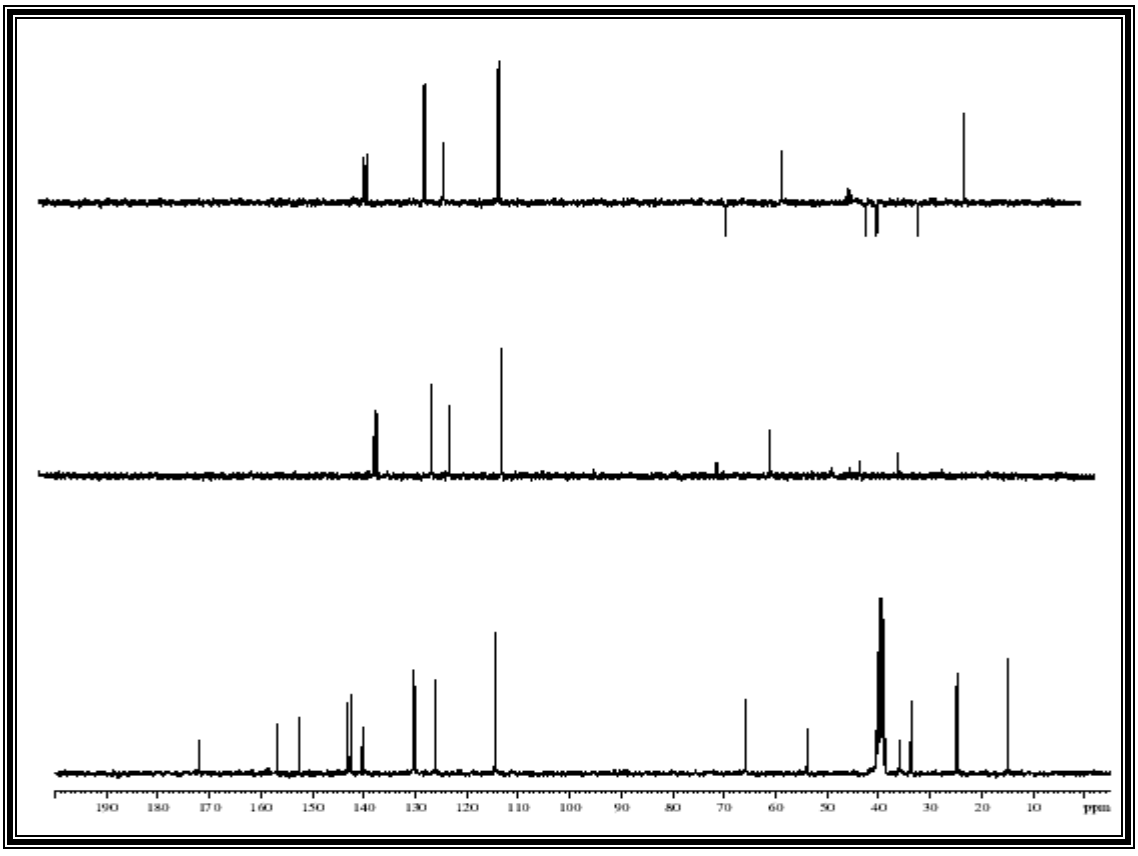


Fig. 29: ^{13}C-DEPT spectrum of Degradation Product-2

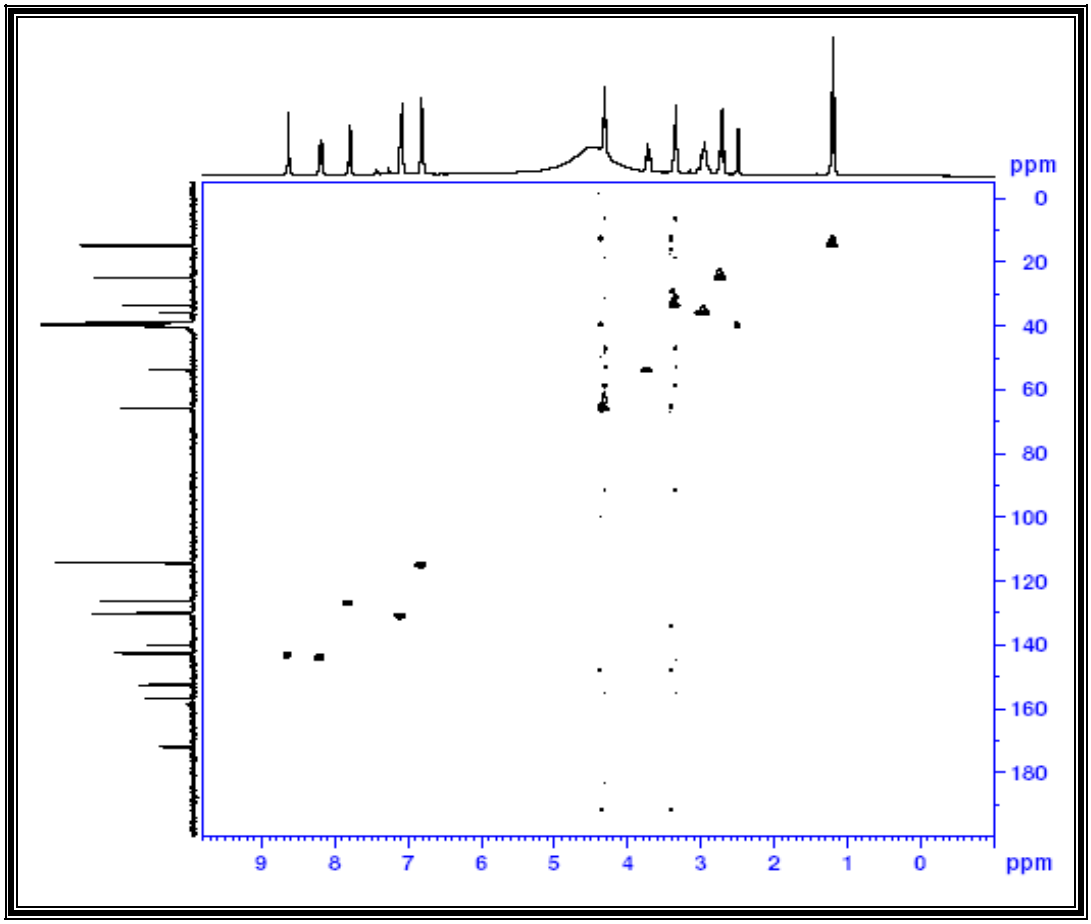
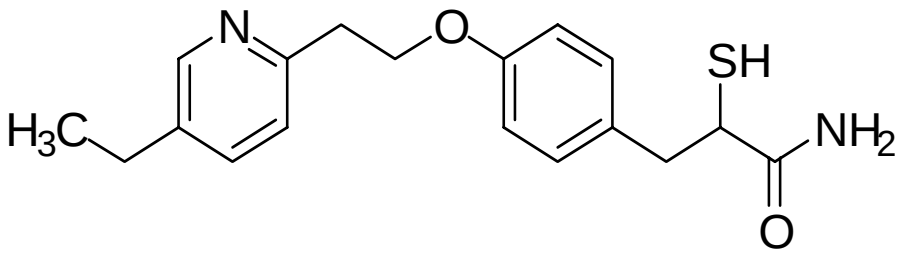
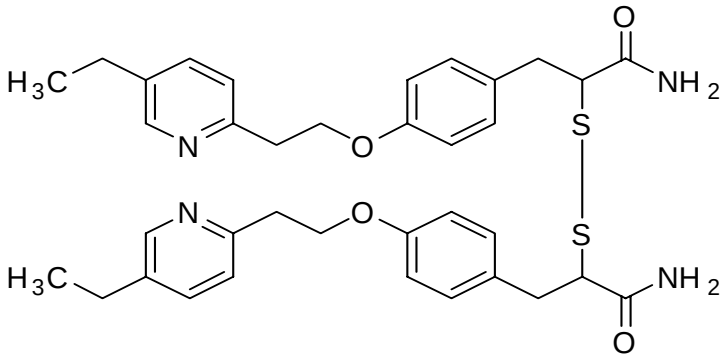


Fig. 30: HMQC spectrum of Degradation Product-2

Form above spectroscopic data of Mass, IR and NMR (^{1}H, ^{13}C, DEPT and HMQC) confirmed the structure of both the degradation products and the values of spectroscopic data are in total agreement with assigned chemical structure. The proposed structures of the degradation products are as below.



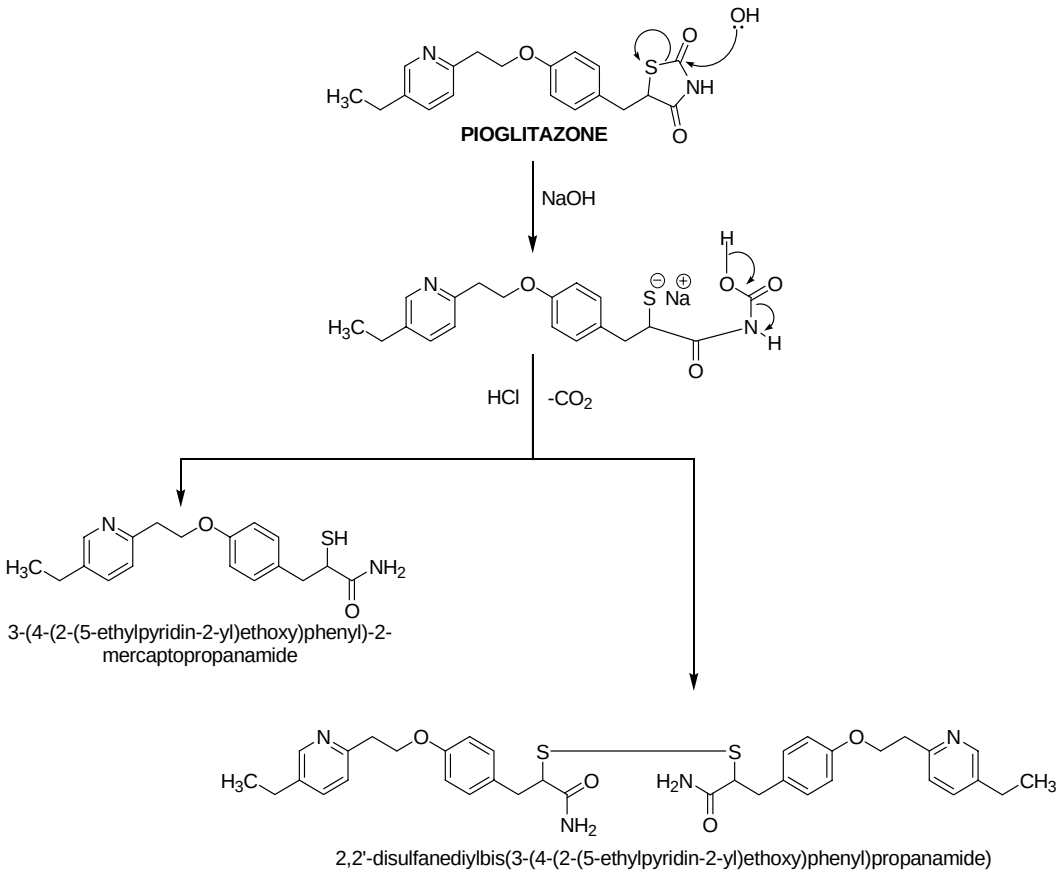
Degradation Product-1



Degradation Product-2

5.4 Proposed Mechanism of Degradation

The above assigned structure of both the degradation products is also supported by mechanistic possibility of probable degradation of pioglitazone under base treatment. The carbonyl group attached to sulphur in pioglitazone is more acidic, therefore anion of hydroxyl group will attach on that position and bond between sulphur and carbonyl group will break, during work-up carbon dioxide will remove and degradation product-1 will form. Additionally, there is obvious possibility of dimerization and formation of disulphite bond because of air oxidation. The probable mechanism is described below.



5.5 Chemical Evidences for the Structure of Degradation Products

The structure of degradation products were assigned by spectroscopic data and mechanistic possibility of the base degradation of pioglitazone. To support our hypothesis chemical evidence of formation of dimer is illustrated.

It is well known that '–S-H' can be oxidize to '–S-S-' by catalytic amount of iodine. Therefore, Degradation Product-1 can be converted in Degradation Product-2 by small pinch of iodine. The chromatogram of the pioglitazone hydrochloride was taken for the study is shown in Fig. 31. The experiment was carried out by taking 200 mg of pioglitazone in 10 mL 5N NaOH and heated for 1 hr at 90° C. Extend of degradation was checked by HPLC. The chromatogram after base degradation is shown is Fig. 32.

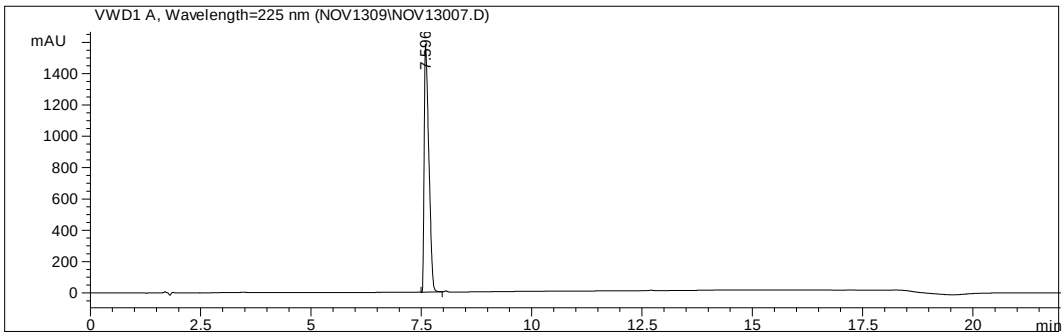


Fig. 31: Chromatogram of Pioglitazone Hydrochloride (For Retention Time)

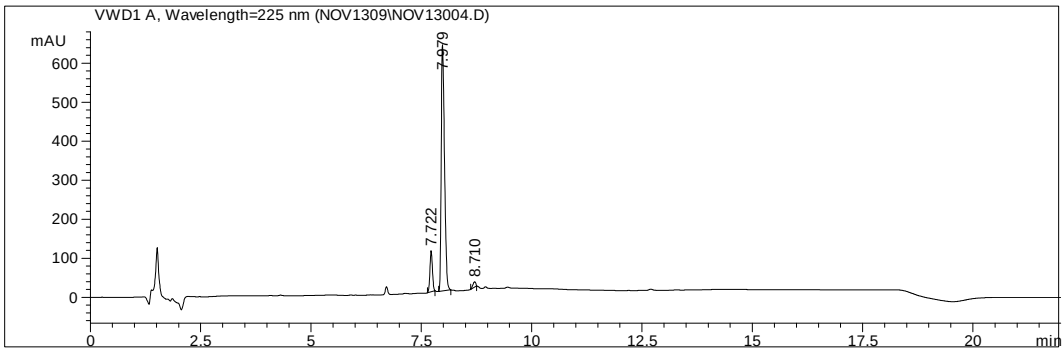


Fig. 32: Chromatogram after base dedgradation

It can be seen from the Fir. 32 that Degradation Product-1 (RRT=1.04) was formed 87.5 %, Degradation Product-2 (RRT=1.12) formed 1.5 % and Pioglitazone remain 11 % by area normalization. The catalytic amount of iodine was added into

reaction mixture and stirred at room temperature for half an hour. Extend of formation of dimer was checked by HPLC. The chromatogram after addition of iodine is shown Fig. 33.

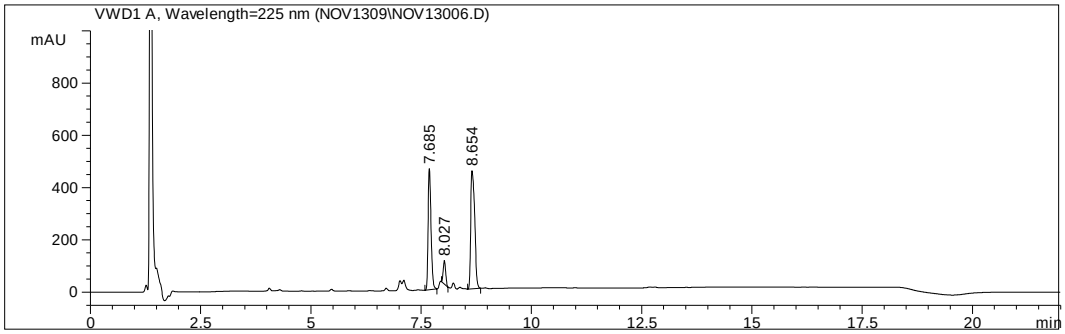


Fig. 33: Chromatogram after addition of iodine

It can be observed from the Fig. 33 that Degradation Product-2 increased very much (52 %) after addition of iodine while peak of Degradation Product-1 is almost disappear compare to Fig. 32. It can be concluded that Degradation Product-2 is a dimer form of Degradation Product-1.

6. Conclusion

The study describes, the isolation and characterization of degradation products in Pioglitazone hydrochloride stress studies.

Systemic degradation of the pioglitazone hydrochloride was studied in five different stress conditions (acid, base, peroxide, UV-light and thermal). The significant degradation was found in base treatment. Two major degradation products were formed at RRT 1.04 and 1.12.

The condition of base degradation was scaled-up and reaction mixture was subjected to preparative HPLC isolation. The degradation products 1 and 2 were successfully isolated with HPLC purity greater than 98 %.

Both degradation products along with pioglitazone was completely characterized by ^{1}H, ^{13}C-NMR and DEPT, IR, UV, Mass and HPLC. The spectroscopic arguments were advanced to support characterization.

The mechanistic support for the proof of structure was also discussed, further support of structure was derived from the oxidative dimerization of R-S-H to R-S-S-R. Additionally, under reductive conditions, the conversion of R-S-S-R to R-S-H was confirmed, which supports the existence of disulfide bridge in degradation product-2.ity. The chemical conversion of degradation product-1 into 2 was studied for additional evidence.

The proposed structure of the degradation products of pioglitazone under base treatment has been confirmed by spectroscopic, mechanistic and chemical evidence.

7. References

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FORMULAS

Mean: $\dfrac{\text{Sum of all Values}}{\text{Number of Values}}$

Standard Deviation (SD ( )):

$$S.D. = \sqrt{\frac{\sum_{i=1}^{n} (y_i - M)^2}{(n-1)}}$$

$$M = \frac{\sum_{i=1}^{n} y_i}{n}$$

Relative Standard Deviation (RSD) [%]: $\dfrac{\text{Standard Deviation x 100}}{\text{Mean}}$

Recovery: $\dfrac{\text{Obtained (Recovered) value X 100}}{\text{True value (Added)}}$

Quantitation from Calibration Curve:

$$x = \frac{\text{y - Intercept}}{\text{Slope}}$$

Limit of Detection (LOD): $= \dfrac{3.3}{S}$

Limit of Quantification (LOQ): $= \dfrac{10}{S}$

LIST OF SYMBOLS AND ABBREVIATIONS

conc	:	Concentration
ppm	:	Parts Per Million
g	:	gram
mg	:	Milligram
min	:	Minute
mL	:	Milliliter
N	:	Normality
nm	:	Nanometer
No	:	Number
UV	:	Ultraviolet
µg	:	Microgram
µL	:	Microliter
m	:	Micrometer
%	:	Percentage
° C	:	Degree Centigrade
r	:	Correlation Coefficient
	:	Greater Than or Equal to
≤	:	Less Than or Equal to
$	:	Dollar
AU	:	Absorbance Unit
mAU	:	Milli Absorbance Unit
6-MP	:	6-Mercaptopurine
ACN	:	Acetonitrile
API	:	Active Pharmaceutical Ingredient
Avg	:	Average
CEC	:	Capillary Electrophoresis Chromatography
cGMP	:	Current Good Manufacturing Practice
EP	:	European Pharmacopoeia
FDA	:	Food and Drug Administration
H_2O_2	:	Hydrogen Peroxide
HCl	:	Hydrochloric acid
HPLC	:	High Performance Liquid Chromatography

ICH	:	International Conference on Harmonisation
IR	:	Infrared
LOD	:	Limit Of Detection
LOQ	:	Limit Of Quantitation
MeOH	:	Methanol
MS	:	Mass Spectroscopy
NaOH	:	Sodium Hydroxide
NCE	:	New Chemical Entity
NLT	:	Not Less Than
NMT	:	Not More Than
NMR	:	Nuclear Magnetic Resonance
ODS	:	Octa Decyl Silane
PDA	:	Photo Diode Array
RT	:	Retention Time
RRT	:	Relative Retention Time
RRF	:	Relative Response Factor
RSD	:	Relative Standard Deviation
SD	:	Standard Deviation
SFC	:	Supercritical Fluid Chromatography
TFA	:	Trifluoro Acetic acid
TLC	:	Thin Layer Chromatography
Temp	:	Temperature
UPLC	:	Ultra Performance Liquid Chromatography
US	:	United State
USP	:	United States Pharmacopoeia.
UV	:	Ultra Violate
WHO	:	World Health Organization