

**ASSURING QUALITY OF PHARMACEUTICALS
THROUGH IMPURITY PROFILING**

A thesis submitted in partial fulfillment for the degree of

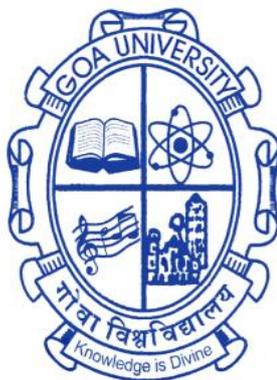
DOCTOR OF PHILOSOPHY

IN

PHARMACY

To

GOA UNIVERSITY



By

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April 2021

DECLARATION

I, Adison fernandes hereby declare that this thesis entitled “**ASSURING QUALITY OF PHARMACEUTICALS THROUGH IMPURITY PROFILING**” represent work which has been carried out by me and that it has not been submitted, either in part or full, to any other University or Institution for the award of any research degree.

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CERTIFICATE

I hereby certify that the above Declaration of the candidate, **Adison Fernandes** is true and the work entitled “**ASSURING QUALITY OF PHARMACEUTICALS THROUGH IMPURITY PROFILING**” was carried out under my supervision.

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ENDORSEMENT

This is to certify that the thesis entitled “**ASSURING QUALITY OF PHARMACEUTICALS THROUGH IMPURITY PROFILING**” is a bonafide research work done by **Adison Fernandes** in the Research Laboratories of Goa College of Pharmacy under the guidance of **Dr. Sanjay Pai PN**, Professor and Head, Dept. of Pharmaceutical Chemistry, Goa College of Pharmacy, Panjim-Goa.

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Dedicated
To
My Beloved Wife
&
Beloved Sons-
Ezekiel, Jeremiah and Daniel

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ABSTRACT

During synthesis of API, its formulation to medicine and subsequent storage, certain unwanted chemicals might remain or develop with time. These are referred to as impurities in pharmaceuticals. The safety and potency of the pharmaceutical product is affected by the presence of these impurities. The quality and safety of a drug is established by tracking and managing the impurities efficiently. Impurity profiling and forced degradation study of drug is considered to be important for ensuring the standard of the drug.

Method development for analysis of drug substance in presence of their specified impurities and degradants is challenging. The search for mobile phases is considered challenging in situations that present a mixture of compounds for separations with diverse physicochemical properties. Varying mobile phase pH is a typical tool for controlling critical chromatographic parameters like retention time, shape of peak and also selectivity. Examining the effect of changes in pH on the separation profiles is recommended to assess the method robustness.

Properties that lead to a change in migration parameters are exploited for identifying a mobile phase that provides complete separation of compounds as seen in their chromatograms with good baseline separation for all the peaks. Certain special techniques like derivatization were applied for identifying compounds with poor chromophoric properties and MS detector exploited for characterization of impurities that arise from stress-induced studies.

Case studies for four drugs were considered viz., Mefenamic acid, Carbimazole, Cyclizine Hydrochloride and Tolfenamic acid for stability indicating HPLC method development. Quantification of Mefenamic acid and its pharmacopoeial impurities (Imp A, Imp C and Imp D) was carried on Waters Sunfire C18 column with acetonitrile: phosphate buffer pH 4 (55:45 % v/v) as mobile phase at detection wavelength of 225 nm, in isocratic mode. Similarly for estimation of Tolfenamic acid and its pharmacopoeial impurities (Imp A and Imp B) by RP HPLC, a simple mobile phase with two components i.e. mixture of acetonitrile and 10 mM ammonium dihydrogen ortho phosphate buffer (pH adjusted to 2.5 with ortho phosphoric acid) in the ratio of 80:20 % v/v at detection wavelength of 205 nm and flow rate of 1 min/ml on C18 column was carried.

An alternative RP HPLC method was developed for quantification of Cyclizine Hydrochloride and its pharmacopoeial impurities (Imp A and Imp B) on Waters Sunfire C18 column with acetonitrile: phosphate buffer pH 6.5 (80:20 % v/v) as mobile phase at detection wavelength of 225 nm, in isocratic mode. Imp A lacked a chromophore in its chemical structure resulting in the need for its derivatization. Derivatized Imp A showed absorption in the UV region. For quantification of Carbimazole and its pharmacopoeial impurity A, a new RP HPLC method was developed on Waters Sunfire C18 column with acetonitrile: phosphate buffer pH 2.5 (50:50 % v/v) as mobile phase at detection wavelength of 260 nm, in isocratic mode with flow rate of 1 min/ml. Through LCMS study, degradation products obtained during stress studies in Carbimazole and Cyclizine Hydrochloride were identified.

Major limitations of existing methods with regard to resolution between peaks, unstable drifting baselines, peaks with reduced areas due to weakly absorbing chromophore and new impurities could be overcome in the proposed methods.

Keywords: Impurity profiling, Mobile phase pH, Stability indicating, RP HPLC, Derivatization, Stress studies, LCMS study.

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LIST OF ABBREVIATIONS

Abbreviations	Full Form
API	Active Pharmaceutical Ingredient
ICH	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
ADME	Absorption Distribution Metabolism and Elimination
LC	Liquid Chromatography
DP	Drug product
DS	Drug Substance
SIAM	Stability Indicating Assay Method
USP	United States Pharmacopoeia
BP	British Pharmacopoeia
IP	Indian Pharmacopoeia
EP	European Pharmacopoeia
PDE	Permitted Daily Exposure
TDI	Tolerable Daily Intake
FDA	Food and Drug Administration
WHO	World Health Organization
DMF	Drug Master File
NDA	New Drug Application
ANDA	Abbreviated New Drug Application
IND	Investigational New Drug Application
MA	Mefenamic Acid
CZ	Carbimazole
CY	Cyclizine Hydrochloride
TA	Tolfenamic Acid
AIBN	2, 2-Azobis Isobutyronitrile
ACVA	Azobis-Cyan Valeric acid
AMPD	Azobis Methyl Propionamidine Dihydrochloride
RPC	Reversed Phase Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
EtOH	Ethanol
THF	Tetrahydrofuran
ACN	Acetonitrile
~	Approximate
pKa	Dissociation Constant
%	Percentage
mg	Milligram
μ	Micron

µg	Microgram
µm	Micrometer
µl	Microliter
ml	Milliliter
mM	Millimolar
N	Normality
mm	millimeter
cm	Centimeter
min	Minute
Conc.	Concentration
nm	Nanometer
Wh/m ²	Watt-hour per square meter
m/z	Mass to Charge Ratio
°C	Degree Centigrade
%	Percentage
w/w	Weight by Weight
v/v	Volume by Volume
w/v	Weight by Volume
amu	Atomic Mass Unit
λ	Lambda
Max	Maximum
δ	Delta
NLT	Not Less Than
NMT	Not More Than
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
COX	Cyclooxygenase
LD	Lethal Dose
ID	Internal Diameter
N	Normality
RT	Room Temperature
RH	Relative Humidity
HCl	Hydrochloric Acid
NaOH	Sodium Hydroxide
H ₂ O ₂	Hydrogen Peroxide
Imp	Impurity
OPA	Ortho Phosphoric Acid
dil HAC	Dilute Glacial Acetic Acid
NDB-Cl	4-Chloro-7-nitrobenzofurazan
Fig	Figure
g/mol	Grams per Mole
AR	Analytical Reagent
ODS	Octadecyl-Silica

SD	Standard Deviation
RSD	Relative Standard Deviation
% RSD	Percentage Relative Standard Deviation
T _f	Tailing Factor
R _t	Retention Time
RRT	Relative Retention Time
R _s	Resolution
n	Number of Sample
TLC	Thin layer chromatography
UV	Ultra Violet
IR	Infra Red
FT-IR	Fourier Transform Infra red
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Liquid Chromatography
NMR	Nuclear Magnetic Resonance
LC-MS	Liquid Chromatography – Mass Spectrometry
ESI	Electro Spray Ionization
TIC	Total Ion Charge
GC-MS	Gas Chromatography-Mass Spectrometry
CE	Capillary Electrophoresis
SFC	Supercritical Fluid Chromatography
PDA	Photo Diode Array Detector
R & D	Research and Development
F & D	Formulation and Development
TTC	Threshold of Toxicological Concern
NA	Not available

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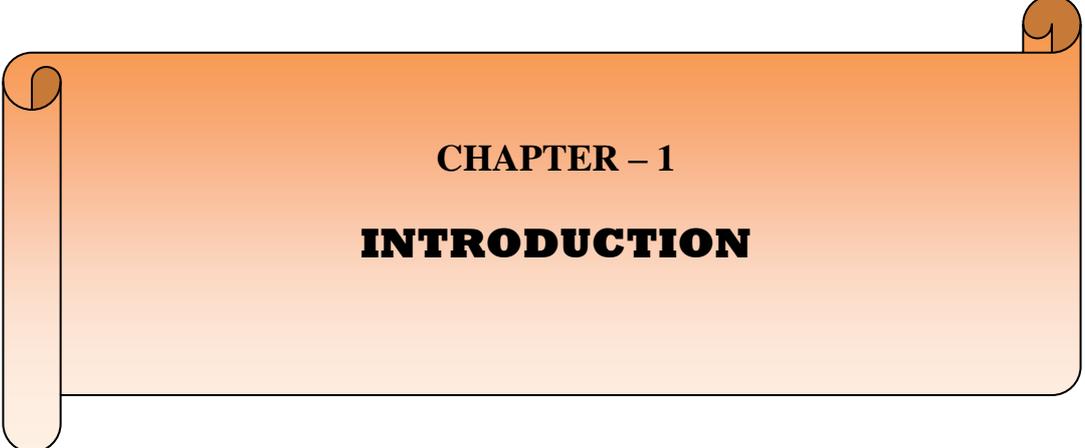
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CHAPTER – 1

INTRODUCTION

1. INTRODUCTION

The bulk drug industry provides active pharmaceutical ingredient (API) of specific quality to all the pharmaceutical industries. Recently great emphasis is laid towards the quality of pharmaceuticals that comes in the market. A drug formulation administered in human body to provide desired pharmacological action contains both active and inactive ingredients. The therapeutic efficacy is due to the active ingredient, i.e. API whereas the inactive ingredient has no pharmacological activity. API present in a formulation is generally not absolutely pure¹.

A big challenge for bulk and pharmaceutical industry is to have quality products. It's absolutely impossible to obtain an absolutely pure API because of its highly expensive processes. Hence it's mandatory to carry out vigorous quality control tests to check for its quality and purity from each industry. Various factors are responsible for purity of API such as raw materials, method of their manufacture, crystallization type and purification processes. Thus, carrying out impurity profiling is very necessary².

During synthesis of API, its formulation to medicines and subsequent storage, certain unwanted chemicals might remain or develop with time. This are referred as impurities in pharmaceuticals. The safety and potency of the pharmaceutical products is affected by the presence of these impurities. It's not necessary that the impurities are always inferior. The purity of drug substance is compromised depending on its usage even if contains another substance with higher pharmacological or toxicological properties³.

Impurity profiling provides details of impurities present in the drug under investigation. It also gives an estimate of the actual amount of different kinds of impurities in the drug. Impurity profiling enlists the types of maximum possible identified or unidentified impurities present in any API produced by a specific controlled manufacturing process. Impurity profiling involves different analytical studies conducted on impurities for its detection, identification/structure elucidation and quantitative in bulk drugs and pharmaceutical formulations.

Potential of impurities with teratogenic, mutagenic or carcinogenic effect have a significant health implications. Due to these various reasons, regulatory authorities like U.S. Food and Drug Administration (FDA) and The International Conference on Harmonization (ICH) has attracted critical attention on impurity profiling.

The United States Pharmacopoeia (USP) and the British Pharmacopoeia (BP) along with other pharmacopoeias, in their API and formulations have started incorporating limits to allowable levels of impurities. One of the goals of analytical activity in the drug development process involves identification of pharmaceutical impurities and to elucidate its chemical structure above a particular threshold. In modern drug analysis it is considered to be the core activity to characterize the quality and stability of bulk drugs and pharmaceutical formulations⁴⁻⁷.

Following are the reasons the drug manufacturers and drug registration authorities have developed an increasing interest of the impurity profiles of bulk drug substances⁸.

a) In the development of a new drug or new process for an existing drug it's important to know the structures of the impurities. With this information synthetic organic chemists is able to do the necessary changes in the reaction conditions so that the formation of the impurity can be avoided or its quantity be reduced to an acceptable level.

b) Once structures are finalized for the impurities, they can be synthesized and final evidence can be provided for their structures initially determined by spectroscopic methods.

c) For development of selective method for quantitative estimation of the impurity and further use of this method as a component of the quality testing of each batch , the impurity synthesized can be used as an 'impurity standard'.

d) The major impurities can be subjected to toxicological studies, contributing to the safety of drug therapy.

e) For drug authorities the impurity profile of a drug substance acts as fingerprint indicating the level and constancy of the manufacturing process of the bulk drug substance.

1.1. IMPURITY

As per ICH guideline, impurity is defined as any component of the new drug substance or the new drug product that is not the chemical entity defined as the new drug substance or not the drug substance or an excipient in the drug product⁹⁻¹⁰.

The safety of a formulation depends on the toxicological properties of the API and also on the impurities if present. Therefore, identification, quantification, and control of impurities in the drug substance and drug product, are an important part of drug development and regulatory assessment.

1.2. GENERAL TERMINOLOGY USED TO DESCRIBE IMPURITIES

Various terms are used to describe the substances that can affect the purity of the API. Following are few terms commonly used in the pharmaceutical industry to describe them⁷.

1.2.1. Starting material

These are the substances that are used to start the synthesis of an API.

1.2.2. Intermediates

The products produced during synthesis of the desired substance are called intermediates especially when they are isolated and characterized.

1.2.3. Penultimate intermediate (Final intermediate)

This is the last compound in the synthesis chain prior to the production of the final desired compound. Generally called as final intermediate

1.2.4. By-products

The unplanned compounds produced in the reaction are generally called by-products. Generally it may or may not be possible to theorize all of them.

1.2.5. Transformation products

Transformation products are found to be very similar to by-products, except this term tends to connote that more is known about the chemical reaction that can lead to these products.

1.2.6. Interaction products

These products could be formed due to interactions between various chemicals involved (intentionally or unintentionally). Two types of interaction products that can be commonly seen are drug substance-excipient interactions and drug substance-container/closure interactions.

1.2.7. Related products

The term related products highlights the similarity of the impurity with the drug substance. It generally has similar chemical structures as the API and might exhibit potentially similar biological activity.

1.2.8. Degradation products

Degradation products (DPs) are produced because of decomposition of the interest substance or active ingredient. It also includes products produced from degradation of other compounds that may be present as impurities in the drug substance.

1.3. COMPENDIAL TERMINOLOGY USED TO DESCRIBE IMPURITIES

The United States Pharmacopoeia (USP) highlights impurities in several sections ^{7, 11}:

- A) Impurities in official articles
- B) Ordinary impurities
- C) Organic volatile impurities

The following terms have been used by the USP to describe impurities;

1.3.1. Foreign substances

Certain materials get incorporated by adulteration or contamination and are not obtained during synthesis or preparation is called foreign substances, e.g., pesticides in oral analgesics.

1.3.2. Toxic impurities

These impurities possess significant biological activity which is undesirable, and hence require specific identification and quantitative estimation by explicit tests.

1.3.3. Concomitant component

These impurities exhibit identical molecular formula and same connectivity between the atoms but differ in the arrangement of its atoms in three dimensional spaces, which lead to differences in pharmacological/toxicological profiles. Hence these impurities needs to be monitored carefully, e.g., chiral impurities.

1.3.4. Signal impurities

These include some degradation products or process-related impurities which provide important information about the process.

These impurities require individual identification and quantification by explicit tests, and differ from ordinary impurities.

1.3.5. Ordinary impurities

The category of impurities in bulk pharmaceutical chemicals which are innocuous and do not possess any serious biological activity in the amount present are called as ordinary impurities.

1.3.6. Organic volatile impurities

These include the solvents used for synthesis or formulation of the drug product.

1.4. ICH TERMINOLOGY USED TO DESCRIBE IMPURITIES

As per ICH guidelines, impurities produced during chemical synthesis in the drug substance are broadly classified into the following categories⁹.

1.4.1. Organic impurities

Starting material, processes related impurities, intermediates, degradation products

1.4.2. Inorganic impurities

Salts, catalyst, ligands, heavy metals or other residual metal

1.4.3. Other materials

Filter aids, charcoal

1.4.4. Residual solvents

Organic and inorganic liquids used during production and / or crystallization.

The organic volatile solvents are classified by ICH¹⁴ as follows.

Class I (to be avoided): benzene, carbon tetrachloride, 1, 2-dichloromethane, 1, 1-dichloroethane, and 1, 1, 1-trichloroethane

Class II (should be limited): acetonitrile, chloroform, methylene chloride, 1, 1, 2-trichloroethane, 1, 4-dioxane, pyridine etc.

Class III (low toxic potential and permitted daily exposure (PDE) of 50 mg or more): acetic acid, acetone, 1-butanol, ethanol, ethyl acetate, formic acid, tetrahydrofuran etc.

Class IV (solvent for which adequate toxic data are not available): 1, 1-diethoxypropane, 2, 2-dimethoxypropane, isopropyl ether, petroleum ether, trifluoroacetic acid etc.

1.5. CLASSIFICATION OF IMPURITIES

Impurities are classified into five main categories based on its sources ^{4, 5,7,12}.

1.5.1. Synthesis related impurities

Impurities in new drug substance or new chemical entity (NCE) generally appear from raw materials, by products, intermediates and solvents during its synthesis process. Raw material used for synthesis is of low purity level than a drug substance; hence they contain components which can react with other chemicals used during synthesis of a drug substance and thus affect its purity.

Some impurities are produced by reaction of impurities present in solvent itself which are used in synthesis, can range from trace levels to high quantity. Intermediates formed during process of synthesis, can form impurity in final product if it is not purified to higher level as in case of drug substance. The purity of such types of impurities is controlled by performing regulatory purity/impurity testing in pharmaceutical synthesis. This frequently entails residual solvents which are not used in further downstream processing or process impurities in cases where they conclusively demonstrate that these moieties are not also degradation products.

Since this step is the last major source of potential impurities, it is therefore desirable that the analytical methods used at this stage be rigorous. Base-to-salt or acid-to-salt conversions can also generate new impurities in the final drug substance. Also it is seen that thermally labile compounds could undergo decomposition if any further processing involves heating.

1.5.2. Formulation related impurities

During the formulation of drug product a number of impurities can arise in drug product due to interaction with excipients during the process. A drug substance is subjected to different conditions during the process of formulation that can steer to its degradation or other deleterious reactions. For example, heat used for drying or for other reasons, can assist degradation of thermally labile drug substances.

Solutions and suspensions are prone to degradation by hydrolysis or solvolysis. Such reactions occur in solid dosage form like capsules and tablets, when any solvent or water is

been used for granulation. Water used in the formulation not only contribute its own impurity but also impart formidable situation for hydrolysis and metal catalysis. Such reactions can occur in other solvents used in formulation. Highly oxidizing materials undergo oxidation if no precautions are taken. Similarly, photochemical reactions are seen in light-sensitive materials. Lyophilization and vortex mixing used during the process of formulation is a high risk operation which can cause impurity formation.

1.5.3. Degradation related impurities

Some impurities are formed due to degradation and /or other interactions on storage of API. Hence it's important to carry out stability studies to predict, evaluate and confirm drug product safety. Stability studies involve assessment of API stability, pre-formulation studies to evaluate harmony of API with the excipients so as to check its stability in the formulation matrix, accelerated stability testing of the drug substance and the drug product, kinetic studies for stability evaluation and determination of expiration date and routine stability studies of drug products in market. These studies are conducted under various exaggerated conditions of light, humidity, and temperature to determine the type of impurities that are generated by degradation reactions.

Kinetic study

In pharmaceuticals most of the degradation reactions occur at finite rates and are chemical in kind. Such reactions are influenced by conditions like solvent, temperature, concentration of reactants, radiation energy, pH of the medium and the presence of catalysts. The reaction rate depends on the concentration of reactant which in turn describes the order of reaction. Mostly the degradation of pharmaceuticals can be categorized as zero order, first order, or pseudo-first order, while they may also degrade by complicated mechanisms, with true expression is of higher order or can be complex and noninteger.

A perception of the restrictions of heat of activation values obtained experimentally is very critical in stability predictions. For example, where two or more mechanisms of degradation are involved the apparent heat of activation of a pH value is not necessarily constant with temperature. Therefore, it is mandatory to acquire the heat of activation for all bimolecular rate constants in a rate-pH profile to speculate degradation rates at all pH values at various temperatures.

1.5.4. Crystallization related impurities

Polymorphism is a phenomenon where a substance exists in different crystal packing arrangements (polymorphs) but has same elemental composition. Whereas Solvatomorphism is a phenomena where the substance exist in different packing arrangements with different elemental composition. Solid state properties are affected by the nature of structure formed by compound on crystallization. The nature of the crystal structure can affect the crystal shape and color, conductivity, dissolution rate, density, hygroscopicity, rate of reaction, melting and sublimation property, solubility, surface tension, refractive index and viscosity. The goal of pharmaceutical manufacturer is to obtain a drug that is in phase pure and maintains its pure phase state during its storage and subsequent manufacturing into drug product and its storage. This causes the need for development and validation of assay methodology for the determination of phase composition.

1.5.5. Stereochemistry related impurities

There is an uppermost importance for stereochemistry-related compounds (compounds that have different spatial orientation but similar chemical structure). Such compounds are considered as impurities in the API. Chiral molecules (enantiomers) are optical isomers having same chemical structure and different spatial arrangement which results in different optical rotation. It's needs careful monitoring because one form of isomer of a compound can have different pharmacological or toxicological profile from that of the other form of isomer of the same compound. Hence the unwanted optical isomer is viewed as a chiral impurity of the API. It is observed that as the number of asymmetrical carbon atoms in a molecule increases, the number of chiral impurities also increase.

1.5.6. Contamination impurities

Contaminant impurities generally are not related to drug and are not part of the synthesis, extraction, or fermentation process but accidentally introduced during storage and processing. These impurities are considered as adulterating compounds the presence of which is reduced significantly by the current manufacturing technology as compared to few decades ago. Few examples includes heavy metal like lead that leach from pipes of manufacturing /storage tanks , agents sprayed in the manufacturing plant to improve the environment, accidental dropping (human hair or paint chips from wall).

For drug molecule obtained from plant the contaminant impurities includes herbicides sprayed in the environment, heavy metal from soil absorbed by plant and polycyclic hydrocarbons present in air absorbed by leaves of the plant. A thorough process involving selection, cleaning, extraction and purification eliminates such types of impurities.

1.6. REGULATORY ASPECT

Purity of drug substance is increased by lowering the level of impurities at the time of release and also sustaining the low levels of degradants during shelf life of the drug substance. For registration applications ICH^{9-10, 13-15} and Food and Drug Administration of USA (FDA)¹⁶⁻¹⁷ lists guidelines on the content and qualification of impurities that may be present in APIs and drug formulations. These guidelines provide details regarding appropriate reporting, identification and qualification thresholds of impurities based upon the total daily intake of drug.

ICH Guidance addresses impurities to accomplish the following in drug substances¹⁸:

- Identify, qualify, classify, set specifications, and discuss analytical methods for impurities.
- To discuss the long-term and accelerated conditions stability evaluation of the drug substance taking into consideration packaging material variety used for storage and distribution.

The methodology for identifying and quantifying impurities is covered under ICH guideline⁹ for the validation of chromatographic methods. Hence it's necessary to evaluate impurities using a variety of techniques and instruments, before setting purity values to the drug substance. ICH and FDA has formulated certain guidelines to handle issues related to stability and impurity as follows (Fig 1.6.1).

Figure 1.6.1: ICH and FDA guidelines related to stability and impurity

Q 1 A (R2)	Stability Testing of New Drug Substance & Products (ICH) ¹⁴
Q 3 A (R2)	Impurities in Drug Substances (ICH) ⁹
Q 3 B (R2)	Impurities in Drug Products (ICH) ¹⁰
Q 3 C	Impurities: Guidelines for Residual Solvent (ICH) ¹⁹
Q 6 A	Specifications: Test procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances (ICH) ¹⁵
NDA's	Impurities in drug substances (FDA) ¹⁶
ANDA's	Impurities in drug products (FDA) ¹⁷

Adoption of FDA's guidelines has assured that the safety profile is considered identical of generic drugs to brand product. Hence it's mandatory for pharmaceutical companies, in dossier of the drug and drug products to include impurity profile of the drug for submission to regulatory authority. It's also an important regulatory requirement in the office of Generic drugs for obtaining the approvability of ANDAs.

1.7. IMPURITY PROFILING

Impurity profiling involves a set of analytical activities concerned with the detection, identification / structure elucidation and quantitative estimation of impurities (organic and inorganic) including residual solvents in APIs and pharmaceutical products. Impurity profiling has become a core activity in drug analysis for characterizing the quality and stability of APIs and pharmaceutical products²⁰.

The effectiveness and welfare of drug products is impacted by presence of impurity even in small amounts. For this reason it has gained critical attention from regulatory authorities like FDA and ICH. Several books^{5, 20} and journal reviews^{3, 21} have addressed this topic recently.

Detailed workable guidelines are formulated by ICH for control of impurities. Pharmacopoeias, such as USP and BP have included limits to allowable levels of impurities present in the drug substance or drug product, which have led to increasing demand for impurity reference standards along with API reference standards for pharmaceutical companies and regulatory authorities.

Impurities are classified by ICH and provide limits for reporting, identification and qualification threshold with respect to maximum daily dose of drug. Identification refers to structural characterization and qualification means evaluation of biological safety. If the limits of impurity exceed its identification / qualification threshold, it has to be isolated and characterized.

The toxicity data of impurities also need to be generated by those involved in manufacturing of pharmaceutical product. Hence it has streamlined the process of impurity profiling.

1.8. IDENTIFICATION AND QUALIFICATION THRESHOULD OF IMPURITIES IN NEW DRUG SUBSTANCE AND NEW DRUG PRODUCTS

According to ICH guidelines Q3A (R2) and Q3B (R2), qualification is the process of establishing biological safety of individual impurity or a given impurity by acquiring and evaluating sufficient data (Q3A (R2) of an individual DP or a given degradation profile (Q3B (R2) at the level(s) specified.

The above ICH guidelines provide identification and qualification threshold for impurities (New drug substance) and degradation products (new drug products) listed in the following Tables.

Table 1.8.1: Threshold for impurities in drug substance

Maximum daily dose ^a	Reporting threshold ^{b c}	Identification threshold ^c	Qualification threshold ^c
≤ 2 g/day	0.05 %	0.10 % or 1.0 mg/day intake (whichever is less)	0.15 % or 1.0 mg/day intake (whichever is less)
> 2 g/day	0.03 %	0.05 %	0.05 %

^a The amount of drug substances administered per day

^b Higher reporting threshold should be scientifically justified

^c Lower threshold can be appropriate if the impurities are unusually toxic

Table 1.8.2: Threshold for degradation products in drug product

Maximum daily dose ^a	Reporting threshold ^{b c}
≤1 g	0.1 %
> 1 g	0.05 %
Maximum daily dose ^a	Identification threshold ^{b c}
< 1 mg	1.0 % or 5 µg TDI, whichever is lower
1 mg – 10 mg	0.5 % or 20 µg TDI, whichever is lower
> 10 mg – 2 mg	0.2 % or 2 mg TDI, whichever is lower
> 2 mg	0.10 %
Maximum daily dose ^a	Qualification threshold ^{b c}
< 10 mg	1.0 % or 50 µg TDI, whichever is lower
10 mg – 100 mg	0.5 % or 200 µg TDI, whichever is lower
>100 mg – 2 g	0.2 % or 3 mg TDI, whichever is lower
> 2 g	0.15 %

^a The amount of drug substances administered per day.

^b Threshold for degradation products are expressed either as a percentage of the drug substance or as total daily intake (TDI) of the degradation product. Lower threshold can be appropriate if degradation product is unusually toxic.

^c Higher reporting threshold should be scientifically justified.

Identification Threshold: A limit above (>) which an impurity or degradation product should be identified.

Qualification Threshold: A limit above (>) which an impurity or degradation product should be qualified.

Reporting Threshold: A limit above (>) which an impurity or degradation product should be reported.

The "Decision tree for identification and qualification of impurities and/or degradation product" (Fig 1.8) given by ICH guidelines highlights various considerations for the qualification of impurities and/or degradation products when thresholds are exceeded²².

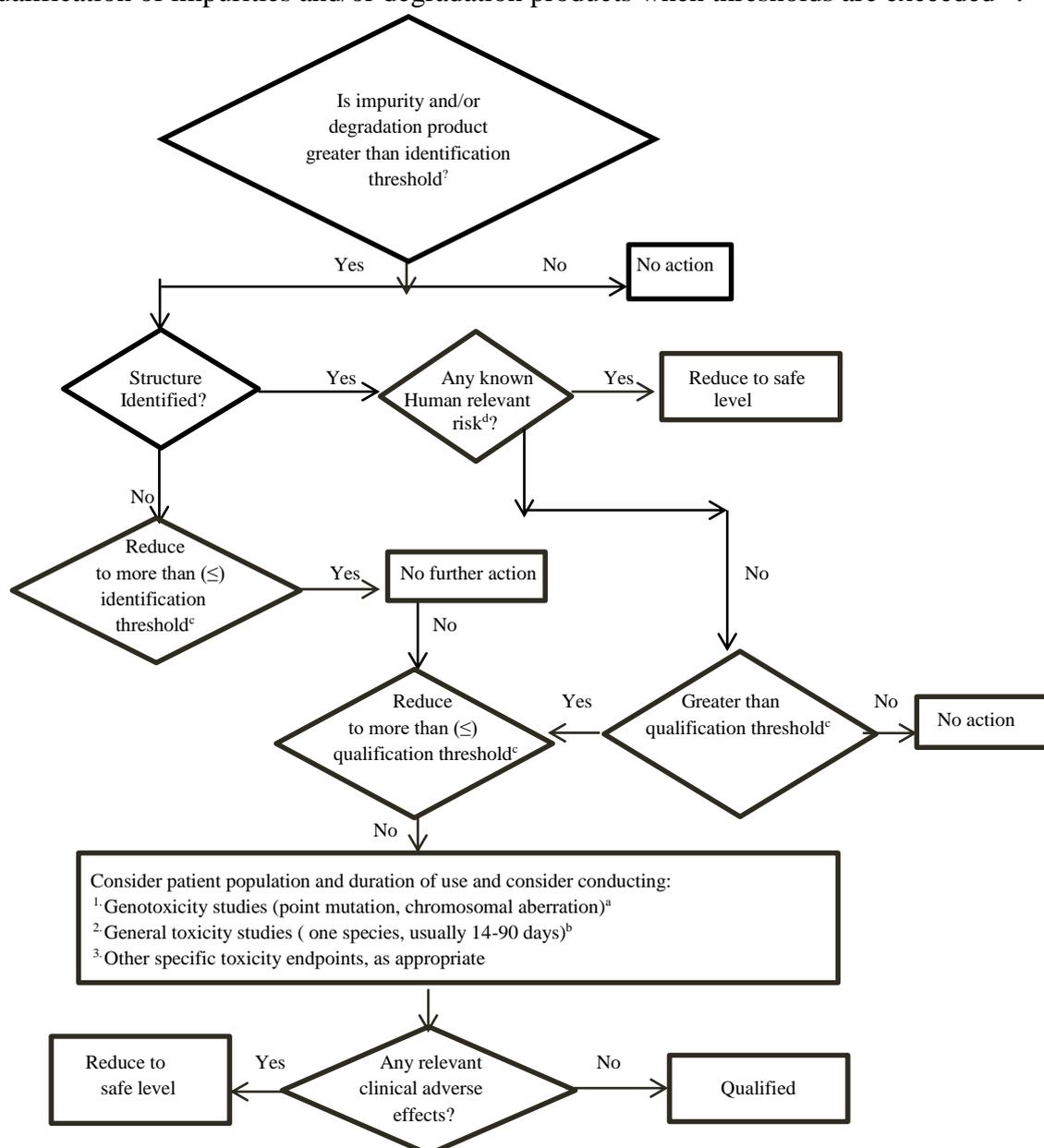


Figure 1.8: Decision tree for identification and qualification of impurities and/or degradation product

- a) A minimum screen (e.g., genotoxic potential) if considered desirable, should be conducted. An appropriate minimum screen study involving detection of point mutation and chromosomal aberration both in vitro should be carried out.
- b) Comparison of unqualified and qualified material should be designed by one or more studies if general toxicity studies are desirable. Based on the accessible appropriate data duration of the study is fixed. The study is performed in the species having potential to detect the toxicity of impurity and/or a degradation product in the species. For single-dose drugs single-dose studies, on a case-by-case basis can be appropriate. Generally minimum period of fourteen days and a maximum duration of ninety days are considered appropriate.
- c) If the impurity and/or degradation product is usually toxic, than lower threshold can be relevant.
- d) For example, does the safety profile of this impurity and/or degradation product or its organisation class prevent human vulnerability at the levels present?

1.9. ISOLATION OF IMPURITIES

Isolation of impurities becomes necessary when the instrumental methods used for analysis of impurity not able to characterize the impurity or when the reference material is required for further confirmation of its structure or its toxicity. Following are the methods used for isolation of impurities⁴.

- Solid- phase extraction methods
- Liquid–liquid extraction methods
- Accelerated solvent extraction methods
- Supercritical fluid extraction
- Column chromatography
- Flash chromatography
- Thin-layer chromatography (TLC)
- Gas chromatography (GC)
- High-pressure liquid chromatography (HPLC)
- Capillary electrophoresis (CE)
- Supercritical fluid chromatography (SFC)

Isolation of impurities involves both chromatographic and non chromatographic methods. Simple methods of isolation are given first preference as it helps in saving time and produce sufficiently a larger quantity of materials with ease. Chromatographic methods should be given first choice when compound of interest to be isolated from complex mixture.

The choice of isolation method depends upon factors like, chemical nature of impurity, complexity of mixture, amount of the impurity required. Mixture containing few impurities with their polarity or pKa is sufficiently different from that of API, the first choice of method is liquid-liquid extraction. In this method of extraction one phase is aqueous and the other is a nonpolar. By adjusting the pH of the aqueous phase, extraction of acidic, basic, or neutral impurities can be done.

Chromatographic techniques are successfully used in isolation of impurities due to better separation efficiency like thin layer chromatography (TLC) and column chromatography. When the resemblance of impurity with API is close and are in low quantity, preparative HPLC becomes the choice of isolation technique.

Capillary electrophoresis is used for those having limited quantities of substances. Mostly this technique is been used for micro-preparative applications.

Supercritical fluid chromatography as an isolation method is used where it is been used as an analytical method for resolving impurities. Advantage of this method involves removal of the mobile phase from the isolated fractions, since the mobile phase is gaseous.

1.10. CHARACTERIZATION OF IMPURITIES

As per ICH there is need to characterize impurity when it is at its identification threshold (0.1%). Characterization is carried out by isolating the impurities and subjecting it to various analytical methods like ultraviolet spectroscopy (UV), raman spectroscopy, infrared spectroscopy (IR), nuclear magnetic resonance (NMR) and mass spectrometric (MS) analysis.

Ultraviolet spectrophotometry (UV) furnishes minimum selectivity of analysis at a single wavelength; however, with the availability of diode array detectors, its possible to obtain at various wavelengths sufficient simultaneous information to assure greater reliability.

Infrared spectrophotometry (IR) provide specific information on limited functional groups which offer selectivity. Raman spectroscopy considered complementary to IR spectroscopy provide complete detail about the vibrational picture of a material. It is not widely used as IR spectroscopy for identification purposes because of its complexity and high cost of instrumentation. However it is a powerful tool for characterizing polymorphs.

The noninvasive and nondestructive nature of NMR spectroscopy makes it a powerful tool for characterization of low-level impurities and degradation products and low-level impurities. In addition to this NMR is considered to be close to a universal detector for atoms like hydrogen and carbon, and also for other magnetically active nuclei. Mass spectrometry provides information about molecular weight of the compound and helps in structure prediction of molecule by fragmentation pattern. Recent advances of the interfaces in terms of its design and efficacy , has opened a door for directly connecting separation techniques with NMR and mass spectrometers for monitoring, characterizing and quantitative estimation of drug and impurities in drug products. Characterization process time is saved by use of hyphenated techniques like LC-NMR, LC-MS/MS and LC-DAD-NMR-MS⁴.

1.11. RELEVANCE OF IMPURITY PROFILING

1.11.1. In synthetic drug research

In synthetic research, compounds are synthesized in small scale and screened for activity, followed by impurity profiling of the selected molecules showing good activity. The organic chemist has to optimize the synthesis and purification process of the substances while reporting the impurities so that substances are scaled up for toxicological, formulation, preclinical and clinical trials. At this stage of development it is not necessary to identify the impurities, but be assured that the impurities are found in and same limit is applicable for all the batches used for the trials²³.

1.11.2. In production of bulk drugs

Following the introduction of new API, it needs to be synthesized in bulk for formulation. It's very important that during the scale up procedure no new impurities appear. The quantity of impurity identified in R&D phase should remain below specification limit.

During scale up procedure it can happen that a new impurity appears or the quantity of the impurity detected (but not identified previously) may exceed threshold limit. In this case it's mandatory to characterize the impurity; hence the organic chemist takes necessary steps to avoid them above threshold limit. Production of bulk drugs has to be carried out in controlled conditions, since minor change in condition of the process can result in substantial changes in impurity profile. In multistep synthesis to identify the origin of the new impurity it's important to estimate the impurities in key intermediates during synthesis²³.

1.11.3. In formulation and development (F&D)

F&D department should possess knowledge of impurity profiling of bulk drugs used for development of formulation, thus helping them to differentiate between synthesis related impurities and degradation products. Generally stability studies are conducted to identify degradation products from synthesis related impurities, the quantity of which increases over time while that of synthesis related impurities remain constant. These findings help the manufacturer in preformulation studies. The exploratory stability studies disclose the degree of vulnerability of the molecule to different environmental conditions (heat, light, humidity, acidic, basic or oxidative). With support of these studies potential degradation products in the drug formulation can be established. Such studies help in identifying the possible interaction between the excipients and the drug substance²³.

1.11.4. In drug registration

A drug master file (DMF) has to be submitted to the regulatory authorities by the manufacturers of the pharmaceutical substances. DMF furnish detailed information on the process facilities used during manufacturing, packaging and storage conditions of the drug. Profile of impurities along with their acceptance criteria are also detailed in the DMF. The main goal of DMF is to aid the regulatory requirements and to demonstrate the quality, safety and efficacy of the drug product so as to obtain Investigational New Drug Application (IND), New Drug Application (NDA) and Abbreviated New Drug Application (ANDA). Tremendous information is available in the area of impurities, because of the efforts of ICH and US-FDA. The comparison of impurities from the same manufacturer of the several batches of the same product gives an idea of consistency maintained in the manufacturing process²³.

Comparison of same drug with other several manufactures highlights the differences in the purity. Since some impurities are specific to the synthetic pathways, impurity profiling of such gives the clue about the synthetic route used by different companies²³.

1.11.5. In stability studies

Efficacy and safety of drug therapy is directly related to the stability of API and drug product. To understand and to predict stability of API and drug product forced degradation studies (Stress degradation studies) are performed. Stability studies help us in identifying and establishing the degradation pathway and intrinsic stability of the molecule. Stress studies are used as a predictive research tool for identifying stability of drug molecule and development of stability indicating method. Stress studies are performed under various exaggerated conditions of humidity, temperature, and light²³.

1.12. AIMS FOR IMPURITY STUDY

Impurity study in pharmaceuticals is challenging and time consuming, and it's important to keep pace with the times as in terms of regulations and technology. The aims fall under two major headings -regulatory requirements and scientific/technical request (Table 1.12.1)²⁴. As per statutory demands, the standard of APIs and formulations is affected by the impurities present, which in turn affect the welfare of the patient.

There might be different views on dealing with impurities between biologists, toxicologists, and analytical chemists, and hence needs to be amalgamated. To conduct an impurity investigation along with regulatory requirements it needs internal and external scientific and technical support. Establishment of impurity and forced degradation studies are fundamental mechanism for the following things.

- a) Prediction of potential degradation products
- b) Developing of analytical method
- c) Understanding of better storage condition
- d) Synthetic processes and formulations
- e) Predicting stability of drug products
- f) Information of degradation products/pathways
- g) Assessment of selectivity of assay method.

Table 1.12: Aims for impurity study

Regulatory requirements	Scientific and technical requirements
<ul style="list-style-type: none"> • Quality and safety of products 	<ul style="list-style-type: none"> • Synthetic and production processes optimization
<ul style="list-style-type: none"> • Method validation, i.e., specificity 	<ul style="list-style-type: none"> • Formulation development and optimization
<ul style="list-style-type: none"> • Acceptance criteria determination 	<ul style="list-style-type: none"> • Efficacy improvement
<ul style="list-style-type: none"> • Expiry date, retest date, and shelf-life evaluation 	<ul style="list-style-type: none"> • ADME and toxicology study
<ul style="list-style-type: none"> • Stability and storage conditions study 	<ul style="list-style-type: none"> • Manufacturing of reference materials
<ul style="list-style-type: none"> • Threshold limits evaluation, i.e., threshold of toxicological concern (TTC), permitted daily exposure (PDE), etc. 	<ul style="list-style-type: none"> • Stability improvement
	<ul style="list-style-type: none"> • DPs and pathways prediction
	<ul style="list-style-type: none"> • Cost consideration

1.13. OBJECTIVES OF IMPURITY INVESTIGATIONS

During investigation of both process and product degradation-related impurities the common objective is to identify from the potential impurities which of them are in fact produced during manufacturing process and which are formed under a given set of storage condition. Table 1.13 highlights the objectives of impurity investigation^{4, 25}.

Table 1.13: Objectives of impurity investigations

Process-related impurities	Degradation-related impurities
Identify significant impurities	Identify potential degradation product through stress testing and actual degradation products through stability studies.
Determine origin of impurities and method for elimination or reduction	Understand degradation pathway and methods to minimize degradation.
Establish a control system for impurities involving: <ol style="list-style-type: none"> 1) Processing/manufacturing conditions 2) Suitable analytical methods/specifications 	Establish a control system for impurities involving: <ol style="list-style-type: none"> 1) Processing/manufacturing conditions 2) Suitable analytical methods/specifications 3) Long term storage conditions including packaging 4) Formulation

1.14. FORCED DEGRADATION STUDIES

Forced degradation studies typically involve exposure of representative samples of drug substance or drug product to the relevant stress conditions of light, heat, humidity, acid / base hydrolysis, and oxidation. The results of forced degradation studies can facilitate SIAM development, drug formulation design, selection of storage conditions and packaging. It provides better understanding of the drug molecule chemistry, and helps in solving stability-related problems²⁶⁻²⁷.

Although the FDA²⁸ and ICH guidelines^{9-10,14} provide useful definitions and general comments about forced degradation studies, their direction concerning the scope, timing, and best practices. Unfortunately, the practical aspects related to stress testing are neither addressed by regulatory authority guidelines, nor by any other document.

1.14.1. Empirical approach towards forced degradation

It is necessary to carry out forced degradation (stress study) for particular drug substance or drug product to develop stability indicating method. Generally desired range of degradation should lie between 5 – 10 %. This is achieved by varying the stress conditions like exposure time, temperature, or concentration of stressing agent (acid, base, oxidizer, etc.). Overstressing may destroy the compound or may lead to further degradation of the relevant primary degradants.

Under-stressing may fail to generate important degradation products. The degradation studies should be terminated after the maximum recommended time/stress conditions, even if sufficient degradation has not been achieved. It is unnecessary and even unwise to try to degrade the drug at all cost as it would only increase the complexity of the method development with little or no benefit in the quality of the data generated by the method²⁹⁻³⁰.

Specific parameters for stress testing of drug substance and drug product are shown in following tables.

Table 1.14.1.1: General protocol for stress testing of drug substances and drug products

Stress condition	Drug substance		Drug product	
	As neat solid	As solution or suspension	Solid dosage form ^a	Liquid ^b
Hydrolysis (Acid, Base, and Thermal)		+		+ ^c
Oxidative		+		+
Photo-degradation	+	+	+	+
Thermal	+		+	+
Thermal/Humidity	+		+	

^aFor tablets, capsules or powder blend.

^bFor oral solutions, oral suspensions, or parenterals.

^cNot required for buffered formulations.

Table 1.14.1.2: Recommended stress conditions for drug substance

Stress type	Conditions	Time
Acid hydrolysis	1 mg/ml in 0.1 N (up to 1.0 N) HCl; RT or higher	1–7 days
Base hydrolysis	1 mg/ml in 0.1 N (up to 1.0 N) NaOH; RT or higher	1–7 days
Oxidative/solution	0.3 % (up to 3 %) H ₂ O ₂ ; RT; protected from light	Few hours to 7 days
Thermal ^a	70 °C	Up to 2 weeks
Thermal/Humidity ^a	70 °C / 75 % RH	Up to 2 weeks
Photo-degradation	Fluorescent and UV light	ICH

^aIf the solid drug substance is unstable to thermal stress at high temperature due to melting, decomposition, etc., use a lower temperature with longer stress time.

^bICH guideline for appropriate light exposure: Fluorescent =1.2 million lx hours, UV = 200 Wh/m².

Table 1.14.1.3: Recommended stress conditions for drug product

Stress type	Conditions	Time
Thermal	70 °C	Up to 3 weeks
Thermal/humidity	70 °C / 75 % RH	Up to 3 weeks
Photo-degradation	Fluorescent and UV light	ICH

Note: As a control, also perform stress testing on placebo to distinguish drug-related degradants from potential non-drug-related degradation products from the excipients or solvents.

1.14.2. Acid and Base hydrolysis of drug substance in solution

Generally, hydrolysis is performed using HCl and NaOH solution. If the compound is poorly water soluble, organic co-solvents may be used in combination with acid or base. Stress is typically initiated at room temperature; if no degradation occurs, an elevated temperature is applied (50–70 °C). A thermal control (i.e., drug in neutral solution at the same stress temperature) should also be run to identify any degradation due to temperature alone. The degraded test samples are often neutralized using acid / base / buffer as per requirement to avoid further decomposition.

1.14.3. Oxidation

Hydrogen peroxide (H₂O₂) is the most commonly used oxidant. The peroxide concentration may be adjusted to obtain 5–20 % degradation. One disadvantage of using H₂O₂ is that it is non-selective and relatively unpredictable in its results. Stress with hydrogen peroxide often leads to secondary degradation of the primary degradants making results interpretation more difficult. Radical initiators such as AIBN (2, 2-azobis isobutyronitrile), ACVA (azobis-cyan valeric acid), and AMPD (azobis methyl propionamide dihydrochloride) are a better choices for oxidation studies, but are less commonly used for oxidative degradation.

1.14.4. Thermal / Humidity

Solid state stability can be evaluated utilizing accelerated storage conditions in general temperature greater than 50 °C and relative humidity > 75 %. The duration of exposure is dependent on the API sensitivity. In the forced degradation thermal / humidity conditions produce a phase change, it is recommended to run conditions below the critical thermal / humidity that produce the phase change.

1.14.5. Photo degradation

According to ICH, for confirmatory studies, overall illumination of light on the sample should be NLT 1.2 million lux hours and integrated near UV energy of NLT 200 Wh/m².

1.15. DEVELOPMENT OF STABILITY INDICATING METHOD

Literature shows various titrimetric, spectrophotometric and chromatographic methods reported for analysis of stability samples. There are also various reports on use of miscellaneous techniques. Very few methods which are reported as stability indicating meet current regulatory requirement. Many studies shown separation of drug from synthetic impurities and / or potential degradation products without subjecting to stress condition. Some methods are developed by subjecting drug to one, two or three stress conditions among acid, base, neutral hydrolysis, photolysis, oxidation and thermal stress. Thus there are very few methods where drug has been exposed to all types of stress condition and attempt was made to separate degradation products and drug from degradation products.

The aim of the most of titrimetric and spectrophotometric methods is to analyze drug of interest alone in the matrix of excipients, additives, degradation products and impurities. The only advantage associated with these methods is their low cost and simplicity. But due to limitation of specificity there are hardly few methods reported as stability indicating. The chromatographic methods have taken precedence over conventional method of analysis because of their high accuracy and sensitivity in separation of multiple components. Various chromatographic methods that have been used are TLC, HPTLC, GC and HPLC. Among these TLC is simple technique. But its disadvantages like variability and non-quantitative nature, limits its use as basic technique for SIAM development. However it is very much used during initial stages of development to study number of degradation products formed, to identify degradants by comparing it with standard, and even for isolation of this products³¹⁻³².

HPTLC overcomes with the disadvantages associated with TLC. It is reliable, fast and accurate for quantitative analysis of drug. Moreover many samples can be run simultaneously using small quantity of mobile phase, thus minimizes time and cost involved in analysis. Due to these advantages large numbers of publications are appeared in the last decade³³⁻³⁶. GC can be used in development of SIAM, but this technique is not versatile and suitable for the analysis of volatile and thermo stable substances. Due to this major limitation there are hardly few SIAM methods established by using GC.

In comparison to all above method HPLC is very widely used in development of SIAM. It became very popular technique due to its high-resolution capacity, sensitivity and specificity. In addition to this, HPLC has a capacity to analyze thermally unstable, polar as well as ionic compounds. Therefore most of the SIAM has been developed using HPLC.

The sensitivity of HPLC analysis can also be increased by coupling it with mass detector (LC-MS or LC-MS/MS) and NMR (LC-NMR), which helps in structural elucidation of unknown impurity. Few miscellaneous methods like NMR, CE are also reported for development of SIAM. Requirements with respect to SIAM is provided in ICH guidelines, but the practical steps to be followed for method development and validation is neither provided in regulatory guidelines nor in pharmacopoeia. So it becomes important to discuss practical steps involved in development of SIAM. Following steps are useful for development of SIAM by HPLC, as most of the methods reported in literature are by this technique³⁷. The processes flow map is given in Fig. 1.15³⁷.

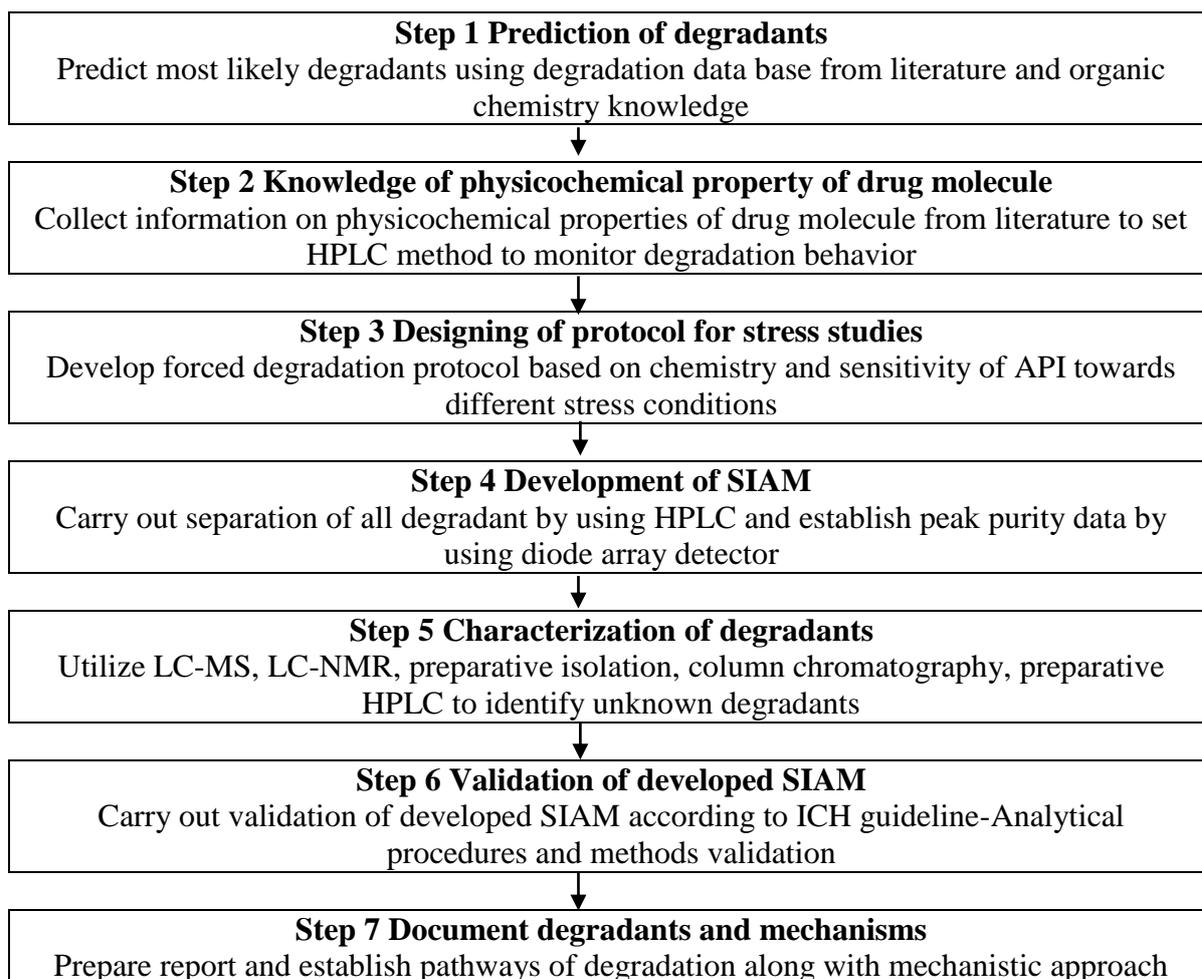


Figure 1.15: Forced degradation processes flow map

Step 1) Critical study of drug structure to predict possible degradation route(s)

Most of new drugs are congeners of existing drug molecules. For these drugs their degradation chemistry can easily predicted on the basis of reported degradation profile. Studies shown that three alpha-adrenergic blockers prazosin, doxazosin and terazosin follow same hydrolysis route which involves breakage of the amide bond. However in some cases congeners follow totally different route of degradation, due to effect of bulky substituent. In case of new drug the information can be gathered from available literature survey or by observing nature of functional groups.

Step 2) Collection of information on physicochemical properties

Before method development it is important to know various physicochemical parameters like pKa, log p, solubility, absorptivity, and wavelength maximum. The knowledge of pKa is important because it leads to change in retention pattern of the drug whenever there is change in pH of mobile phase ± 1.5 units of the pKa value. The knowledge of log p value for the drug and degradants helps in selection of stationary phase. Information of solubility data in aqueous, organic and commonly used HPLC solvents and their combinations is very useful in selection of solvent and mobile phase.

As most of HPLC analysis is carried out by using UV detector. The knowledge of wavelength maximum and extinction of drug and degradation products in different solvent and in different pH helps in developing method, where all the components show very good absorbance. In case of unknown degradation products the wavelength maxima can be determined with the help of photo diode array (PDA) detector.

Step 3) Stress (forced decomposition) studies

Stress studies are carried out to generate possible degradation products. The ICH guidelines suggest following conditions to conduct stress studies¹⁴.

- 1) Thermal stress – 10 °C increments above accelerated temperatures (50 °C, 60 °C etc.)
- 2) Humidity – 75 % RH or greater where appropriate
- 3) Hydrolysis – across a wide range of pH values
- 4) Oxidation
- 5) Photolysis - according to ICH Q1B guidelines

However guidelines do not provide any practical information on how stress studies to be carried out. On the other hand some articles³⁸ and book^{5, 39} addressed this issue and has given practical approach to conduct stress study. It is generally recommended that four samples (blank solution stored under normal condition, blank subjected to stress in the same manner as the drug solution, zero time sample containing drug which is stored under normal condition and drug solution subjected to stress treatment) to be generated for every stress conditions. Furthermore, it is advised to withdraw samples at different time periods for each reaction condition and should be analyzed by HPLC. It provides clear idea about number of degradant formed under different stress conditions and their relative stability. This information is essential in establishment of SIAM.

Step 4) Development of SIAM and optimization

Preliminary analysis of stress samples were carried out to know number and type of degradation products formed. This can be done by using reverse phase octadecyl column along with mobile phase which is employed previously in the development of assay method for the drug. The final method is developed by using mixture of degradants. The mobile phase can be changed if peak shape or separation problems are seen. Majority of time degradation leads to formation of polar as well as non-polar degradants. Separation of such types of degradants is carried out by using gradient programme. The detection wavelength is set based on the spectral behavior of degraded samples. The injection volume, column temperature and flow rate can be adjusted to achieve better separation. After proper separation retention time (RT) and relative retention time (RRT) is tabulated. Special attention is paid to those components whose RT or RRT is very close. PDA spectra or LC-MS profile of such components are critically evaluated to ascertain whether the degradants are same or different.

Step 5) Identification and characterization of degradation products

According to ICH it is necessary to identify and qualify the degradation products if these degradants are up to identification and qualification threshold. Identification of degradants also helps to establish degradation pathways. Once the structure of degradant is identified it can be synthesized and purified to be used as impurity standard. Traditionally, degradant products were identified by isolation and characterization. The characterization of the compound is carried out by subjecting it to spectral (UV, IR, NMR, MS) and elemental analysis.

However this approach is tedious and time consuming when multiple degradation products are formed. Against it, the modern approach is to use hyphenated LC techniques coupled with mass spectrometry (LC-MS and / or LC-MS/MS) and nuclear magnetic resonance spectroscopy (LC-NMR). It provides a fair idea about chemical structure of degradants.

Step 6) Validation of stability indicating assay method

Validation of analytical methods, in general, has been extensively covered in the ICH guidelines Q2 (R1) ⁴⁰, in FDA guidance ⁴¹, and by United States Pharmacopoeia (USP). In general, analytical method validation must include studies on specificity, linearity, range, accuracy, precision, detection limit, quantitation limit and robustness⁴⁰⁻⁴³.

6.1. Specificity

As per ICH guidelines specificity is the capacity to evaluate explicitly of the substance in company of other components like impurities, degradants, matrix etc. which may be expected to be present. For chromatographic method developing separations involves the demonstration of specificity. In case of SIAM, the only effort involved is to develop a method that separates components from a physical mixture of drug and degradation products.

6.2. Accuracy

Intimacy of the test outcome with the true or accepted reference value obtained by a method is referred as the accuracy of an analytical method. The accuracy of any analytical method should be established across its range. Accuracy is calculated by recovery study, determining the known added amount in the sample. The ICH recommends minimum nine determinations over a minimum three concentration levels to assess its efficiency, covering the specified range. Accuracy of method is determined by four different ways.

6.2.1. First accuracy can be assessed by analyzing a sample of known concentration (reference materials) and comparing the measured value to the true value.

6.2.2. The second approach is to compare test results from the new method with results from an existing alternate well characterized procedure that is known to be accurate.

6.2.3. The third approach, based on the recovery of known amounts of analyte, it is performed by spiking analyte in blank matrices. The analyte levels in the spiked samples

should be determined using the same quantitation procedure as will be used in the final method procedure. The percent recovery should then be calculated.

6.2.4. The fourth approach is the technique of standard additions, which can also be used to determine recovery of spiked analyte. This approach is used if it is not possible to prepare a blank sample matrix without the presence of the analyte.

6.3. Precision

It involves the intimacy of harmony with series of measurements, from different specimens of the same equivalent sample under stipulated conditions. Repeatability, intermediate precision and reproducibility are considered as levels of precision. Precision is expressed usually as variance, standard deviation or coefficient of variation of number of estimations.

6.3.1. Repeatability

Repeatability is the result of the method running over a short time interval under the same conditions (intra-assay precision). It should be determined from a minimum of nine determinations covering the specified range of the procedure (for example, three levels, three repetitions each) or from a minimum of six determinations at 100 % of the test or target concentration.

6.3.2. Intermediate precision

Intermediate precision is the results from within lab variations due to random events such as different day, analysts, equipment, etc. In determining intermediate precision, experimental design should be employed so that the effects (if any) of the individual variables can be monitored.

6.3.3. Reproducibility

It is determined by testing homogeneous samples in multiple laboratories, is often a part of inter laboratory crossover studies. The evaluation of reproducibility results often focuses more on measuring bias in results than on determining differences in precision alone. As far as precision is concerned there are no special requirements for stability indicating methods.

6.4. Limit of detection (LOD)

LOD of a method is the lowest amount of the sample that can be noticed but not generally quantified to an exact value. The injected amount that results in a peak of height twice or three times as high as baseline noise level is considered to be the detection limit in chromatography. The ICH also lists two other options to determine LOD, these are as follows.

6.4.1. Based on visual evaluation

Visual evaluation is used for non-instrumental and instrumental methods. Analysis of defined concentrations of analyte is done to estimate the detection limit and representing the minimum level of analyte which could be detected reliably and quantified.

6.4.2. Based on standard deviation of the response and the slope

LOD is calculated using the formula: $LOD = 3.3 (SD/S)$, where SD (Standard deviation of response) and S (Slope of the standard curve of the analyte). SD estimation can be done in various ways, for example:

6.4.2.1. Based on standard deviation of the blank

Appropriate number of blank samples is analyzed to measure the magnitude of analytical background responses and thus calculating SD of these responses.

6.4.2.2. Based on calibration curve

A specific calibration curve is plotted with analyte samples in the range of detection limit. For determining standard deviation, the standard deviation of y-intercept of regression and the residual standard deviation of a regression line can be used.

6.4.3. Recommended data

Proper presentation of the method used for determining detection limit should be done. Relevant chromatograms display is considered acceptable for justification if detection limit is estimated depending on visual evaluation or based on signal to noise ratio. When the value obtained for detection limit by extrapolation or by calculation, it has to be validated subsequently with suitable number of samples which are near or prepared at the detection limit by an independent analysis.

6.5. Limit of quantitation (LOQ)

The limit of quantitation is defined as the lowest concentration of an analyte determined in a sample under the stated operational conditions of the method with acceptable precision and accuracy. According to ICH a signal-to-noise ratio of 10:1 is used to determine LOQ. Like LOD, the ICH also lists two other options to determine LOQ, these are as follows.

6.5.1. Based on visual evaluation

Visual evaluation is used for non-instrumental and instrumental methods. Estimation of quantitative limit is done by analyzing samples with defined concentrations of analyte and further constituting the minimum level where analyte could be quantified with acceptable accuracy and precision.

6.5.2. Based on standard deviation of the response and the slope

LOQ is calculated using the formula: $LOQ = 10 (SD/S)$, where SD (Standard deviation of response) and S (Slope of the standard curve of the analyte). SD estimation can be done in various ways, for example:

6.5.2.1. Based on Standard Deviation of the Blank

Standard deviation of the responses is calculated by analysis of appropriate number of blank samples to measure the magnitude of analytical background responses.

6.5.2.2. Based on Calibration Curve

A specific standard curve is plotted with analyte samples in the range of detection limit. For determining standard deviation, the standard deviation of y-intercept of regression and the residual standard deviation of a regression line can be used.

6.5.3. Recommended Data

Proper presentation of the method used for determining quantitation limit should be done. By analysis the value obtained for quantitative limit has to be validated subsequently with suitable number of samples which are near or prepared at quantitative limit. In case of SIAM detection and quantitation limits are not important for active drug substances as their concentration is not expected to fall to such a low level during shelf-life of their formulations. However, these limits should be established for the degradation products.

6.6. Linearity

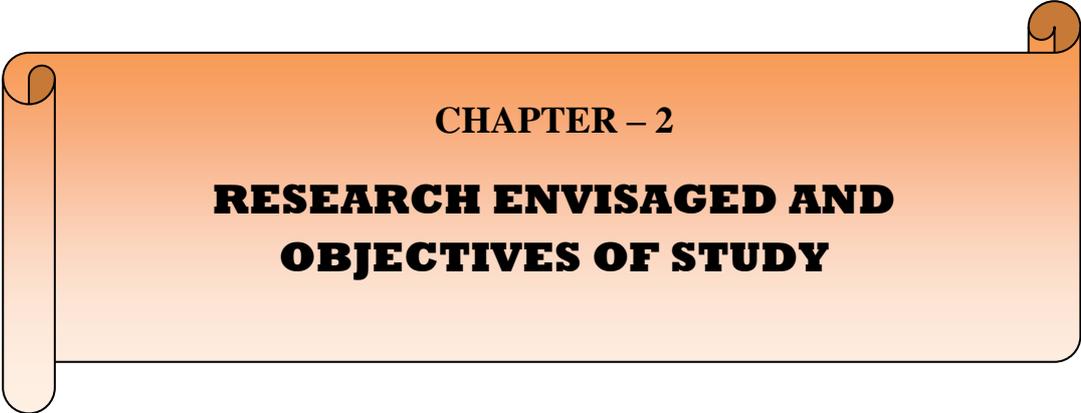
Linearity is the competence of the method to obtain test results equivalent to the analyte concentration within a given range, directly or by well-defined mathematical transformation. The ICH guidelines specify a minimum of five concentration levels, along with certain specified ranges. In practice the linearity study should be designed to be appropriate for the intended analytical method. Acceptability of linearity data is often judged by examining the correlation coefficient and y-intercept of the linear regression line for the response versus concentration plot. A correlation coefficient of 0.999 is generally considered as evidence of acceptable fit of the data to the regression line.

6.7. Range

Range is the interval between the upper and lower levels of analyte (inclusive) that have been demonstrated by using appropriate method with utmost precision and accuracy. For assay, the minimum specified range is from 80 to 120 % of the target concentration. For an impurity test, the minimum range is from the reporting level of each impurity to 120 % of the specification. For content uniformity testing, the minimum range is from 70 to 130 % of the test or target concentration, and for dissolution testing ± 20 % over the specified range of the test. The linearity for SIAM should be established initially in the range of 0 – 100 %, as the drug may fall to very low concentrations during forced degradation studies. The final validation range, however, can be narrowed based upon the form in which drug substance or formulation is dispensed. Validation range for degradation products during stability studies usually should vary from 0 to 20 %.

6.8. Robustness

Robustness of an analytical method is the measurement of the method's ability to remain unchanged by minute but intended alterations in the method's variables. It is evaluated by varying method parameters such as percent organic solvent, pH of buffer in mobile phase, ionic strength, different HPLC columns (lots and/or suppliers), column temperature, flow rate etc. As documented in the ICH guidelines, robustness should be considered early in the development of a method. In addition, if the results of a method or other measurements are susceptible to variations in method parameters, these parameters should be adequately controlled and a precautionary statement should be included in the method documentation.



CHAPTER – 2

**RESEARCH ENVISAGED AND
OBJECTIVES OF STUDY**

2. RESEARCH ENVISAGED AND OBJECTIVES OF STUDY

HPLC method development offers a number of learning experiences while handling intricate situations, depending on the complexity of drug selected for study. Certain situations throw challenges that require a systematic approach in new method development superseding the arbitrary trial and error method during development. Systematic approach involves a thorough study of chemistry, physiochemical properties and such other parameters that could influence orderly movement of the analytes through the column offering appropriate innervations and consequently fault free separations of components.

Method development for drug substances in presence of its impurities requires procedures that are sensitive, robust and powerful to detect and quantify them irrespective of their number and concentration. Current monographs in the pharmacopoeia presents a number of specified impurities for each drug and in several cases the similarities in structure and their physiochemical properties present delicate situations to explore method development through routine trial and error method. In addition, ability of the drug substance to disintegrate and lose its structural integrity or degrade during various stages of analytical procedures envisages search for rational method development.

Following are few situations proposed to be engaged during the proposed research activity on the basis of which the objectives of study has been laid upon.

- a) Drug substance that come with a possibility of a large number of specified impurities with insignificant differences in physical attributes and chemical properties including dissociation constant- a key attribute used in mobile phase optimization process.
- b) Drug that have potential to undergo degradation readily in working medium/atmospheric condition, unexpectedly breaking down during analysis; demonstrate ability to react with components of the mobile phase or disintegrate at higher temperature and/or humidity.
- c) Drug/impurities that go undetected even with most versatile PDA detector- necessitating the non-absorbing compound/s to be converted to an absorbing entity without interfering with the chemistry of all other compounds present in the solution.

- d) Analysis of a precursor or prodrug with its active form simultaneously, in situations wherein they could be present together. Such methods could find application in pharmacokinetic studies too.

2.1 OBJECTIVES OF THE PROPOSED RESEARCH PROJECT

The core of the project work embarks upon the following objectives of study-

- a) To investigate trait or source of pharmacopoeial impurities mainly 'process related' for drug substance. Systematic analytical method development is planned after thorough study of chemistry for all the compounds involved and appropriate innervations done for modifying the retention profiles.
- b) To develop and optimize appropriate chromatographic method for determination of drug substance in presence of identified impurities that could be influenced by modifying the strength and pH of the concerned mobile phase. The explorative study to be conducted on Mefenamic acid and Tolfenamic acid.
- c) To develop a simultaneous method for analysis of a drug substance along with it's pro-drug, with focus on carbimazole method development.
- d) Identify impurities that go undetected due to lack of sensitive chromophore and propose appropriate derivatization process to analyse them, taking Cyclizine Hydrochloride as the case study.
- e) To study the degradation potential of drugs of research interest and develop methods to separate them from potential process related impurities and also degradation products, if formed (Stability indicating analytical method).
- f) To validate the optimized "stability indicating analytical method" in accordance with ICH guidelines.
- g) To apply LC-MS method for characterization of degradation products formed in stress samples of selected drugs- Carbimazole and Cyclizine Hydrochloride.

The objective of the present research work involves profiling of impurities in four drug substances, namely- Mefenamic acid, Tolfenamic acid, Carbimazole and Cyclizine Hydrochloride. The study is schematized as follows.

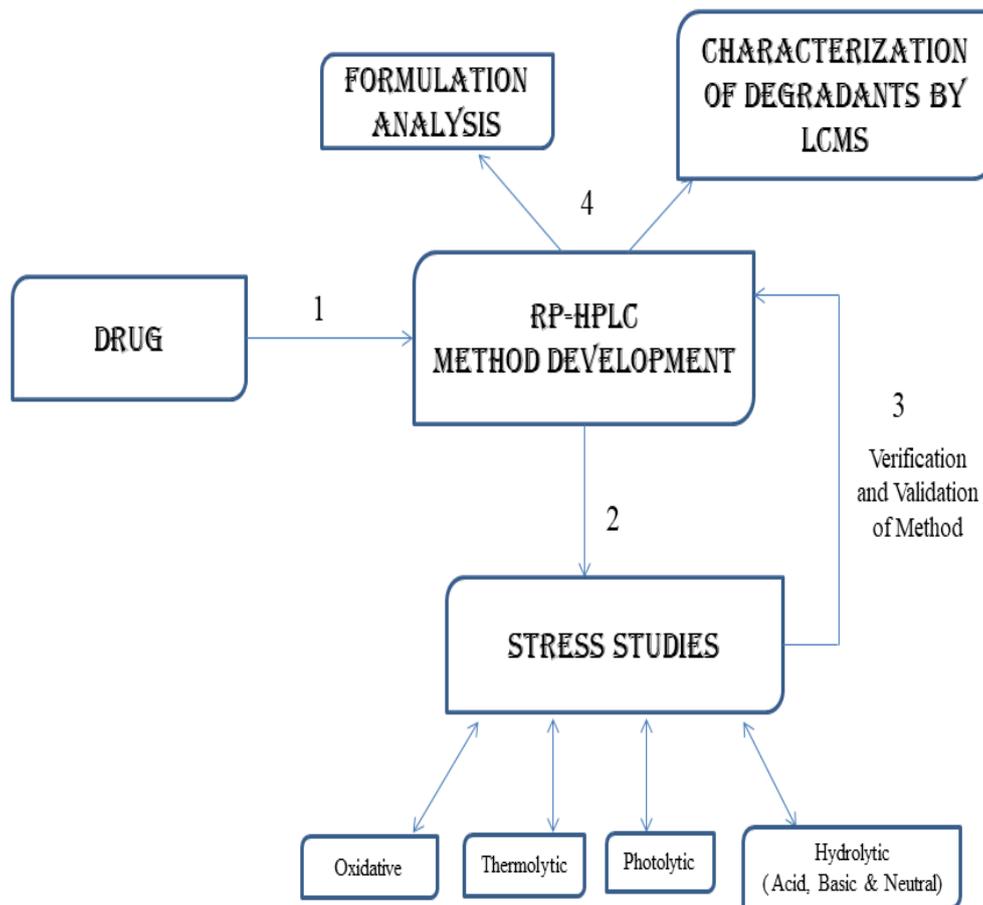


Figure 2.1: Schematized study of the proposed research work

CHAPTER – 3

**OPTIMIZING SEPARATION OF COMPOUNDS
WITH CLOSER pKa VALUES.**

**CASE STUDY- IMPURITY PROFILING OF
MEFENAMIC ACID**

3. OPTIMIZING SEPARATION OF COMPOUNDS WITH CLOSER pKa VALUES. CASE STUDY- IMPURITY PROFILING OF MEFENAMIC ACID

3.1. INTRODUCTION

A RP HPLC method is developed for estimation of Mefenamic acid (MA) in presence of its pharmacopoeial specified impurities, and to verify the developed method's stability indicating power by analyzing the stressed samples of MA.

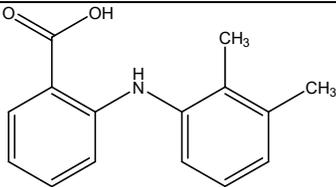
The following objectives were set to carry out the research activity:

- 1) To identify the pharmacopoeial specified impurities which are processes related for MA API.
- 2) To develop and optimize a RP HPLC method for estimating MA as API in presence of their pharmacopoeial specified impurities.
- 3) To carry out stress induced studies for drug in accordance with ICH guidelines.
- 4) To validate the optimized method for detection and estimation of MA in presence of its pharmacopoeial specified impurities and degradation products.

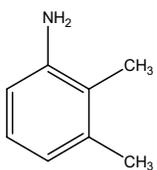
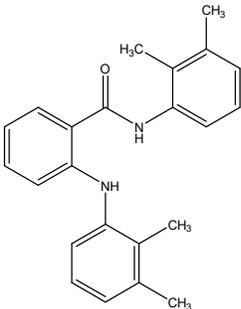
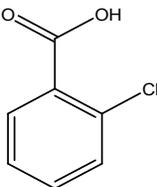
3.2. PROFILE

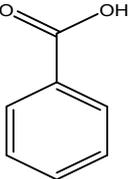
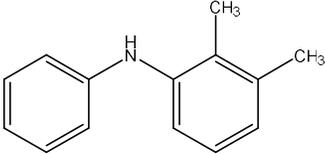
3.2.1. Drug profile

Mefenamic acid is official in IP⁴⁴, BP⁴⁵, USP⁴⁶ and EP⁴⁷

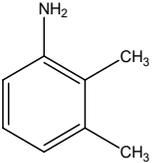
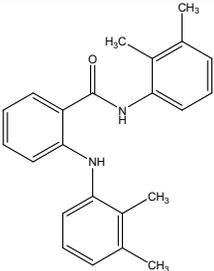
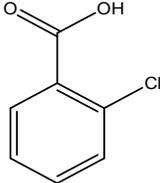
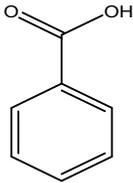
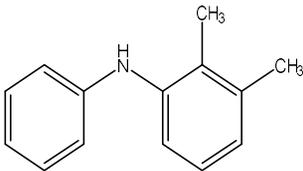
General Name	Mefenamic acid
Chemical Structure	
Chemical Name	2-[(2,3-Dimethylphenyl)amino]benzoic acid
Molecular Formula	C ₁₅ H ₁₅ NO ₂
Molecular Weight	241.3 g/mol
Melting Point	230-231 °C
Description	White or almost white, microcrystalline powder
Solubility	Practically insoluble in water, slightly soluble in ethanol (96 per cent) and in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.
pKa	4.2
Drug Category	Cyclooxygenase inhibitor, analgesic; anti-inflammatory

<p>Clinical Pharmacology</p>	<p>MA, an anthranilic acid derivative, is a member of the fenamate group of nonsteroidal anti-inflammatory drugs (NSAIDs). It exhibits anti-inflammatory, analgesic, and antipyretic activities. Similar to other NSAIDs, MA inhibits prostaglandin synthetase. MA binds the prostaglandin synthetase receptors COX-1 and COX-2, inhibiting the action of prostaglandin synthetase. As these receptors have a role as a major mediator of inflammation and/or a role for prostanoid signaling in activity-dependent plasticity, the symptoms of pain are temporarily reduced^{48, 49}</p>
<p>Pharmacokinetics</p>	<p>MA is absorbed from the gastrointestinal tract. Peak plasma concentrations occur about 2 to 4 hours after ingestion. The plasma elimination half-life is reported to be about 2 to 4 hours. MA is more than 90 % bound to plasma proteins. It is distributed into breast milk. MA is metabolised by the cytochrome P450 isoenzyme CYP2C9 to 3-hydroxymethyl mefenamic acid, which may then be oxidised to 3 -carboxymefenamic acid. Over 50 % of a dose may be recovered in the urine, as unchanged drug or, mainly, as conjugates of mefenamic acid and its metabolites⁵⁰.</p>
<p>Toxicity</p>	<p>Symptoms of overdose of the drug may include severe stomach pain, coffee ground-like vomit, dark stool, ringing in the ears, change in amount of urine, unusually fast or slow heartbeat, muscle weakness, slow or shallow breathing, confusion, severe headache or loss of consciousness⁴⁹.</p>

<p style="text-align: center;">Impurity Specified impurities A,C, D</p>	<p>A</p> <div style="text-align: center;"><p>2,3-Dimethylaniline</p></div>
	<p>B</p> <div style="text-align: center;"><p>N-(2,3-Dimethylphenyl)-2-[(2,3 dimethyl phenyl) amino] benzamide</p></div>
	<p>C</p> <div style="text-align: center;"><p>2-Chlorobenzoic acid</p></div>

	<p>D</p>  <p>Benzoic acid</p> <p>E</p>  <p>2,3 -dimethyl-N-phenylaniline</p>
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3.2.2. Profiles of Impurities

Impurity	A	B	C	D	E
Chemical Structure					
Chemical Name	2,3-Dimethylaniline	N-(2, 3-Dimethylphenyl)-2-[(2, 3 dimethyl phenyl) amino] benzamide	2-Chlorobenzoic acid	Benzoic acid	2, 3-Dimethyl-N-phenylaniline
Molecular Formula	C ₈ H ₁₁ N	C ₂₃ H ₂₄ N ₂ O	C ₇ H ₅ ClO ₂	C ₇ H ₆ O ₂	C ₁₄ H ₁₅ N
Molecular Weight	121.18 g/mol	344.45 g/mol	156.57 g/mol	122.12 g/mol	197.27 g/mol
Boiling point/ Melting Point	221.5 °C	191-194 °C	142 °C	122.3 °C	319.8 ± 11.0 °C

Description	Clear, dark red liquid	White to off-White Solid	White to off white Powder or powder with chunk(s)	White crystalline solid or powder	NA
Solubility	Soluble in alcohol, ether, carbon tetrachloride. Slightly soluble in water.	Slightly soluble in DMSO and Ethyl acetate.	Soluble in water, acetone benzene and methanol, freely soluble in alcohol, ether.	Soluble in water, alcohol, chloroform, acetone, carbon tetrachloride benzene.	NA
pKa	4.70	-1.28 & - 4.48	2.89	4.19	0.8
Toxicity	May cause Respiratory tract irritation. Prolonged exposure may result in dizziness and general weakness. May cause methemoglobinemi, (chocolate-brown colored blood), headache, weakness,	May be harmful if inhaled. May cause respiratory tract irritation. Harmful if swallowed. May be harmful if absorbed through skin. May cause skin irritation. May cause eye	This compound may be harmful by inhalation, ingestion or skin absorption. It is irritating to eyes, skin, mucous membranes and the upper respiratory tract. When heated to decomposition	The substance can be absorbed into the body by inhalation and by ingestion resulting in redness, burning sensation, Itching, Cough and, Sore throat. Redness Pain in eye and Abdominal pain, Nausea. Vomiting.	NA

	<p>dizziness, breath shortness, cyanosis (bluish skin due to deficient oxygenation of blood), rapid heart rate, unconsciousness and possible death. Chronic exposure may cause liver damage. Chronic exposure may cause blood effects. Limited evidence of a carcinogenic effect.</p>	<p>irritation.</p>	<p>this compound emits toxic fumes. No component of this product present at levels greater than or equal to 0.1% is identified as probable, possible or confirmed human carcinogen by IARC</p>		
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3.3. LITERATURE SURVEY

Literature survey quotes diverse analytical methods for the estimation of MA in drug product or biological fluids either individually or with in combination with other drugs, including viz., spectrophotometry⁵¹⁻⁵⁶, HPLC⁵⁷⁻⁶⁶, HPTLC⁶⁷, and Gas Chromatography⁶⁸. Method involving simultaneous determination of MA and two of its pharmacopoeial specified impurities by RP-HPLC has been reported⁶⁹. A few RP HPLC stability indicating method has been outlined in the literature for MA individually and in combination⁷⁰⁻⁷³. Limited literature is found on stability indicating RP HPLC method for MA in presence of its three pharmacopoeial specified impurities. Following Table 3.3 encapsulates few chromatographic conditions used in HPLC methods for MA reported in literature.

Table 3.3: Summary of reported chromatographic conditions used for determination of Mefenamic acid by RP HPLC.

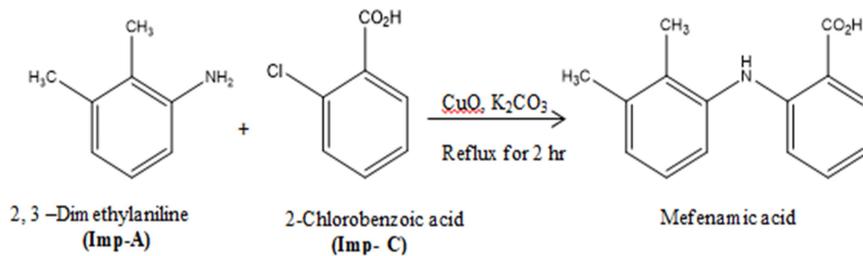
Sr.no	Column type	Mobile phase composition	Flow rate	Detector used	R _t of drug	References
1	BP method					
	C18 (5 µm)	50 mM Phosphate buffer (pH adjusted to 5 with dilute ammonia): Acetonitrile: THF (40:46:14 % v/v)	1.0 ml/min	UV fixed wavelength (254 nm)	8 min	45
2	Application of photodiode array UV detection in the development of stability indicating LC methods: Determination of mefenamic acid					
	Nova-Pak C18 (5 µm)	Acetonitrile: THF : Water: Glacial acetic acid (15:40:45:2 % v/v)	1.0 ml/min	UV fixed wavelength (278 nm)	4.0 min	57
6	Rapid and sensitive liquid chromatographic assay of mefenamic acid in plasma					
	Cyano C18	Water:acetonitrile:methanol:17 M acetic acid (69:15:15:1 % v/v)	1.0 ml/min	UV fixed wavelength (290 nm)	6.9 min	58
7	Liquid chromatography method for determination of mefenamic acid in human serum.					
	Tech sphere C8 (3 µm)	Acetonitrile: Water (50:50 % v/v), adjusted to pH of 3 by phosphoric acid	1.0 ml/min	UV fixed wavelength (280 nm)	7.4 min	60
5	Simple, rapid and sensitive reversed phase high performance liquid chromatographic method for the determination of mefenamic acid in plasma					

	Li Chrosorb C-18 (7 µm)	6.5 mM phosphoric acid: acetonitrile (45: 55 % v/v).	2.0 ml/min	UV fixed wavelength (280 nm)	6.6 min	61
8	Validated RP-HPLC method for the estimation of mefenamic acid in formulation					
	C18 (5 µm)	Acetonitrile:0.05 M ammonium phosphate buffer: Tetrahydrofuran (6:40:14 % v/v)	1.0 ml/min	UV fixed wavelength (254 nm)	10.59 min	62
4	Different chromatographic methods for simultaneous determination of mefenamic acid and two of its toxic impurities					
	C18 (5 µm)	0.05 M KH ₂ PO ₄ buffer: acetonitrile (40:60 % v/v)	1.0 ml/min	UV fixed wavelength (225 nm)	7.7 min	69
3	Stability indicating method for the determination of mefenamic acid in pharmaceutical formulations by HPLC					
	SPHER-100 C8 (5 µm)	0.05 M Ammonium dihydrogen orthophosphate buffer (pH=5, with ammonia) : acetonitrile + THF mixture(460+140) (55:45 % v/v)	1.0 ml/min	UV fixed wavelength (285 nm)	18.3 min	73

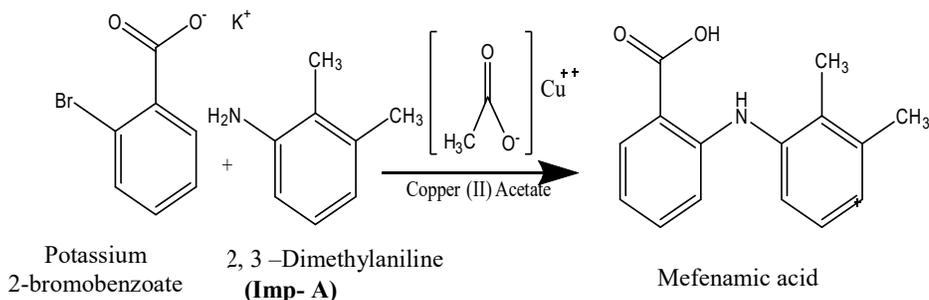
3.4. LOCATING SOURCE OF IMPURITIES IN MEFENAMIC ACID

Synthetic schemes of MA from the available literature were collected for identifying the possible impurities which can remain with the final product of Mefenamic acid API during its chemical synthesis.

A) Commonly used method for MA synthesis involves the Ullman condensation involving 2-Chlorobenzoic acid and 2, 3-Dimethylaniline in presence of CuO , K_2CO_3 ⁷⁴.

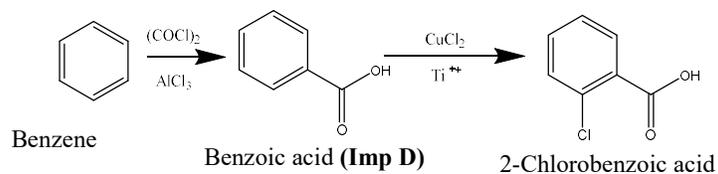


B) Another popular method involves reaction of potassium 2-bromobenzoate with 2, 3-Dimethylaniline in presence of copper acetate⁷⁵.



From the above synthetic schemes, it is observed that 2, 3-Dimethylaniline and 2- Chlorobenzoic acid are the common starting material for the synthesis of MA. The trace amounts of these compounds in the final product are listed as specified impurity A and C respectively in the BP monograph of MA.

Benzoic acid the raw material for 2- Chlorobenzoic acid is listed as specified impurity D in the monograph. The Friedel Craft's condensation of benzene with oxalyl chloride in presence of AlCl_3 in carbon disulfide yields benzoic acid which is chlorinated with cupric chloride and thallium (III) trifluoroacetate in trifluoroacetic acid to give 2-Chlorobenzoic acid⁷⁶.



Presence of Imp A during synthesis might react with Imp D giving rise to Imp E, whereas reaction of Imp A with MA may possibly give rise to Imp B. It was inferred that Imp A, Imp C and Imp D listed in the BP monograph of MA as specified impurities are process related impurity which might remain with MA during its chemical synthesis. These compounds as Imp A, Imp C and Imp D were considered for developing a new RP HPLC method for separation and estimation of MA.

3.5. DEVELOPMENT AND OPTIMIZATION OF RP HPLC METHOD FOR ESTIMATION OF MEFENAMIC ACID IN PRESENCE OF ITS PHARMACOPOEIAL SPECIFIED IMPURITIES

3.5.1. Selection of Chromatographic Method

The most popular technique of chromatography is Reversed phase chromatography (RPC). Around 70 % of the separations involving HPLC are carried out using (RPC). The principle of separation is based on analytes' partition coefficient between polar mobile phase and hydrophobic (nonpolar) stationary phase in RPC. Official methods and methods published in literature for MA are based on RPC separation. So RPC method was selected for estimation of MA in presence of its three pharmacopoeial specified impurities.

3.5.2. Selection of Stationary Phase

BP monograph⁴⁵ of MA recommends C18 column for related substances estimation by RP HPLC. Various literature studies for estimation of MA individually, in combination and for stability studies of MA, C18 column is used.

3.5.3. Selection of Wavelength for Analysis

The overlain spectrum involving MA and its three specified pharmacopoeial impurities (A, C and D) was recorded in acetonitrile (ACN) as shown in Fig 3.5.3. The solutions of concentration used 10 µg/ml of MA and 1 µg/ml of each impurity (A, C and D) for study. All the components showed different wavelength maximum. For the study one specific wavelength was selected where the absorptivity of A, C and D was comparatively higher

than for MA, enhancing sensitivity. Wavelength of 225 nm was selected for the study and also found mentioned in literature⁶⁹.

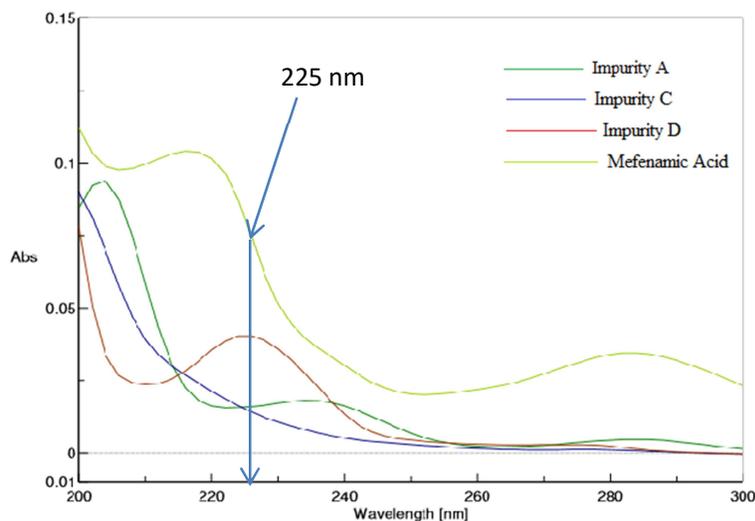


Figure 3.5.3: UV overlain spectrum of MA and impurities (A, C and D)

3.5.4. Application of BP monograph method for study

Exploratory study involving BP monograph method for MA was carried out. The chromatographic parameters applied are listed in Table 3.5.4 and the obtained chromatogram is shown in Fig 3.5.4.

Table 3.5.4: Chromatographic conditions for separation of MA and impurities (A, C and D) as per BP method

Mobile phase	50 mM Phosphate buffer (pH adjusted to 5 with dilute ammonia): Acetonitrile: THF (40:46:14 % v/v)
Column	Waters -Sunfire ODS C18 (250 x 4.6 mm ,5 µm) column
Flow rate	1 ml/min
Wavelength	254 nm
Standard solution	MA- 50 µg/ml; Imp A- 5 µg/ml; Imp C- 5 µg/ml: Imp D- 5 µg/ml

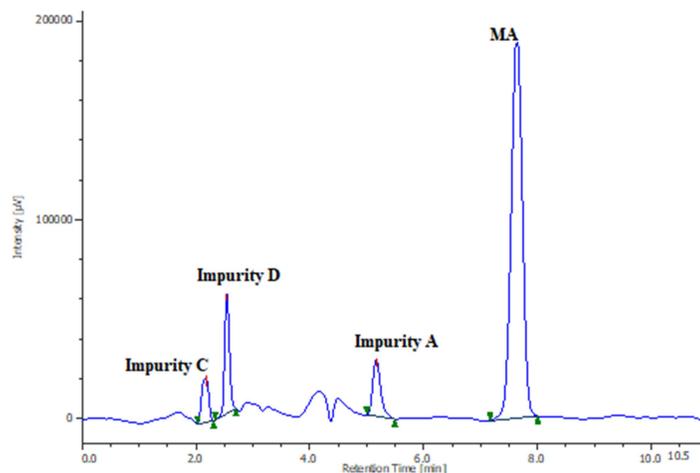


Figure 3.5.4: Chromatogram of MA and impurities (A, C and D) as per BP method.

Several noise peaks in the vicinity of impurity peaks are seen. Base line stabilization was not satisfactory and unconventional solvent like tetrahydrofuran (THF) is used in the method. Mobile phase composition containing ternary solvent did not provide any specific advantage nor produce satisfactory smooth baselines necessitating improvisation. Also use of THF as a component of the mobile phase was proposed to be avoided, minimizing noise peaks interfering with the peaks of impurities and drug was major objective.

3.5.5. Selection and optimization of mobile phase for separation of MA and its impurities

A specific mobile phase that could provide complete separation of MA from its specified pharmacopoeial impurities, and also monitor degradation behavior of MA under varied stress conditions was required to be developed. In BP monograph of MA ternary system comprising of acetonitrile and THF is used as organic phase and buffer solution (ammonium dihydrogen phosphate, adjusted to pH = 5.0 with dilute ammonia) as aqueous phase, the flow rate 1 ml/min, injection volume of 10 μ l and detection wavelength of 254 nm, on C18 column (4.6 x 250 mm, 5 μ m). Based on the pKa values of concerned components MA (pKa 4.2), Imp A (pKa 4.70), Imp C (pKa 2.89) and Imp D (pKa 4.2), separate trials were carried out. Mobile phase composition comprising of acetonitrile and 10 mM ammonium dihydrogen orthophosphate buffer was varied over pH varying from 3 to 6. Observations are recorded and presented in Table 3.5.5.1.

Table 3.5.5.1: Exploratory trials for optimization of mobile phase composition on Sunfire C-18 column (250 x 4.6 mm, 5 µm) for MA and impurities (A, C and D)

Trial no	Mobile Phase composition (ACN and 10 mM potassium dihydrogen orthophosphate Buffer) in % v/v	pH of Mobile Phase	Remark	Figure
1	60:40	6	Only 3 peaks are seen instead of 4 and 1 peak is seen in dead volume	3.5.5.1.1
2	55:45		Only 3 peaks are seen instead of 4 and 1 peak is seen in dead volume	3.5.5.1.2
3	50:50		Only 3 peaks are seen instead of 4 and 1 peak is seen in dead volume	3.5.5.1.3
4	40:60		Only 3 peaks are seen instead of 4 and 1 peak is seen in dead volume	3.5.5.1.4
5	30:70		Out of 4 peaks first 3 peaks are very close and peaks are seen in dead volume	3.5.5.1.5
1	60:40	5	All 4 peaks are seen with 2 peak appearing in dead volume and merging each other	3.5.5.1.6
2	55:45		All 4 peaks are seen with 2 peak merging each other and appearing in dead volume	3.5.5.1.7
3	50:50		All 4 peaks are seen with 2 peak appearing in dead volume and very close to each other	3.5.5.1.8
1	60:40	4	All 4 peaks are well separated with 1 peak below 3min.	3.5.5.1.9
2	55:45		All 4 peaks are well separated with all peak after 3min.	3.5.5.1.10
1	60:40	3	Unclear Peaks	3.5.5.1.11
2	55:45		Unclear Peaks	3.5.5.1.12

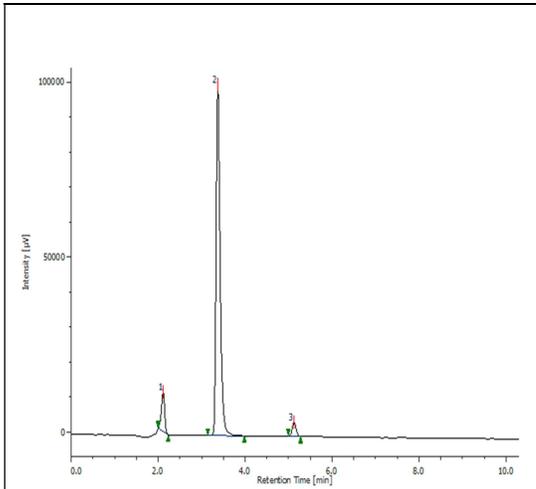


Figure 3.5.5.1

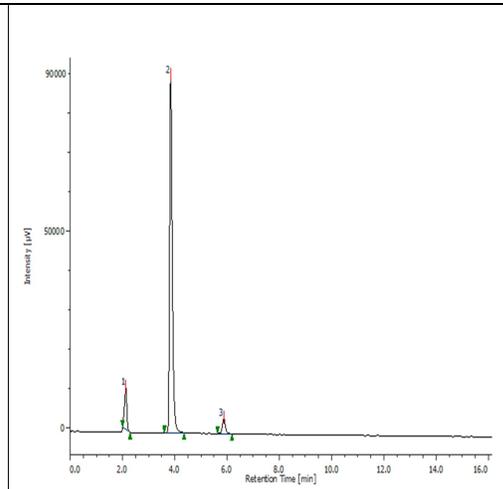


Figure 3.5.5.2

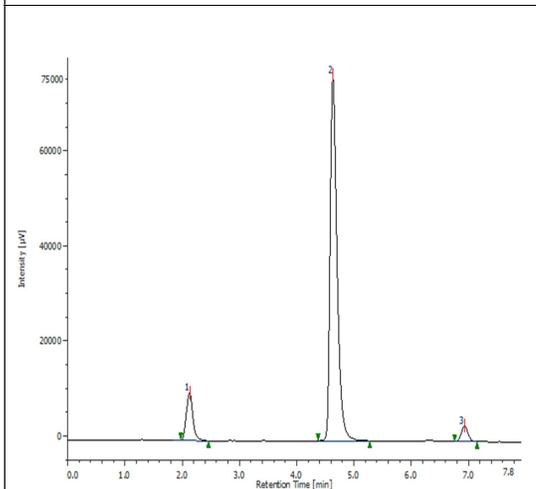


Figure 3.5.5.3

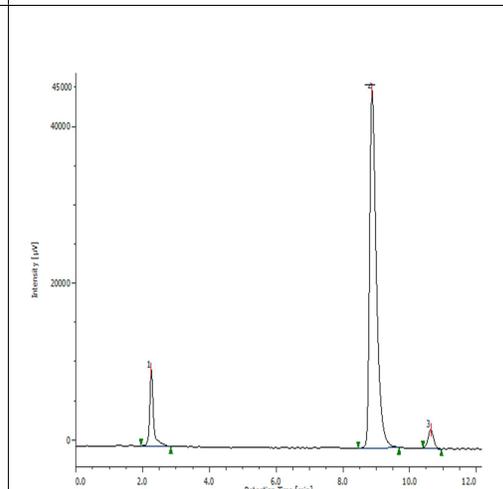


Figure 3.5.5.4

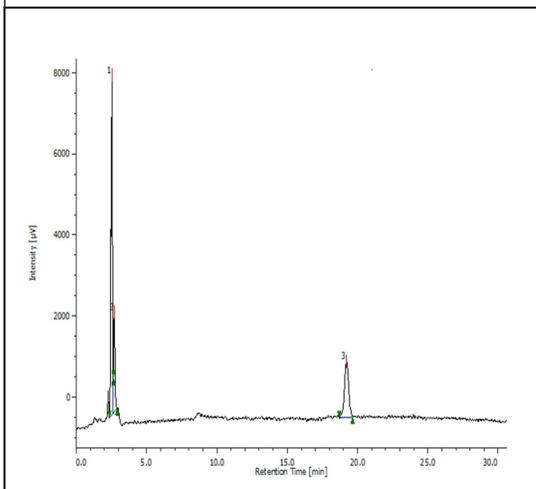


Figure 3.5.5.5

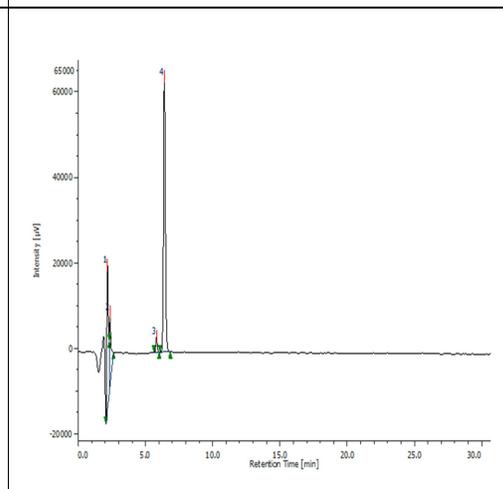


Figure 3.5.5.6

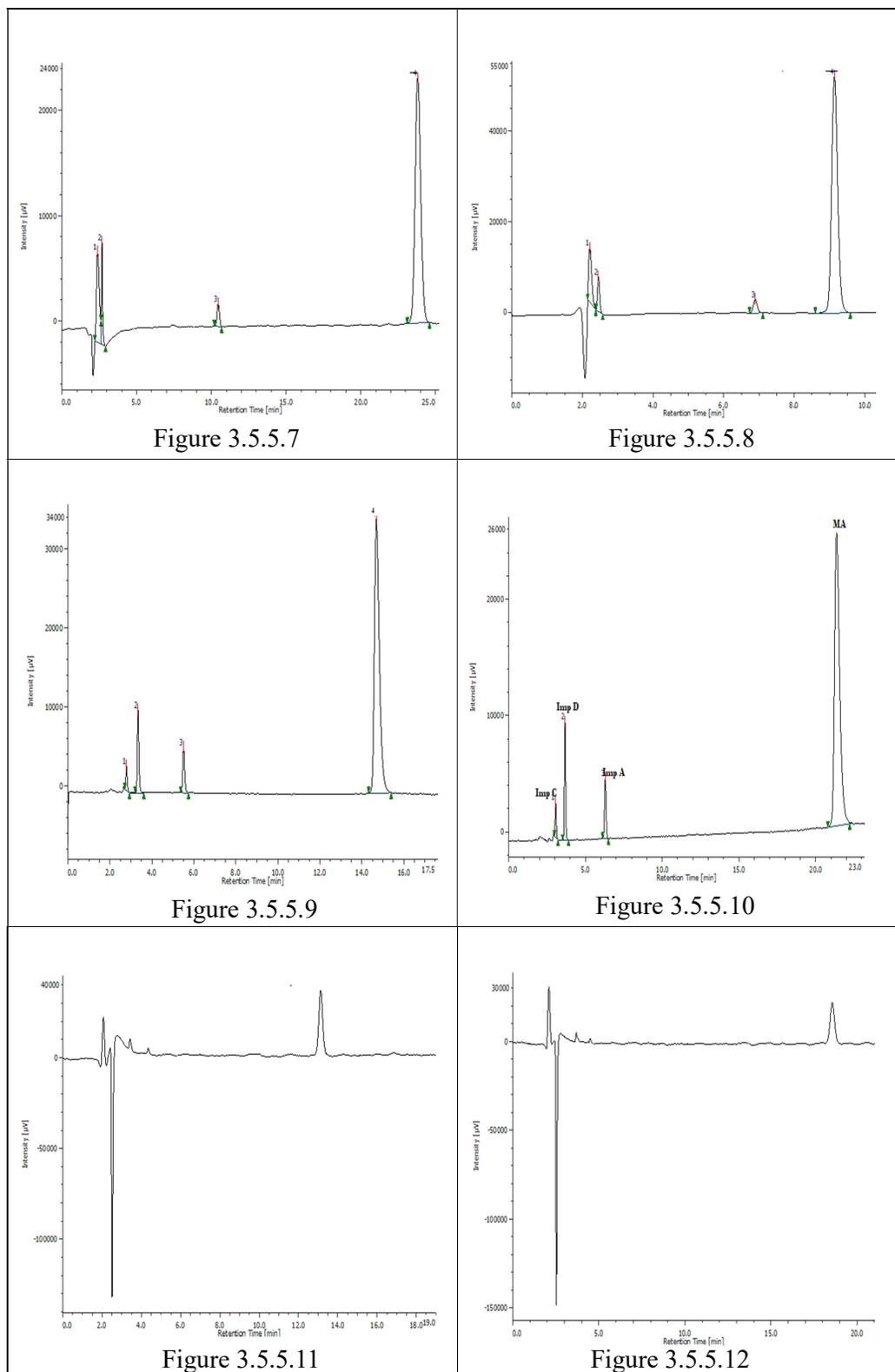


Figure 3.5.5: Representative chromatograms of exploratory trials for optimization of mobile phase composition for MA and impurities (A, C and D)

It was observed that when the pH of the buffer was maintained at 3, 5 and 6 incomplete separation due to peak merging or partial elution seen or early elution (peaks eluted in dead volume) was seen. However at pH (pKa of Mefenamic acid) separation was satisfactory. All the peaks were well resolved from each other and from drug peak as seen in Fig 3.5.5.10.

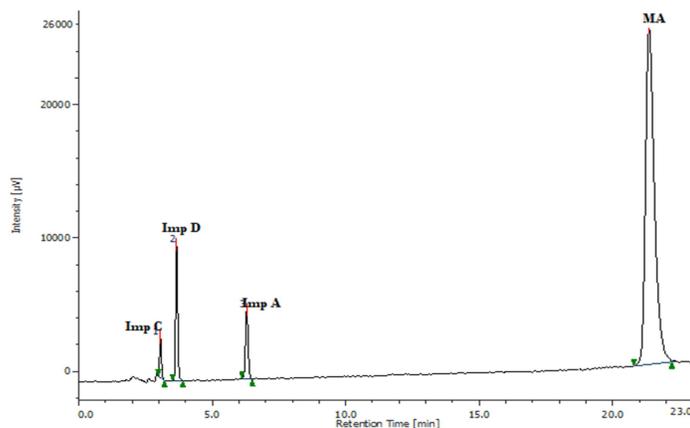


Figure 3.5.5.10: Optimized Chromatogram of Mefenamic acid and impurities (A, C and D)

Chromatographic conditions were optimized from exploratory trial on Waters Sunfire C-18 column. Best fit mobile phase for separation of MA from Imp A, Imp C and Imp D was selected best of satisfactory resolution of peaks on smooth baseline. Final optimized chromatographic condition is shown in Table 3.5.5.2.

Table 3.5.5.2: Optimized Chromatographic Conditions for separation of MA and impurities (A, C and D)

Mobile phase	Acetonitrile: 10 mM Ammonium dihydrogen ortho phosphate buffer (pH adjusted to 4 by dilute glacial acetic acid) in the ratio of 55:45 % v/v.
Column	Waters -Sunfire ODS C18 (250 x 4.6 mm ,5 µm)
Flow rate	1 ml/min
Wavelength	225 nm
Injector loop size	10 µL

3.6. FORCED DEGRADATION STUDIES

The forced degradation studies conducted for the drug MA was exposed to hydrolysis (HCl, NaOH and neutral), oxidation, elevated temperature and photolytic stress. Series of samples were prepared for each condition as per routine protocols (Table 3.6.). Sample subjected to stress were obtained and analyzed by the proposed method.

Table 3.6: Protocol for stress degradation of MA

Samples	Hydrolysis			Oxidative Deg.	Thermal Deg.	Photo Deg.
	Acid	Alkaline	Neutral			
Blank stored under normal condition	√	√	√	√	-----	-----
Blank subjected to stress condition	√	√	√	√	-----	-----
Drug / drug solution stored under normal condition	√	√	√	√	√	√
Drug / drug solution subjected to stress condition*	√	√	√	√	√	√

* To get desired degradation, initially degradation was carried out at room temperature and if necessary samples were subjected to higher temperature or strength of stress reagent was increased.

3.6.1. Hydrolytic Degradation

Hydrolytic studies were carried with HCl, NaOH and water to simulate acidic, alkaline and neutral conditions respectively. The acid and base degradation was carried out using hydrochloric acid and sodium hydroxide, initially with strength of N/10 and followed by 1N if no degradation is seen at lower strength. The studies were performed initially at room temperature followed by heating the solution of drug with acid, base and water at 70 °C on water bath equipped with thermostat for extended time intervals, if no degradation was seen at RT.

The drug solution treated with acid and base was neutralized by using base and acid respectively of the same strength and made up to the mark by diluent. These solutions were then subjected to analysis by the proposed method.

3.6.1.1. Acid Degradation

Acid degradation was carried out by using hydrochloric acid. Samples were prepared by taking 0.1 ml of stock solution of drug (1000 $\mu\text{g/ml}$) and 1 ml of HCl in 10 ml V.F and kept at RT and at higher temperature of 70 $^{\circ}\text{C}$ for extended time interval around 6 hr. Further the study was conducted using 1 ml of 1N HCl under the RT and 70 $^{\circ}\text{C}$. The samples were cooled and then neutralized by 1 ml of same strength of NaOH and volume was made with diluent up to the mark. The solutions were then analyzed by the proposed method. Observation of hydrolytic study of MA with varied concentration of HCl at different temperature and time is presented in Table 3.6.1.1 and representative chromatogram as Fig 3.6.1.1.

Table 3.6.1.1: Degradation study of MA with HCl

Concentration of HCl	Temperature	Time	% of active drug after degradation	% degradation
0.1N	RT	6 hr	96.88	-
1N	RT	6 hr	93.11	-
0.1N	70 $^{\circ}\text{C}$	6 hr	91.31	5.47
1N	70 $^{\circ}\text{C}$	6 hr	90.04	1.39

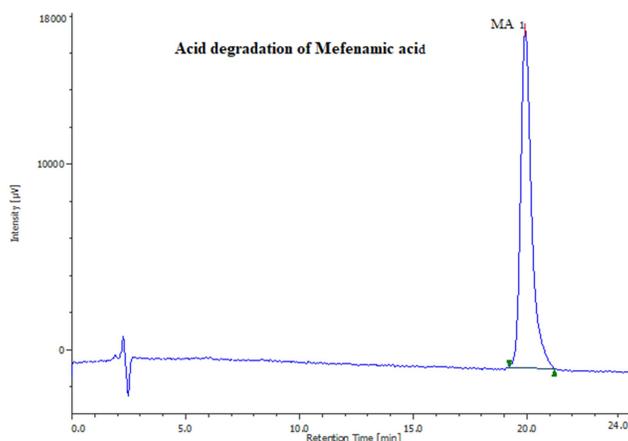


Figure: 3.6.1.1: Chromatogram of MA (10 $\mu\text{g/ml}$) treated with 1N HCl for 6 hr at 70 $^{\circ}\text{C}$

Peak name	CH	R _t (min)	Area($\mu\text{V}\cdot\text{sec}$)	NTP	Symmetry
MA	1	20.19	529260	8351	1.42
Control	1	20.33	587779	8192	1.48

3.6.1.2. Base Degradation

Base degradation was carried out by using sodium hydroxide. Samples were prepared by taking 0.1 ml of stock solution of drug (1000 µg/ml) and 1 ml of N/10 NaOH in 10 ml V.F and kept at RT and at higher temperature of 70 °C for extended time interval around 6 hr. The samples were cooled and neutralized by 1 ml of same strength of HCl and volume was made with diluent up to the mark. The solutions were then analyzed by the proposed method. Observation of hydrolytic study of MA with varied concentration of NaOH at different temperature and time is presented in Table 3.6.1.2 and representative chromatogram as Fig 3.6.1.2.

Table 3.6.1.2: Degradation study of MA with NaOH

Concentration of NaOH	Temperature	Time	% of active drug after degradation	% degradation
0.1N	RT	6 hr	98.94	-
1N	RT	6 hr	97.11	-
0.1N	70 °C	6 hr	97.39	1.56
1N	70 °C	6 hr	92.64	4.60

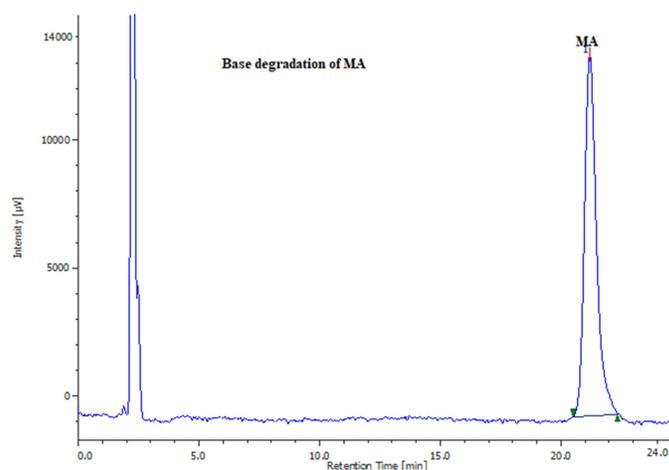


Figure: 3.6.1.2: Chromatogram of MA (10 µg/ml) treated with 1N NaOH for 6 hr at 70 °C

Peak name	CH	R _t (min)	Area(µV-sec)	NTP	Symmetry
MA	1	21.18	485086	9628	1.37
Control	1	21.61	523624	9550	1.26

3.6.1.3. Neutral Degradation

Neutral degradation was carried out by using distilled water. Samples were prepared by taking 0.1 ml of stock solution of drug (1000 µg/ml) and 1 ml of distilled water in 10 ml V.F and kept at RT and at higher temperature of 70 °C for extended time interval around 6 hr. The samples were cooled and volume was made with diluent up to the mark. The solutions were then analyzed by the proposed method. Observation of hydrolytic study of MA with water at different temperature and time is presented in Table 3.6.1.3 and representative chromatogram as Fig 3.6.1.3.

Table 3.6.1.3: Degradation study of MA with water

Temperature	Time	% of active drug after degradation	% degradation
RT	6 hr	95.25	-
70 °C	6 hr	93.08	2.27

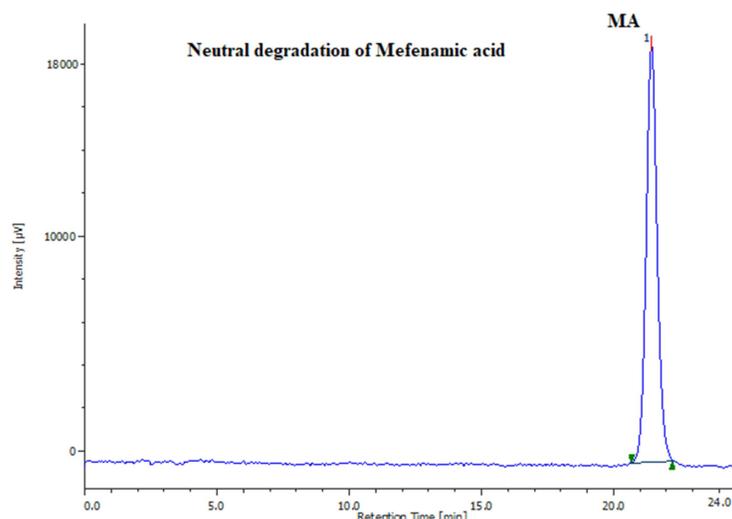


Figure 3.6.1.3: Chromatogram of MA (10 µg/ml) treated with water for 6 hr at 70 °C

Peak name	CH	R _t (min)	Area(µV-sec)	NTP	Symmetry
MA	1	21.42	479635	13250	1.07
Control	1	21.10	515330	9281	1.45

3.6.2. Oxidative Degradation

Oxidative degradation was carried out by using hydrogen peroxide. Samples were prepared by taking 0.1 ml of stock solution of drug (1000 µg/ml) and 1 ml of hydrogen peroxide (10 % v/v) in 10 ml V.F at RT for specified time interval (7 days) to obtain sufficient degradation. On repeated analysis, it was found that results of 3rd day were consistently obtained till 7th day. After required exposure samples were diluted up to the mark by mobile phase and subjected for analysis by the proposed method. Observation of oxidative study of MA with 10 % v/v H₂O₂ is presented in Table 3.6.2 and representative chromatogram as Fig 3.6.2.

Table 3.6.2: Degradation study of MA with H₂O₂

Temperature	Time	% of active drug after degradation	RRT of additional peak formed
RT	3 days	62.56	1) 0.46 (DP I) 2) 0.74 (DP II)

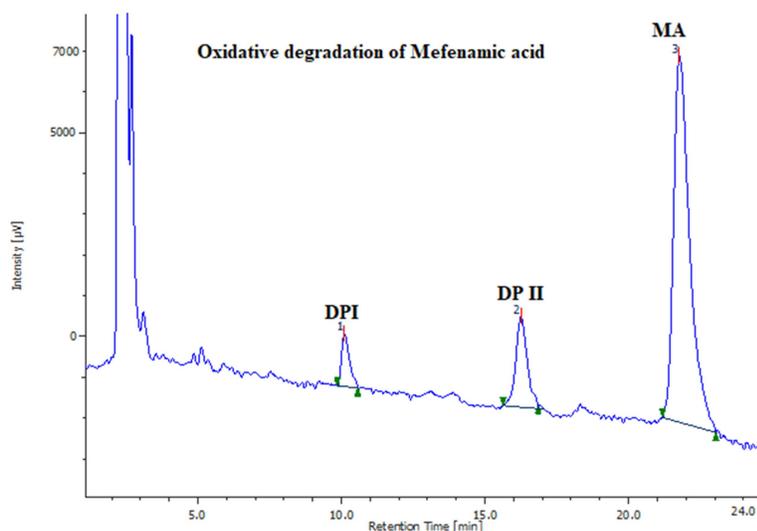


Figure 3.6.2: Chromatogram of MA (10 µg/ml) treated with 10 % v/v H₂O₂ at RT for 3 days

Peak name	CH	R _t (min)	Area(µV-sec)	NTP	Symmetry
MA	1	21.76	351931	7912	1.64
DPI	1	10.1	24701	8654	1.06
DP II	1	16.25	62325	6701	1.66

3.6.3. Thermal Degradation

Drug sample 10 mg each was taken in two 10 ml V.F and closed with stopper. One flask was exposed to dry heat in hot air oven for specified temperature (80 °C) and time interval (4 days) and another was kept as control. After required exposure two separate solutions were prepared by weighing appropriate amount of sample exposed to thermal stress and control to produce concentration of 10 µg/ml. The samples were diluted up to the mark with mobile phase and injected separately for analysis by the proposed method. Observation of thermal study of MA in hot air oven (80 °C) is presented in Table 3.6.3 and representative chromatogram as Fig 3.6.3.

Table 3.6.3: Degradation study of MA in hot air oven

State	Temperature	Time	% of active drug after degradation
Solution	70 °C	6 hr	93.08
Solid	80 °C	4 days	89.61

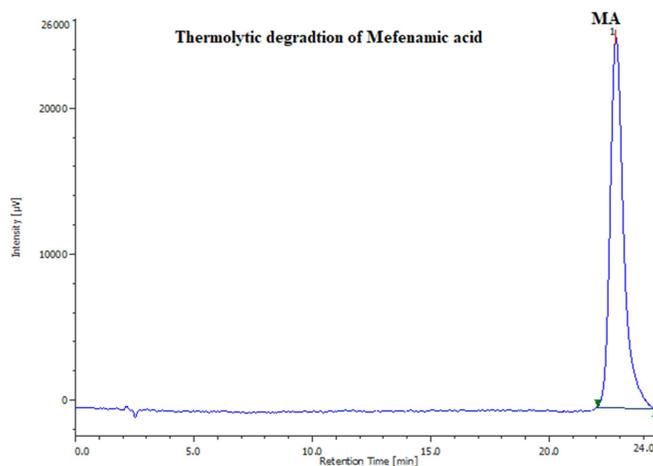


Figure 3.6.3: Chromatogram of MA (10 µg/ml) in oven for 4 days at 80 °C

Peak name	CH	R _t (min)	Area(µV-sec)	NTP	Symmetry
MA	1	22.74	533414	9162	1.39
Control	1	22.23	595230	7490	1.56

3.6.4. Photo Degradation

Drug in sufficient amount was taken in 10 ml volumetric flask and was exposed to direct sunlight for a specified time period (7 days). Another flask containing drug was kept as control. After required exposure two separate solutions were prepared by weighing appropriate amount of sample exposed to stress and control to produce concentration of 10 µg/ml. The samples were diluted up to the mark with mobile phase and injected separately for analysis by the proposed method. Observation of photo light study of MA after direct exposure to sunlight is presented in Table 3.6.4 and representative chromatogram as Fig 3.6.4.

Table 3.6.4: Degradation study of MA with direct exposure to sunlight

Temperature	Time	% of active drug after degradation
Sunlight	7 days	96.38

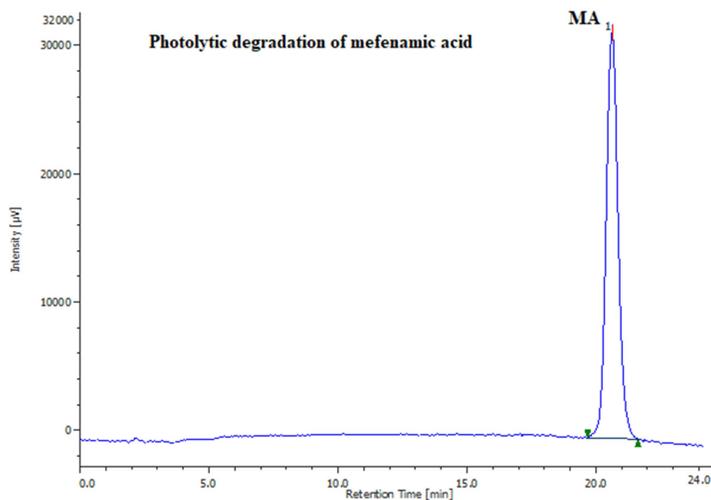


Figure 3.6.4: Chromatogram of MA (10 µg/ml) exposed to direct sunlight for 7 days

Peak name	CH	R _t (min)	Area(µV-sec)	NTP	Symmetry
MA	1	20.61	509570	9777	1.10
Control	1	21.02	528709	11043	1.08

Forced degradation study of MA showed that there were formation of two degradation products (DP I and DP II) under oxidative stress conditions. DP I showed an RRT value of 0.464 and DP II showed an RRT value of 0.746. The RRT values of DP I and DP II are different from the RRT values of all the specified impurities after application of the developed method. Hence the RP HPLC developed for separation of MA and its pharmacopoeial impurities is a stability indicating method for MA.

3.7. VALIDATION OF DEVELOPED STABILITY INDICATING ANALYTICAL METHOD

Validation is an act of confirming that a method does what it is intended to do. According to USP SIAM is grouped under category II (Analytical methods for determination of impurities in APIs or for determination of degradation products in final drug products). Parameters recognized by ICH for method validation (Table: 3.7).

Table 3.7: Validation parameters and acceptance criteria

Sr.No.	Validation Parameters	Acceptance Criteria
1	Specificity	Peak (s) of degradation products and analyte should be pure and well separated from one other.
2	Linearity	Correlation coefficient not less than 0.999
3	Accuracy (across the specified range)	Recovery (%) between 98 % to 102 %
4	Precision	
	4.1) Intra-day precision	RSD (%) of replicate injections not more than 2.0 of peak area
	4.2) Inter-day precision	
5	Robustness	
6	System Suitability Test	1) Resolution \nless 2.0 2) % RSD of replicate injections \nless 2.0 3) Theoretical plate number \nless 2000 4) Asymmetry of peak should not be more than 2.0

3.7.1. System suitability parameters

Results of the study for system suitability parameters when proposed method was applied for analysis of MA in presence of Imp A, Imp C, Imp D, and Degradation products (DP I and DP II) is presented in Table 3.7.1. Values obtained meet the acceptance criteria Table 3.7.

Table 3.7.1: System suitability testing parameters of the proposed RP HPLC method

Sr.no	Components	RRT	Resolution	Peak asymmetry	Theoretical plate
1	MA	1.00	NA	1.45	21212
2	Imp A	0.29	39.37	1.05	19264
3	Imp C	0.14	4.8	1.30	8696
4	Imp D	0.17	16.29	1.19	10387
5	DP I	0.46	10.35	1.66	6701
6	DP II	0.74	6.55	1.06	8654

3.7.2. Specificity and Selectivity

Results of specificity and selectivity study undertaken on MA along with Imp A, Imp C Imp D , DP I and DP II are presented in Table 3.7.2 and are in agreement with acceptance criteria defined in Table 3.7.

Table 3.7.2: Selectivity of the HPLC method

Component	Peak (RRT)
MA	1.00
Imp A	0.29
Imp C	0.14
Imp D	0.17
DP I	0.46
DP II	0.74

3.7.3. Linearity and Range

Study involving determination of linearity range was undertaken with working/standard solution of MA, Imp A, Imp C and Imp D diluted to appropriate concentrations as mentioned under 3.9.6.2. Results of the study are tabulated and linearity graphs presented by various figures as follow.

Table 3.7.3.1: Linearity Range

Compound	Linearity range ($\mu\text{g}/\text{ml}$)	R ²	Reference
MA	12.42-100	0.9990	Fig 3.7.3.1
Imp A	0.67-10	0.9997	Fig 3.7.3.3
Imp C	1.66-10	0.9982	Fig 3.7.3.5
Imp D	1.04-10	1.0000	Fig 3.7.3.7

Table 3.7.3.2: Linearity data of MA

Sr.No	Conc. ($\mu\text{g}/\text{ml}$)	Peak Area				RSD (%)
		Injection 1	Injection 2	Injection 3	Average of 3 injection	
1	10	530877	523185	534942	529668	1.12
2	20	1024480	1034615	1015133	1024743	0.95
3	40	2000730	2001279	2010463	2004157	0.27
4	60	3025211	3020751	3020860	3025211	0.08
5	80	4135517	4127797	4107798	4123704	0.34
6	100	5233434	5317863	5261211	5270836	0.81

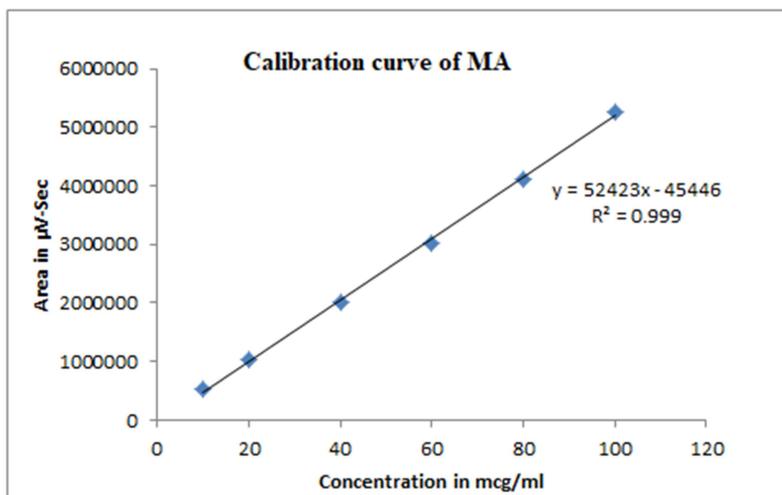


Figure: 3.7.3.1 Linearity graph of MA

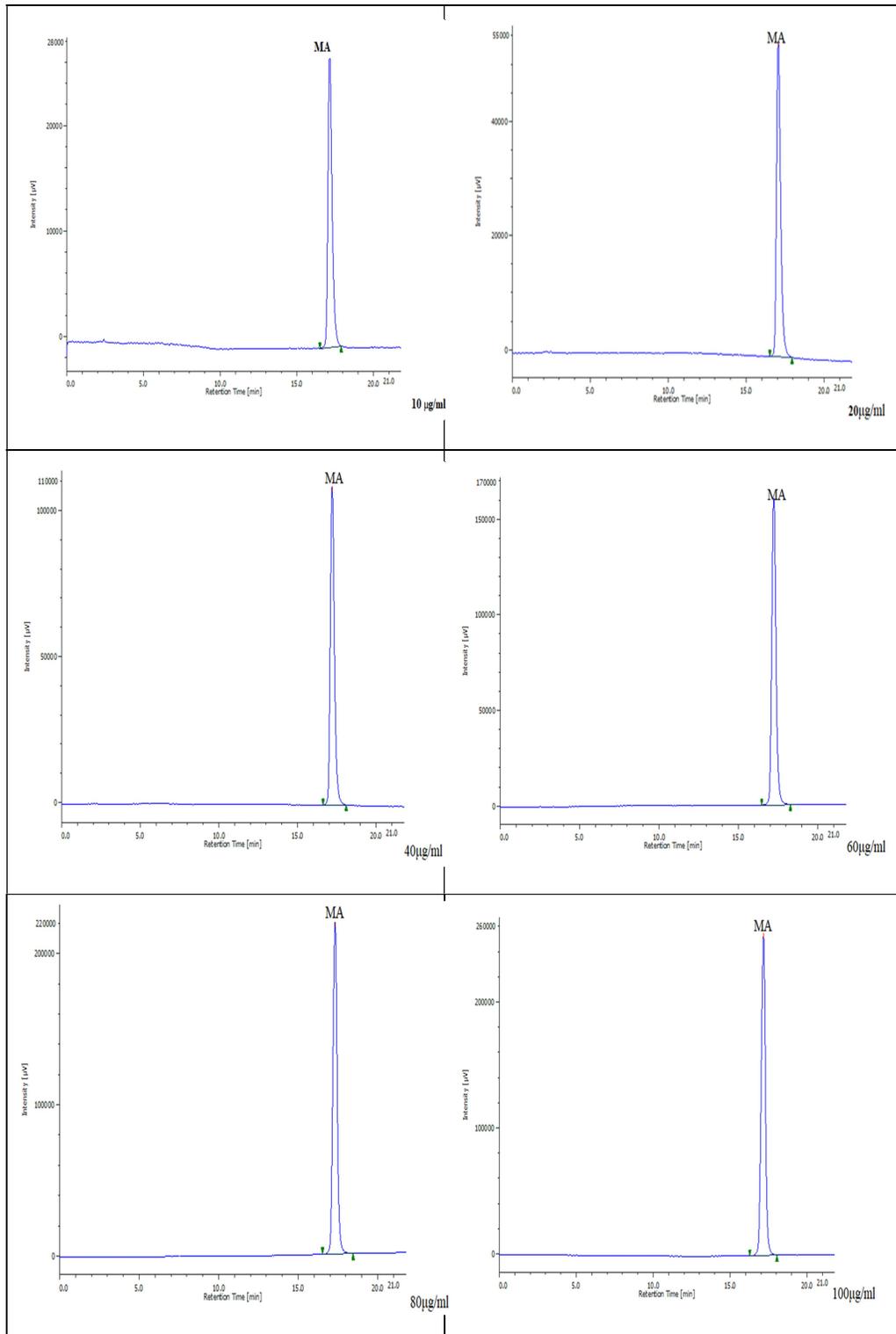


Figure 3.7.3.2: Representative chromatograms of MA (Conc. 10, 20, 40, 60, 80, 100 µg/ml) after first injection

Table 3.7.3.3: Linearity data of Imp A

Sr.No	Conc. (µg/ml)	Peak Area				RSD (%)
		Injection 1	Injection 2	Injection 3	Average of 3 injections	
1	0.5	17928	17854	18002	17928	0.41
2	1	30997	31278	30637	30971	1.04
3	2	61503	60750	62352	59535	1.30
4	4	126726	126003	126210	126313	0.29
5	8	252098	250486	253000	251861	0.51
6	10	320092	317281	317484	318286	0.49

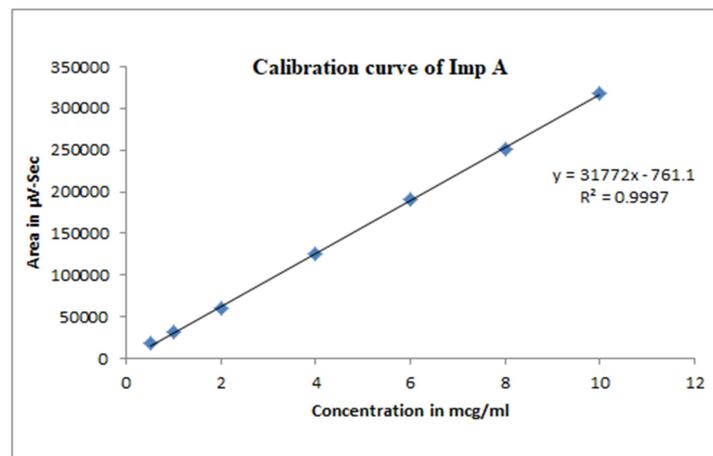
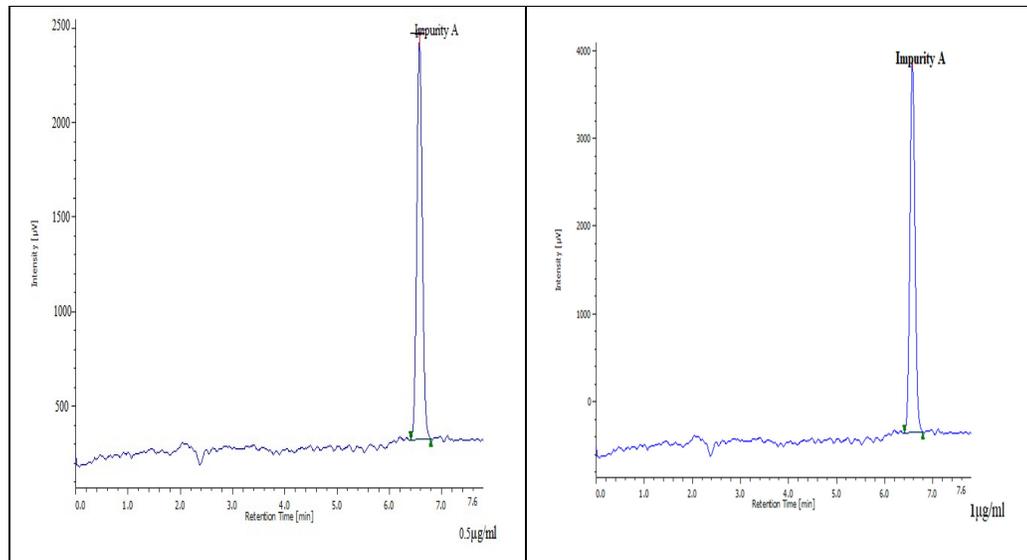


Figure 3.7.3.3: Linearity graph of Imp A



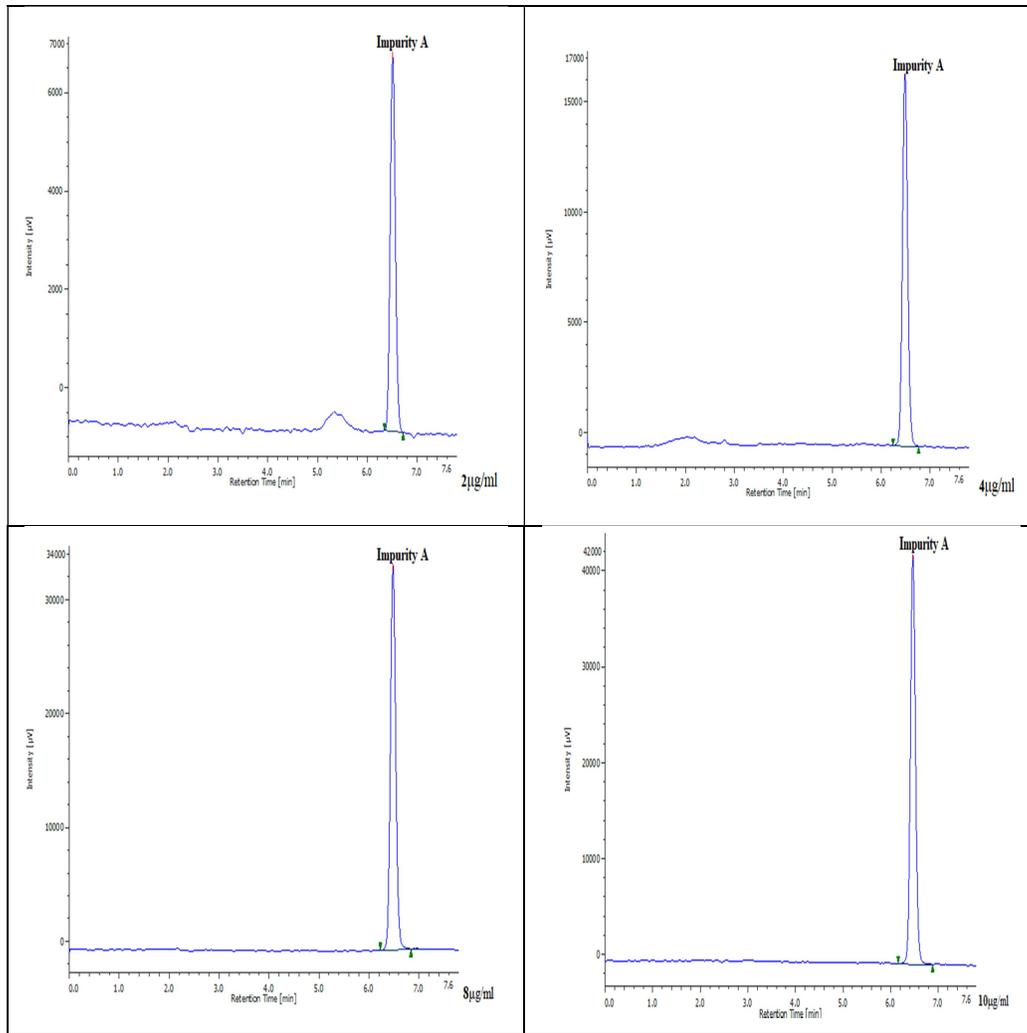


Figure 3.7.3.4: Representative chromatogram of Imp A (Conc. 0.5, 1, 2, 4, 8, 10 µg/ml) after first injection

Table: 3.7.3.4: Linearity data of Imp C

Sr.No	Conc. (µg/ml)	Peak Area				RSD (%)
		Injection 1	Injection 2	Injection 3	Average of 3 injections	
1	1	15783	15810	16054	15882	0.94
2	2	29699	30278	30338	30105	1.17
3	4	54477	54787	54477	54580	0.32
4	6	89077	89844	90030	89650	0.56
5	8	115909	116116	116258	116094	0.15
6	10	142881	143419	143693	143330	0.28

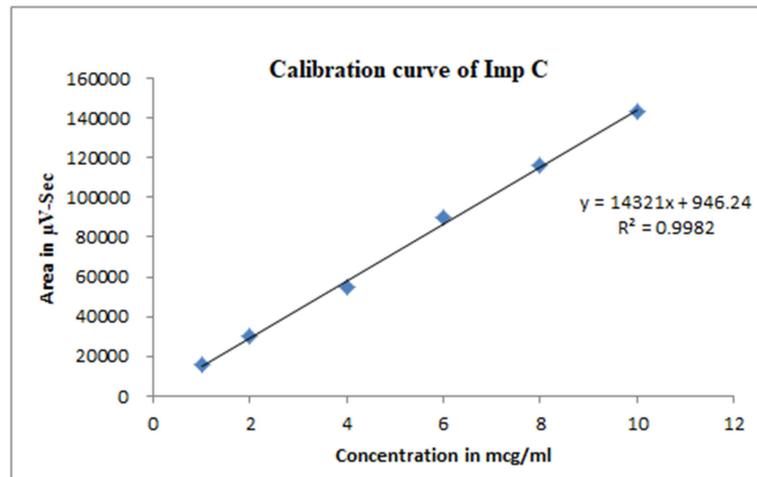
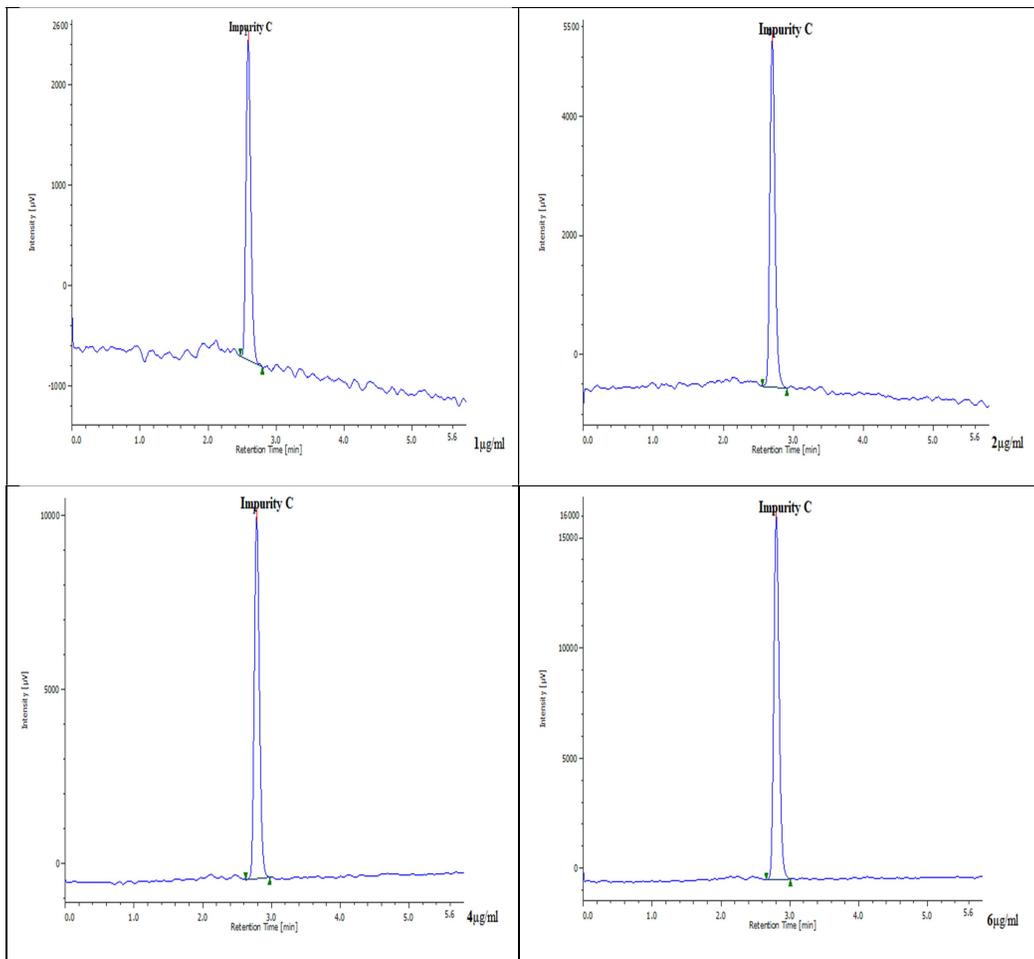


Figure 3.7.3.5: Linearity graph of Imp C



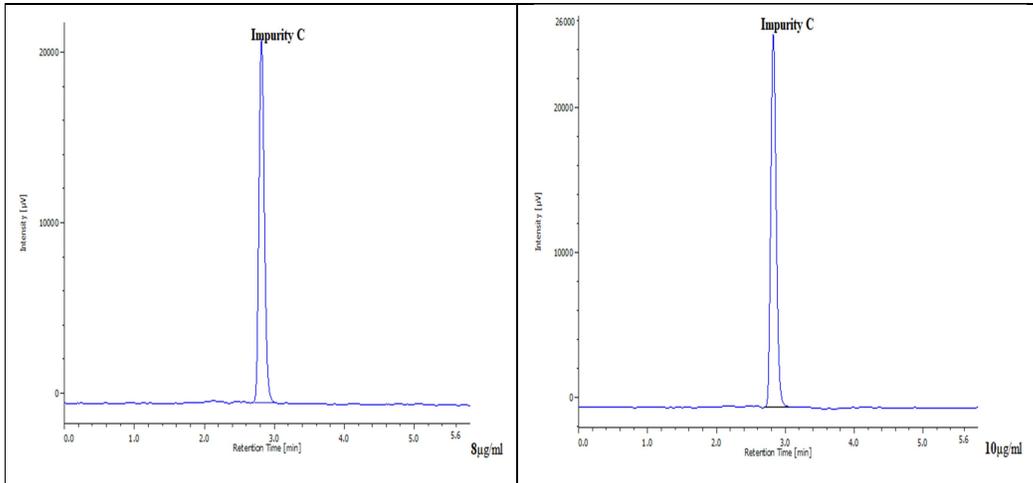


Figure 3.7.3.6: Representative chromatogram of Imp C (Conc, 1, 2, 4, 6, 8, 10 µg/ml) after first injection

Table 3.7.3.5: Linearity data of Imp D

Sr.No	Conc. (µg /ml)	Peak Area				RSD (%)
		Injection 1	Injection 2	Injection 3	Average of 3 injections	
1	1	53166	53016	52173	52785	1.01
2	2	105716	103457	105716	104963	1.24
3	4	205678	206261	205893	205944	0.14
4	6	310052	309881	311706	310546	0.32
5	8	413681	414687	413927	414098	0.12
6	10	516880	513889	516872	515880	0.33

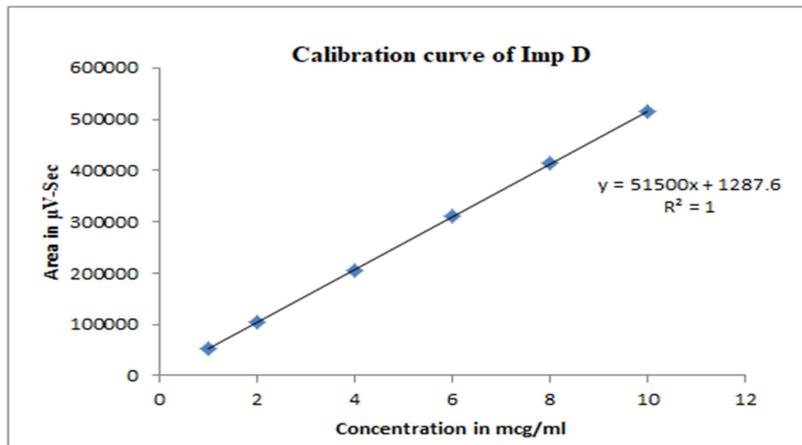
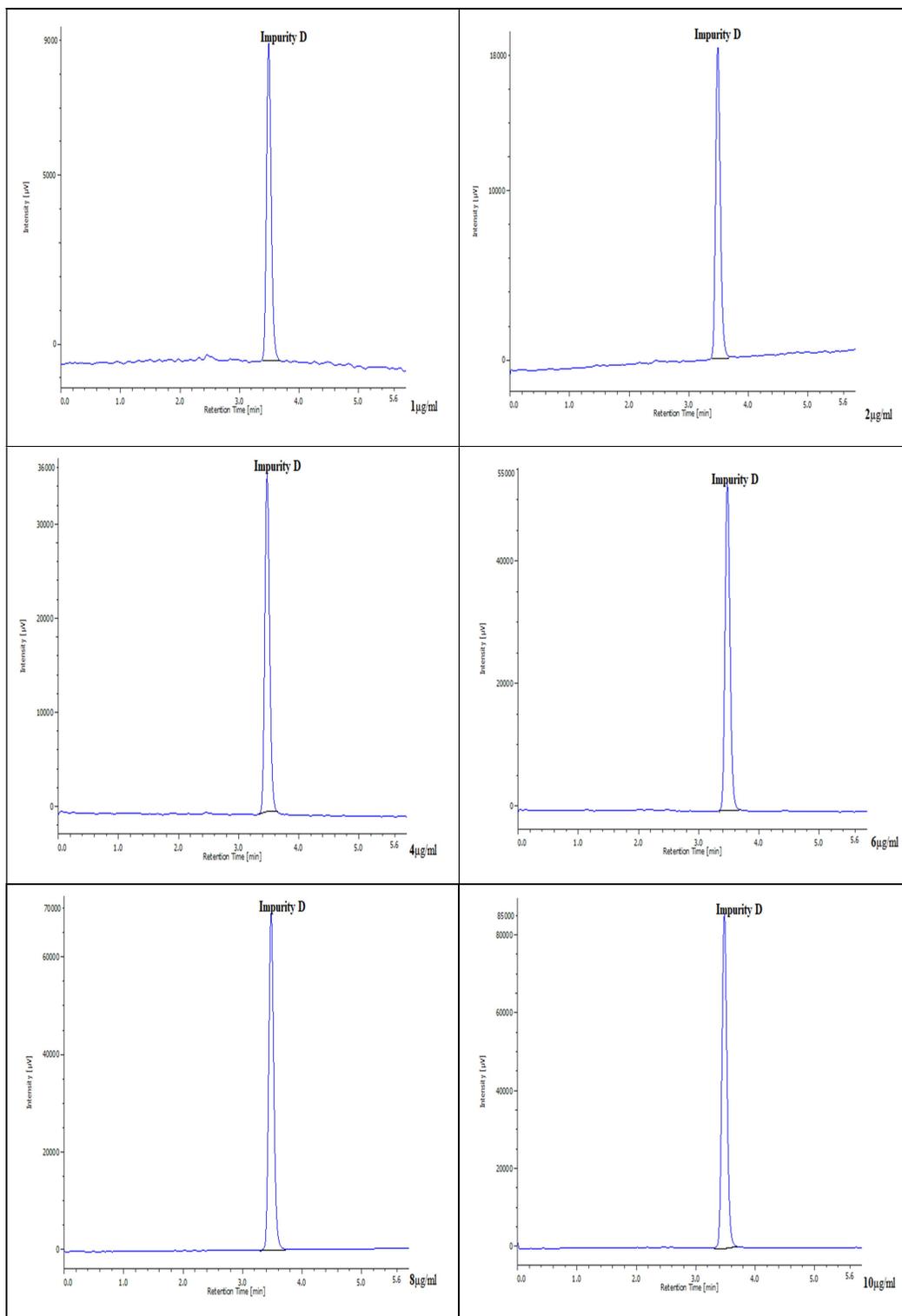


Figure 3.7.3.7: Linearity graph of Imp D



**Figure 3.7.3.8: Representative chromatogram of Imp D
(Conc. 1, 2, 4, 6, 8, 10 $\mu\text{g/ml}$) after first injection**

3.7.4. Recovery / Accuracy

Accuracy of the proposed method was determined by recovery studies using standard addition method. The percentage recovery studies of MA, Imp A, Imp C and Imp D was carried out in triplicate at 3 different levels ranging from 80 % - 120 %, by spiking standard samples as described under procedure at section 3.9.6.3. Result of recovery study is presented in Table 3.7.4.

Table 3.7.4: Recovery studies (n=3)

Component	Amount of sample (µg/ml)	Average peak area of sample	Level of spiking (%)	Amount of standard spiked (µg/ml)	Average Peak area* after standard addition	Peak area of recovered standard	Amount Recovered (µg/ml)	% Recovery
MA	40	2004157	80	32	3626549	1622392	32.38	99.08
			100	40	4007314	2003157	39.98	99.95
			120	48	4415831	2411674	48.13	100.27
Imp A	4	126313	80	3.2	226113	99800	3.16	98.76
			100	4	251426	125113	3.96	99.04
			120	4.8	279340	153027	4.84	100.95
Imp C	4	54580	80	3.2	98770	44190	3.28	98.95
			100	4	111160	56580	4.14	100.66
			120	4.8	122690	68110	4.99	101.99
Imp D	4	205944	80	3.2	371474	165530	3.21	98.99
			100	4	409888	203944	4.05	100.45
			120	4.8	453631	247687	4.81	101.22

*Average of three readings

3.7.5. Precision and Sensitivity

Intraday repeatability of the method was evaluated by analyzing three concentrations of MA (30, 50 and 70 µg/ml), and three concentration of Imp A, Imp C and Imp D (3, 5 and 7 µg/ml). Interday precision was evaluated by assaying the three chosen same sets of concentration of MA, Imp A, Imp C and Imp D in triplicates on two successive days using the procedure stated under section 3.9.6.4 and 3.9.6.5. Results of the study are presented in Table 3.7.5.1 and Table 3.7.5.2.

Table 3.7.5.1 Intra and Interday Precision data (n=3)

Compound	Concentration (µg/ml)	Inter-day		Intra-day	
		mean peak area ± SD	% RSD	mean peak area ± SD	% RSD
MA	30	1514450 ± 3030	0.20	1289450 ± 4572	0.35
	50	2512564 ± 2682	0.37	2038633 ± 8437	0.41
	70	7145978 ± 16188	0.22	2921366 ± 12363	0.42
Imp A	3	93924 ± 481	0.51	90593 ± 674	0.74
	5	158456 ± 230	0.78	154125 ± 1389	0.90
	7	221230 ± 812	0.36	217802 ± 2140	0.98
Imp C	3	42342 ± 229	0.54	48709 ± 259	0.53
	5	72115 ± 294	0.99	81632 ± 754	0.92
	7	102872 ± 338	0.32	112458 ± 353	0.31
Imp D	3	310907 ± 1035	0.33	149787 ± 1326	0.88
	5	516490 ± 981	0.45	257520 ± 790	0.30
	7	724644 ± 952	0.13	350317 ± 1167	0.33

Table 3.7.5.2 Sensitivity of the method (LOD and LOQ)

Parameters	MA	Imp A	Imp C	Imp D
LOD(µg/ml)	4.09	0.22	0.54	0.34
LOQ(µg/ml)	12.42	0.67	1.66	1.04

3.7.6. Robustness

Robustness of an analytical method is the measurement of the methods ability to remain unchanged by minute but intended alterations in the methods variables and provide reliability during normal use. The robustness of the method was studied by making deliberate changes in flow rate, and composition of mobile phase ratio.

3.7.6.1. Variation in the flow rate (± 0.1 ml/ min of the optimized flow rate)

The optimized flow rate for the method was set at 1ml/min. For robustness study, the flow rate was varied to 0.9 ml/min and 1.1 ml/min. The solutions were then injected into the chromatograph equilibrated with the flow rate of 0.9 ml/min and 1.1 ml/min. The results

obtained upon the flow rate variation are displayed in Table 3.7.6.1.1 with representative chromatogram (Fig 3.7.6.1.1 and Fig 3.7.6.1.2).

Table 3.7.6.1.1: Results of flow rate variation

	MA	Imp A	Imp C	Imp D
Flow rate	Average area (n=3)	Average area (n=3)	Average area (n=3)	Average area (n=3)
0.9 ml	2261437	141117	73500	259198
1 ml	2221497	137451	72447	258846
1.1 ml	2204200	138724	74501	254653
% RSD	1.31	1.33	1.39	0.98

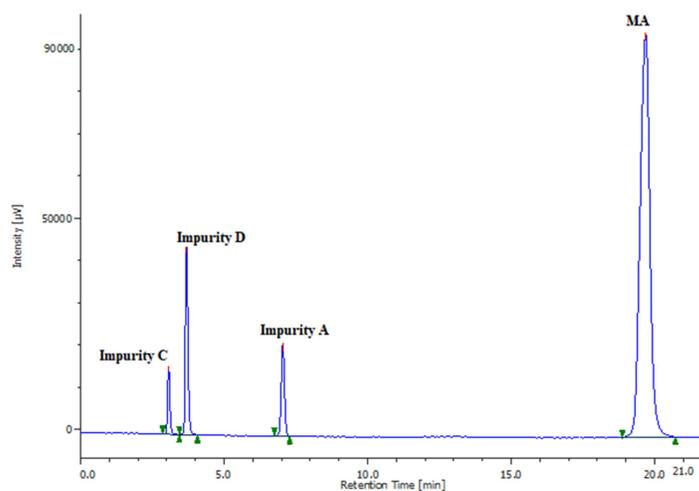
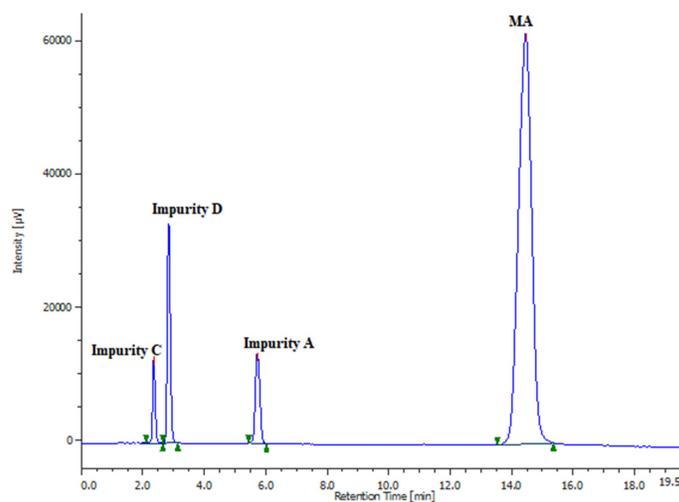


Figure 3.7.6.1.1: Chromatogram with flow rate 0.9 ml/min (Optimized 1.0 ml/min)



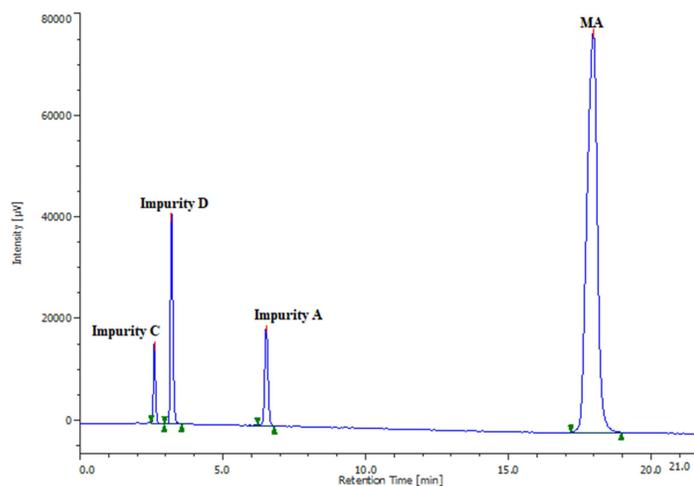
**Figure 3.7.6.1.2: Chromatogram with flow rate 1.1 ml/min
(Optimized 1.0 ml/min)**

3.7.6.2. Variation in the mobile phase composition ($\pm 2\%$ of the optimized ratio)

The optimized isocratic elution program was acetonitrile: ammonium dihydrogen ortho phosphate buffer (pH adjusted to 4 with OPA acid) in ratio of 55:45 % v/v. For robustness study, organic phase ratio was varied to 53:47 % v/v (- 2 % of the optimized ratio) and 57:43 % v/v (+ 2 % of the optimized ratio). The results obtained for robustness study wherein organic phase ratio of mobile phase was varied is presented in Table 3.7.6.2.1 with representative chromatograms (Fig 3.7.6.2.1 and Fig 3.7.6.2.2).

Table 3.7.6.2.1: Results of mobile phase variation

	MA	Imp A	Imp C	Imp D
Composition	Average area (n=3)	Average area (n=3)	Average area (n=3)	Average area (n=3)
53:47 % v/v	2002461	153439	81568	254916
55:45 % v /v	2036931	156937	81237	258631
57:43 % v/v	2013820	153439	81507	255312
% RSD	0.87	1.04	0.21	0.79



**Figure 3.7.6.2.1: Chromatogram with organic phase ratio altered to 53:47 % v/v
(Optimized 55:45 % v/v)**

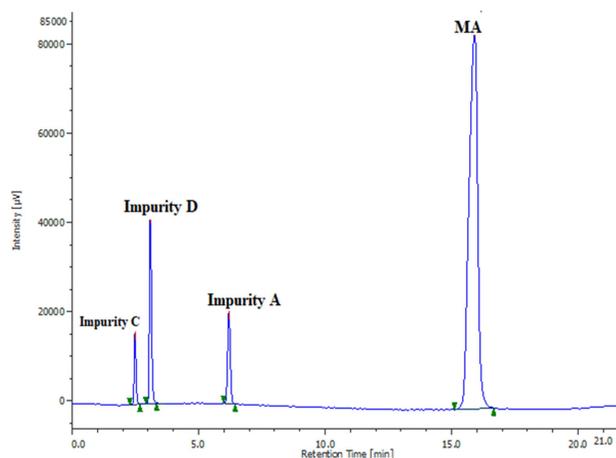


Figure 3.7.6.2.2: Chromatogram with organic phase ratio altered to 57:43 % v/v (Optimized 55:45 % v/v)

3.8. ANALYSIS OF MARKETED PRODUCT

Further new validated HPLC method was extended for the determination of MA in marketed formulation MEFTAL-250 DT (Mfg. Blue Cross Laboratories Pvt Ltd) as per the procedure under section 3.9.7. The assay result obtained was 99.02 %. Results are summarized in Table 3.8. No detectable peaks were found at the RRTs of Pharmacopoeial impurities (A, C and D) and degradation products, signifying their absence in the detectable concentration (Fig 3.8).

Table 3.8: Result of MA in marketed product (MEFTAL -250 DT)

Sr. No	Conc. of sample solution ($\mu\text{g/ml}$)	Peak Area	Mean Peak Area	% Assay
1	50	2483902	2487941	99.02
2		2490939		
3		2488983		

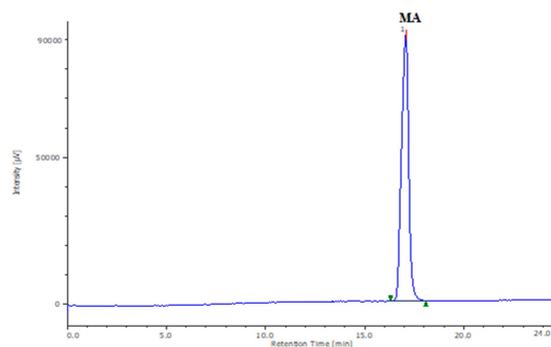


Figure 3.8: Chromatogram of MA sample (MEFTAL -250 DT)

Thus proposed new RP HPLC is validated and demonstrated to be stability indicating analytical method for the determination of MA, and can be a useful alternate for the sensitive determination of the drug as API and in formulations (Tablets) in presence of its possible specified impurities.

3.9. METHODOLOGY FOR DETERMINATION OF MEFENAMIC ACID AND ITS IMPURITIES

3.9.1. Instrumentation

Instrument	Source
HPLC System	Jasco LC-4000 series quaternary pump system (PU-4180), an on-line degasser, an auto-sampler (AS-4050), a column temperature controller (CO-4061) and a diode array detector (MD-4010). Software: Jasco Chrom NAV software
Column	Waters -Sunfire ODS C18 (4.6 x 250 mm, 5 µm) column.
Sonicator	Citizon Ultrasonic cleaner
pH meter	Digital pH Meter, Labtronics, LT-10
Electronic balance	Wensar Digital Electronic Balance MAB 220
HPLC water purification system	Bio-age Water purification system
Hot air oven	Universal Hot air Owen
Constant temperature water bath	Tempo

3.9.2. Chemicals and Reagents

- 1) Acetonitrile (HPLC grade), Rankem, India.
- 2) Water (HPLC grade), obtained from Bio age water purification system.
- 3) Ammonium Dihydrogen Ortho Phosphate (AR grade), S.D. Fine-Chem Ltd., Mumbai, India.
- 4) Glacial acetic acid (AR grade), Qualigens fine chemicals, Mahape, India.
- 5) Hydrochloric acid (AR grade), Qualigens fine chemicals, Mahape, India.
- 6) Sodium Hydroxide (AR grade), Qualigens fine chemicals, Mumbai, India.
- 7) Hydrogen peroxide, S.D. Fine-Chem Ltd., Mumbai, India.

3.9.3. Working standard

Mefenamic acid (Blue cross laboratories Ltd, Verna, Goa), 2,3-Dimethylaniline (MA- Imp A), 2-Chlorobenzoic acid (MA- Imp C) and Benzoic acid (MA- Imp D) (Sigma Aldrich, USA).

3.9.4. Solution Preparation

3.9.4.1. Preparation of Stock Solution of MA

Mefenamic acid (100 mg) was weighed and transferred into 100 ml V.F., dissolved in 50 ml of acetonitrile and volume made up to the mark to obtain solution concentration 1000 µg/ml. Further dilutions were made from stock as per the requirements.

3.9.4.2. Preparation of Stock solution of Impurities

An amount of 100 mg of MA- Imp A, MA- Imp C and MA- Imp D was weighed and transferred into three separate 100 ml V.F. 75 ml of acetonitrile added, mixed well and sonicated for 10 min. Final volume was made up to the mark to obtain concentration of each impurity 1000 µg/ml.

3.9.4.3. Preparation of working standard solution of Impurities

1 ml of stock solution of each impurity was transferred into three different 10 ml V.F. Acetonitrile was added in the flasks to the mark to get concentration of 100 µg/ml of each impurity.

3.9.4.4. Preparation of buffer – 10 mM Ammonium dihydrogen orthophosphate (pH = 4.0)

Ammonium dihydrogen orthophosphate (1.15 gm) was dissolved in 1000 ml of HPLC grade water and pH adjusted to 4.0 with dil HAc. The solution was filtered through 0.45 µ membrane filter.

3.9.4.5. Preparation of 1N sodium hydroxide solution

Sodium hydroxide flakes (4 gm) were dissolved in 50 ml of water in 100 ml V.F and volume made up to the mark with water to get 1N sodium hydroxide solution.

3.9.4.6. Preparation of 1N hydrochloric acid solution

Hydrochloric acid (1N) was prepared by diluting 8.5 ml of Conc. HCl to 100 ml with water in V.F.

3.9.4.7. Preparation of 10 % v/v hydrogen peroxide solution

Hydrogen peroxide (10 % v/v) was prepared by appropriately diluting 30 % v/v H₂O₂ to 100 ml with water in V.F.

3.9.4.8. Diluent

Mixture of ACN and ammonium dihydrogen orthophosphate buffer (pH adjusted to 4 with dil HAc) in the ratio 55:45 % v/v was used as diluent for dilution of samples.

3.9.5. Stress studies

The forced degradation studies were carried out with objectives to achieve substantial degradation of the drug. The drug was exposed to hydrolytic, oxidative, thermal and photolytic stress. The results were obtained by comparing four samples which were generated for every stress condition except thermal and photolytic stress viz., the blank stored under normal condition (blank untreated), the blank subjected to stress in same manner as that of drug solution (blank treated), zero time sample containing the drug (standard untreated) and the drug solution subjected to stress treatment. For thermal and photolytic stress conditions, only two samples were generated one sample exposed to stress condition and other is control.

3.9.5.1. Hydrolytic degradation

The hydrolytic degradations were carried out in acidic, alkaline and neutral conditions. Samples were prepared by taking 0.1 ml of stock solution of drug (1000 µg/ml) and 1 ml of hydrolytic agent (HCl / NaOH / water) in 10 ml volumetric flask. Initially 0.1N strength of HCl and NaOH was used for study followed by 1N strength, if no degradation was seen in 0.1N strength of hydrolytic agent. The study was carried out at room temperature. If required, the samples were heated on constant temperature water bath at 70 °C for specified time intervals. After required exposure samples were neutralized by using equal strength of acid or alkali which ever was required. Finally volume was made up to the mark by using diluent and subjected for HPLC analysis by proposed method.

3.9.5.2. Oxidative degradation

Oxidative degradation was carried out using H₂O₂. Samples were prepared by using 0.1 ml of stock solution of drug (1000 µg/ml) and 1 ml of hydrogen peroxide (10 %) in 10 ml V.F

at RT for varied time intervals (7 days) Further the samples were processed by diluting up to the mark using diluent and subjected to HPLC analysis by proposed method.

3.9.5.3. Thermal degradation

Drug sample 10 mg each was taken in two 10 ml V.F and stoppered. One flask was exposed to dry heat in hot air oven for specified temperature (80 °C) and time interval (4 days) and another was kept as control. After exposure two separate solutions were prepared by weighing appropriate amount of sample exposed to thermal stress and control to produce concentration of 10 µg/ml. The samples were diluted up to the mark with the help of diluent and subjected to analysis by proposed HPLC method.

3.9.5.4. Photo degradation

Drug in sufficient amount was taken in 10 ml V.F and was exposed to direct sunlight for a specified time period (7 days). Another flask containing drug was kept as control. After required exposure two separate solutions were prepared by weighing appropriate amount of sample exposed to stress and control to produce concentration of 10 µg/ml. The samples were diluted up to the mark with the help of diluent and injected and analyzed by proposed HPLC method.

3.9.6. Validation studies

Validation of optimized stability indicating method was carried out with respect to parameters recommended under ICH guideline Q2 (R1).

3.9.6.1. Specificity and Selectivity

Establishment of resolution factor between the drug peak, pharmacopoeial impurity peaks and other obtained degradation peak was explored to determine the specificity of the method. Method Specificity was evaluated by comparing relative retention time (RRT) of MA, Imp A, Imp C and Imp D and stressed samples of MA on the developed method. Chromatograms were recorded and RRT were calculated.

3.9.6.2. Linearity and Range

To establish linearity and range, stock solution of drug (1000 µg/ml) was further diluted with the help of diluent to get the drug concentration range of 10-100 µg/ml.

The samples introduced into HPLC in triplicate. For impurities, each impurity was diluted from working standard solutions to get a concentration range of 0.5-10 µg/ml. The samples were introduced into HPLC in triplicate.

3.9.6.3. Accuracy

Accuracy of the proposed method was determined by recovery studies using standard addition method. The percentage recovery studies of MA, MA-Imp A, MA-Imp C and MA-Imp D was carried out in triplicate at 3 different levels of 80 %, 100 % and 120 %, by spiking standard samples. Standard drug solutions of concentration 32 µg/ml (80 %), 40 µg/ml (100 %) and 48 µg/ml (120 %) were prepared by transferring 0.32 ml, 0.4 ml and 0.48 ml respectively from stock solution of drug into a series of 10 ml V.F each containing 0.4 ml of sample stock solution. The volume up to the mark was made with mobile phase. The solutions prepared were injected (n=3) into the chromatograph and the concentration were determined from the peak areas obtained from the chromatograms. Similarly study was carried out for individual impurity by preparing solutions of concentration 0.32 µg/ml (80 %), 0.4 µg/ml (100 %) and 0.48 µg/ml (120 %), from respective working standard of each impurity.

3.9.6.4. Precision

Intraday repeatability of the method was evaluated by analyzing three concentrations of MA (30, 50 and 70 µg/ml), and three concentration of MA-Imp A, MA-Imp C and MA-Imp D (3, 5 and 7 µg/ml). Three different sets were prepared each containing MA, MA-Imp A, MA-Imp C and MA-Imp D. The first set contained 30 µg/ml of MA and 3 µg/ml of each impurity. The second set contained 50 µg/ml of MA and 5 µg/ml of each impurity and the third set contained 70 µg/ml of MA and 7 µg/ml of each impurity. Interday precision was evaluated by assaying the three chosen sets of MA, MA-Imp A, MA-Imp C and MA-Imp D in triplicate on two successive days using the same procedure stated under chromatographic conditions. % RSD was calculated.

3.9.6.5. Sensitivity

The sensitivity of the developed method was determined by calculating LOD and LOQ for MA and its three impurities. LOD and LOQ were calculated for MA and its three impurities based on the Standard deviation of the Response and the Slope as mentioned below.

LOD = 3.3 x Standard deviation of the response/ Slope of the calibration curve

LOQ = 10 x Standard deviation of the response/Slope of the calibration curve

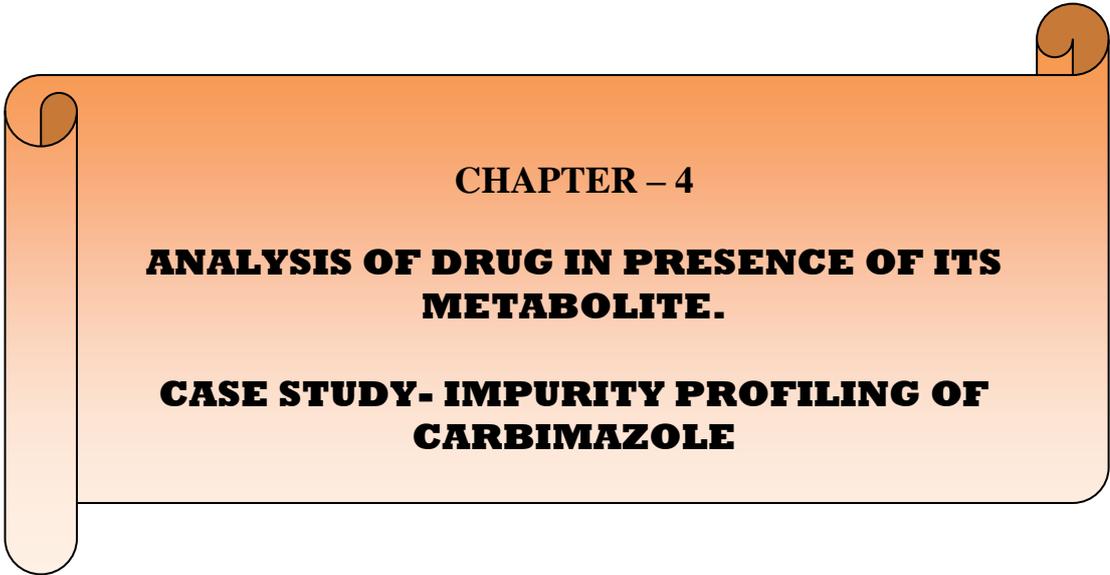
3.9.6.6. Robustness

Robustness of the developed method was determined by making three injections of mixture containing MA and pharmacopoeial impurities and by making deliberate change in method parameters like flow rate, and composition of mobile phase ratio. The values of percent relative standard deviation (% RSD) of peak area was recorded. Required aliquots were taken from stock solution of MA and working standard solution of each impurity in 10 ml V.F. Volume was made up to the mark using diluent to get concentration of solution containing 50 µg/ml of MA and 5 µg/ml of each impurity. To study the effect of variation in flow rate on the method, ± 0.1 ml of the optimized flow rate (1 ml) was studied on the method. The prepared solution was analyzed in triplicate by the proposed method by altering the flow rate of the proposed method to 0.9 ml (- 0.1 ml) and 1.1 ml (+ 0.1 ml).

To analyze the effect of change in composition of mobile phase ratio on the proposed method, the composition of organic phase in the mobile phase was altered by ± 2 % of the optimized composition. Hence the mobile phase used for study was ACN: buffer at 53: 47 % v/v (- 2 %) and 57:43 % v/v (+ 2 %). The prepared solution was analyzed in triplicate by the proposed method, carrying out the necessary changes in the mobile phase composition.

3.9.7. Analysis of marketed product

For analysis of tablets, 10 tablets were weighed individually and their average weight determined. Tablets were then crushed to fine powder and powder equivalent to 50 mg was transferred to 50 ml V.F and dissolved in 40 ml acetonitrile with vigorous shaking for 15 minutes. The solution was sonicated for 10 min. Finally the volume up to the mark was made with ACN. The solution was then filtered through Whatman filter paper (#1). 5 ml of the filtered solution was transferred into 100 ml V.F and the volume up to the mark was made with diluent to obtain a concentration of 50 µg/ ml. The solution prepared were injected (n=3) into the chromatograph to determine the peak area. From the peak area of the standard solution and the tablet sample solution, the percentage content of MA in the marketed tablets was calculated.



CHAPTER – 4

**ANALYSIS OF DRUG IN PRESENCE OF ITS
METABOLITE.**

**CASE STUDY- IMPURITY PROFILING OF
CARBIMAZOLE**

4. ANALYSIS OF DRUG IN PRESENCE OF ITS METABOLITE. CASE STUDY- IMPURITY PROFILING OF CARBIMAZOLE

4.1. INTRODUCTION

A RP HPLC method is developed for estimation of Carbimazole (CZ) in presence of its pharmacopoeial specified impurity, and to verify its stability indicating power by analyzing the stressed samples of CZ.

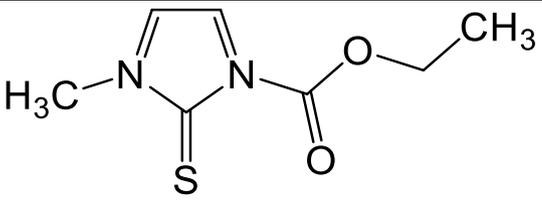
The following objectives were pursued through laboratory studies:

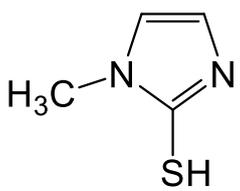
- 1) To identify the pharmacopoeial impurity, process related for CZ.
- 2) To develop and optimize a RP HPLC method for estimation of CZ in presence of its pharmacopoeial impurity.
- 3) To perform stress induced studies for CZ in accordance with ICH guidelines and generate suitable data to understand stability of CZ.
- 4) To validate the optimized method for detection and estimation of CZ in presence of its pharmacopoeial impurity and degradation products, if any.

4.2. PROFILE

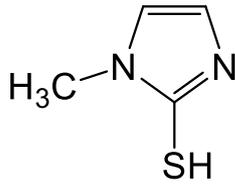
4.2.1. Drug profile

Carbimazole is official in IP⁴⁴, BP⁴⁵, USP⁴⁶ and EP⁴⁷

General Name	Carbimazole
Chemical Structure	
Chemical Name	Ethyl 3-methyl-2-thioxo-2,3-dihydro-1H-imidazole-1-carboxylate
Molecular Formula	C ₇ H ₁₀ N ₂ O ₂ S
Molecular Weight	186.2 g/mol
Melting Point	122 °C to 125 °C
pKa	-0.3 (strongest basic) by Marvin Sketch (Ver 19.9.0, 2019, www.chemaxon.com)
Description	White or yellowish-white, crystalline powder,
Solubility	Slightly soluble in water, soluble in acetone and in ethanol (96 per cent).
Drug Category	Thionamide antithyroid drug.
Clinical Pharmacology	<p>CZ is a thiourea antithyroid agent that decreases the uptake and concentration of inorganic iodine by thyroid. It also reduces the formation of di-iodotyrosine and thyroxine. Its active form methimazole (MZ), prevents the thyroid peroxidase enzyme from coupling and iodinating the tyrosine residues on thyroglobulin, hence reducing the production of the thyroid hormones T₃ and T₄. It is used in the management of hyperthyroidism, including the treatment of Graves' disease, the preparation of hyperthyroid patients for thyroidectomy, as an adjunct to radio-iodine therapy, and in the treatment of thyroid storm. CZ has been preferred in some patients compared to</p>

	methimazole, as it may have fewer side effects, like less frequent GI problems ⁷⁷⁻⁷⁸ .
Pharmacokinetics	The pharmacokinetics of CZ and MZ can be considered together since CZ is rapidly and completely metabolised to MZ in the body. The antithyroid activity of CZ is dependent upon this conversion to MZ. Both are rapidly absorbed from the gastrointestinal tract with peak plasma concentrations occurring about 1 to 2 hours after oral doses. MZ is concentrated in the thyroid gland. MZ is moderately bound to plasma proteins. MZ has an elimination half-life from plasma of about 3 to 6 hours and is metabolised, probably by the liver, and excreted in the urine. Less than 12 % of a dose of methimazole may be excreted as unchanged drug. 3-Methyl 2-thiohydantoin has been identified as a metabolite of MZ. The elimination half-life may be increased in hepatic and renal impairment. MZ crosses the placenta and is distributed into breast milk ⁷⁷ .
Toxicity	Cholestatic Hepatitis in Toxic Multinodular Goiter ⁷⁹ .
Impurity Specified impurity A	A  1-methyl-1H-imidazole-2-thiol (thiamazole)

4.2.2. Impurity Profile (Imp A of CZ)

Impurity	A
Chemical Structure	
Chemical Name	1-methyl-1H-imidazole-2-thiol (Thiamazole)
Molecular Formula	C ₄ H ₆ N ₂ S
Molecular Weight	114.17 g/mol
Melting Point	143-146 °C
pKa	a) 10.41 (strongest acidic) b) -3 (strongest basic) by Marvin Sketch (Ver 19.9.0, 2019, www.chemaxon.com)
Description	White to slightly cream-coloured crystalline powder.
Solubility	Freely soluble in water, alcohol and in acetone
Toxicity	Rashes are most commonly. Agranulocytosis syndrome is rare. Arthritis and toxic hepatitis have been reported. Neuropathy associated with foot drop has been reported ⁸⁰⁻⁸¹ .

4.3. LITERATURE SURVEY

Literature on review has shown few methods for determination of CZ, its impurity (MZ) or both of them. The methods include potentiometric^{82, 85}, spectroscopic^{83, 86-91, 93-96}, densitometric⁹¹, polarographic^{84,97}, voltammetric⁹⁷, titrimetric⁸⁸, HPTLC⁹⁸ and HPLC⁹⁹⁻¹⁰¹ methods. As conventional potentiometric, spectroscopic, densitometric, polarographic, voltammetric, and titrimetric methods are not expected to analyse compounds present in microgram quantities, HPLC methods were only reviewed in depth to understand the mechanism of separation to enable development of newer method. Following Table 4.3 summarizes few chromatographic conditions used for determination of CZ by HPLC.

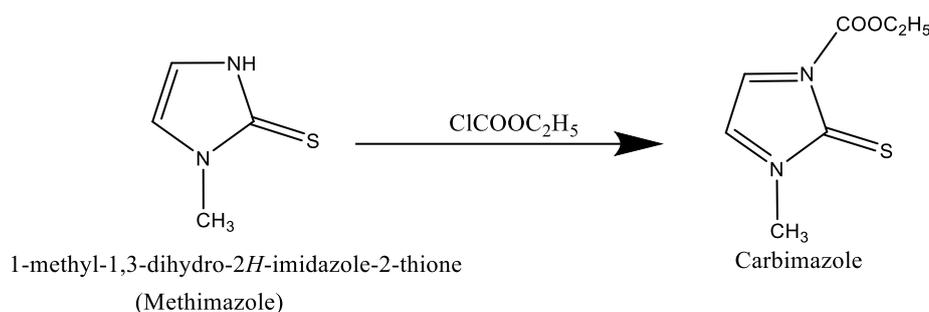
Table 4.3: Summary of reported chromatographic conditions used for determination of CZ by HPLC

Sr.No.	Column Type	Mobile Phase Composition	Flow Rate	Detector Used	R _t of drug	References
1	BP method					
	C18 (5 μm)	Acetonitrile: water (10:90 % v/v)	1.0 ml/min	UV fixed wavelength (254 nm)	6.0 min	45
2	Chromatographic methods development, validation and degradation characterization of the anti-thyroid drug Carbimazole					
	CN (5 μm)	Acetonitrile: 0.05 M KH ₂ PO ₄ (20: 80 % v/v)	1.0 ml/min	PDA (254 nm and 290 nm)	4.1 min	99
3	Estimation of carbimazole in presence of its degradants using RP-HPLC					
	C18 (5 μm)	Water: acetonitrile (75:25 % v/v)	1.5 ml/min	UV fixed wavelength (298 nm)	5.9 min	100
4	Formulation of an oral liquid solution of Carbimazole and validation of a stability indicator assay method					
	C18 (5 μm)	Acetonitrile: water (10:90 % v/v)	1.0 ml/min	UV fixed wavelength (254 nm)	5.2 min	101

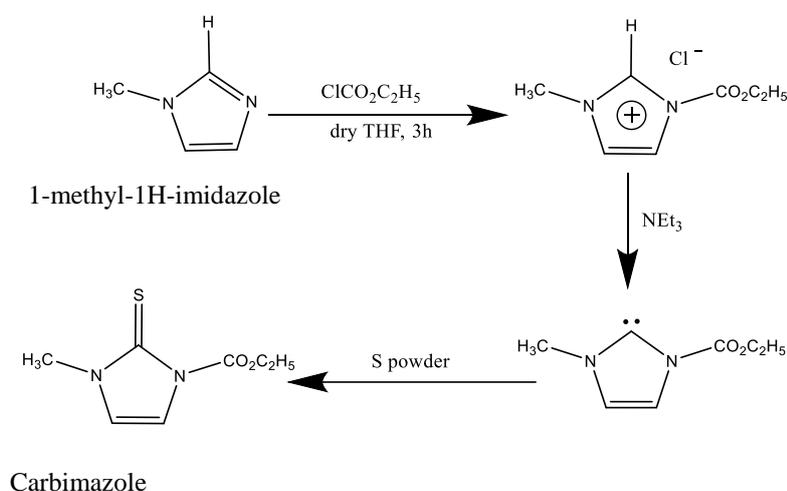
4.4. LOCATING SOURCES OF IMPURITIES IN CARBIMAZOLE

To predict possible impurities, various synthetic schemes of CZ were identified from literature. The starting materials and intermediates for CZ in its synthetic schemes were considered to be the potential impurities in the final product.

A) A commonly used synthetic route includes use of Methimazole as starting material¹⁰³.



B) Another synthesis route involved reaction of 1-methyl-1H-imidazole with ethyl carbonochloridate and dry tetrahydrofuran for 3 hr followed by triethylamine and sulphur powder¹⁰⁴.



Pharmacopoeial monograph consider MZ as an Imp A as justified from synthetic scheme where MZ is used as a starting material for synthesis of CZ. Hence Imp A, listed in the monograph of CZ is a process related impurity. A new RP HPLC method for separation and estimation of CZ from Imp A was developed. Also, CZ was subjected to stress to enable formation of degradants if any for purpose of developing an analytical method, where the drug peak appears as a distinctly separate peak from process related and degradant peaks, if any.

4.5. DEVELOPMENT AND OPTIMIZATION OF RP HPLC METHOD FOR ESTIMATION OF CARBIMAZOLE IN PRESENCE OF ITS PHARMACOPOEIAL IMPURITY

4.5.1. Selection of Chromatographic Method

The most popular technique of chromatography is reversed phase chromatography (RPC). The principle of separation is based on analytes distribution based on partition coefficient between polar mobile phase and hydrophobic (nonpolar) stationary phase. Methods published in literature for CZ were mostly reverse phased.

4.5.2. Selection of Stationary Phase

BP⁴⁵ recommends use of C18 column for estimation of CZ and its related substance by RP HPLC. Literature revealed various studies for estimation of CZ individually or in combination and for stability studies using C18 column.

4.5.3. Selection of detection wavelength

The overlain spectrum of CZ and its specified pharmacopoeial Imp A was recorded in acetonitrile and shown as Fig 4.5.3. Solution containing 10 µg/ml of CZ and 1 µg/ml of Imp A was used for study. At λ_{\max} of CZ, Imp A impurity showed insignificant absorbance whereas at λ_{\max} of Imp A, drug showed significant absorptivity recordable such that trace of Imp A if present can be conveniently detected in CZ. Hence wavelength of 260 nm was selected for the study.

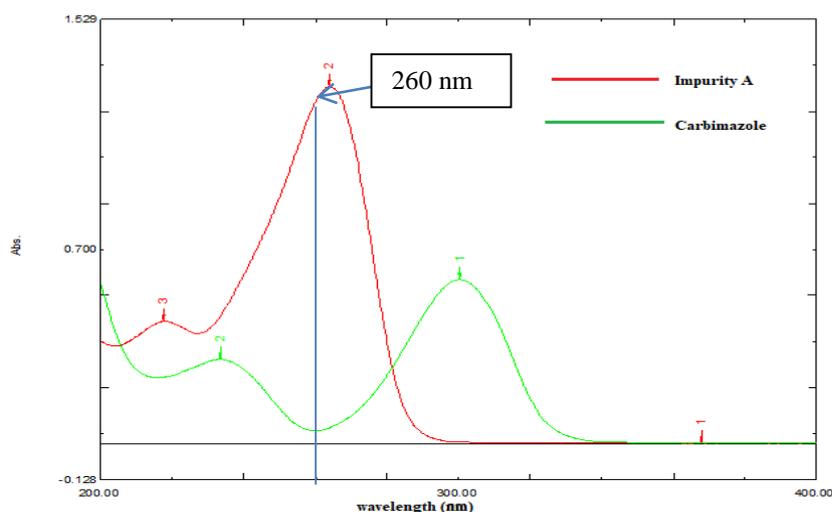


Figure 4.5.3: UV Overlain spectrum of CZ and Imp A.

4.5.4. Application of BP monograph method for study

A study involving pharmacopoeial method for CZ as per monograph was carried out. The chromatographic parameters are listed in Table 4.5.4 and the obtained chromatogram is shown in Fig 4.5.4.

Table 4.5.4: Chromatographic conditions for separation of CZ and Imp A as per BP method

Mobile phase	Acetonitrile: water (10:90 % v/v)
Column	Waters -Sunfire ODS C18 (250 x 4.6 mm ,5 μ m)
Flow rate	1 ml/min
Wavelength	254 nm
Standard solution	CZ- 50 μ g/ml; Imp A- 10 μ g/ml

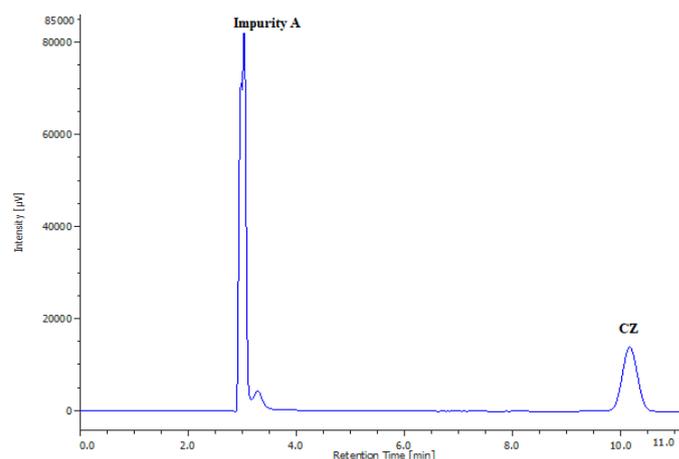


Figure 4.5.4: Chromatogram of CZ and Imp A as per BP method.

The presence of an additional peak attached to Imp A peak was observed. Absence of buffer of specific pH was expected to promote variation in pH of mobile phase during the course of study when exposed to different environmental atmospheric conditions. There is a need to develop an appropriate mobile phase for optimum resolution of peaks minimizing the run time being major objective.

4.5.5. Selection and optimization of mobile Phase for separation of CZ and its impurity

A desirable mobile phase that would provide efficient separation of CZ from its impurity, and also to study the degradation behavior of CZ under varied stress conditions was

required to be developed. In BP monograph of CZ, binary system comprising of acetonitrile as organic phase and water as aqueous phase, with the flow rate 1 ml/min, injection volume of 10 μ l and detection wavelength of 254 nm, on C18 column (3.9 x 150 mm, 5 μ m) is used.

Discrete trials were conducted with mobile phase composition comprising of different composition of acetonitrile and 10 mM ammonium dihydrogen orthophosphate buffer over pH range varying from 2.5 to 6.5. Observations are recorded and presented in Table 4.5.5.1.

Table 4.5.5.1: Exploratory trials for optimization of mobile phase composition on Sunfire C-18 column (250 mm x 4.6, 5 μ m) for CZ and Imp A

Trial no	Mobile Phase composition (ACN and 10 mM ammonium dihydrogen orthophosphate Buffer) in % v/v	pH of Mobile Phase	Remark	Figure
1	90:10	2.5	Resolution between the peaks was less than 2 and symmetry was 0.5 for Imp A	4.5.5.1.1
2	80:20		Resolution was close to 3 with both peaks appearing before 3 min with Imp A peak appearing in dead volume	4.5.5.1.2
3	70:30		Resolution was close to 4 with both peaks appearing before 3 min with Imp A peak in dead volume	4.5.5.1.3
4	60:40		Resolution was close to 5 with both peaks appearing before 4 min with Imp A peak appearing close to dead volume	4.5.5.1.4
5	50:50		Resolution was close to 6 with both peaks appearing before 4 min and appearing after dead volume	4.5.5.1.5
6	40:60		Theoretical plates of Impurity A were less than 2000.	4.5.5.1.6
1	70:30	3.5	Resolution was close to 4 with both peaks appearing	4.5.5.1.7

			before 3 min with Imp A peak appearing in dead volume	
2	60:40		Resolution was close to 5 with both peaks appearing before 4 min with Imp A peak appearing close to dead volume	4.5.5.1.8
3	50:50		Resolution was close to 5 with both peaks appearing before 4 min and after dead volume with theoretical plates close to 2000	4.5.5.1.9
4	40:60		Theoretical plate of Imp A is less than 2000	4.5.5.1.10
1	50:50	4.5	Theoretical plates of both peaks are close to 2000	4.5.5.1.11
1	50:50	5.5	Resolution was close to 6 with both peaks appearing before 4 min with Imp A peak appearing close to dead volume	4.5.5.1.12
1	50:50	6.5	Resolution was close to 7 with both peaks appearing before 4 min with Imp A peak appearing close to dead volume	4.5.5.1.13

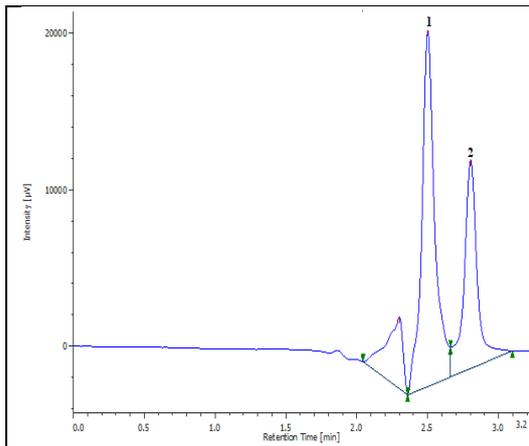


Figure 4.5.5.1

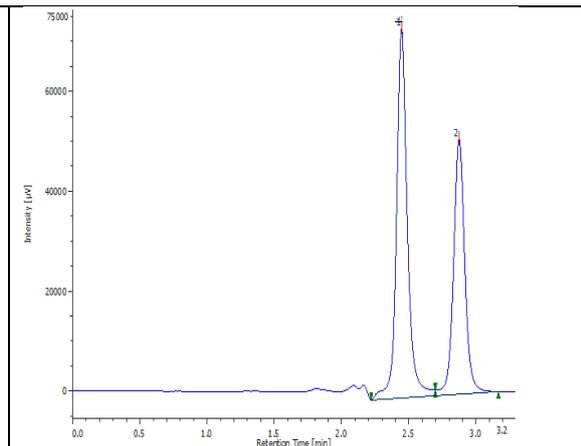


Figure 4.5.5.2

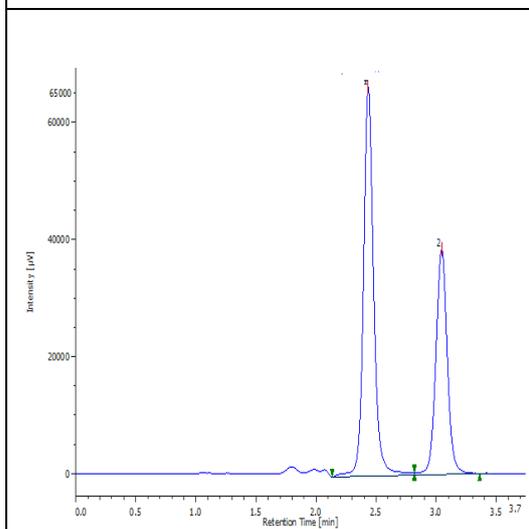


Figure 4.5.5.3

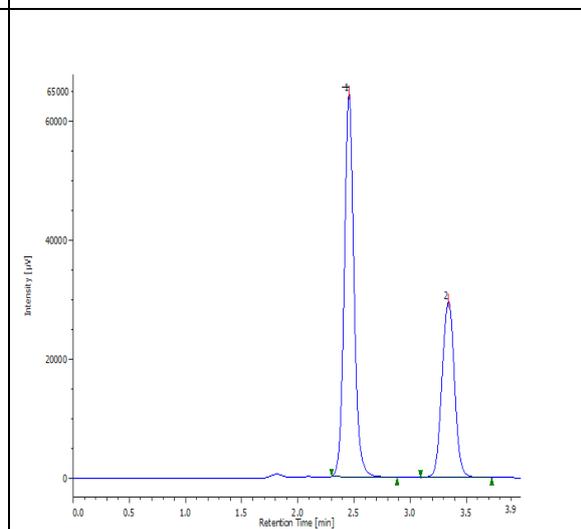


Figure 4.5.5.4

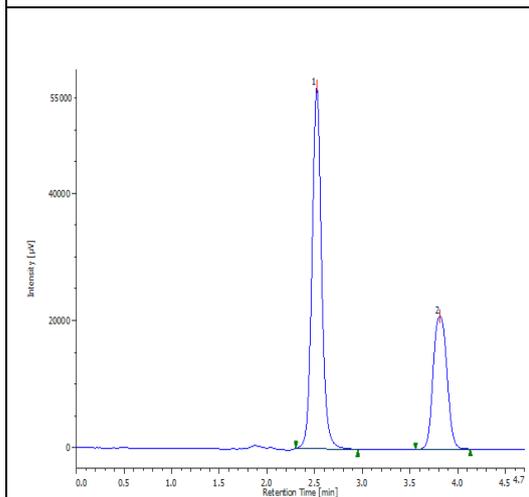


Figure 4.5.5.5

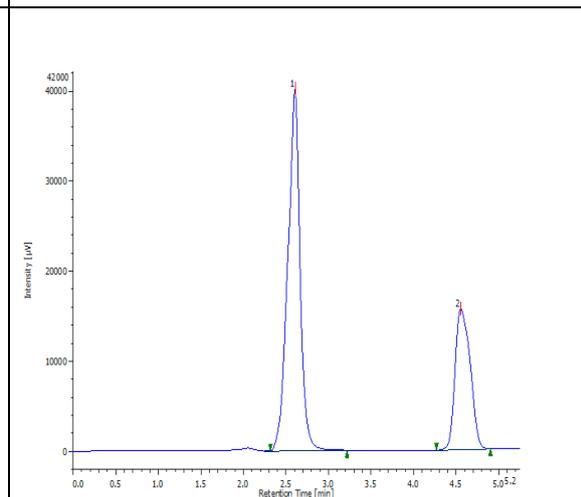


Figure 4.5.5.6

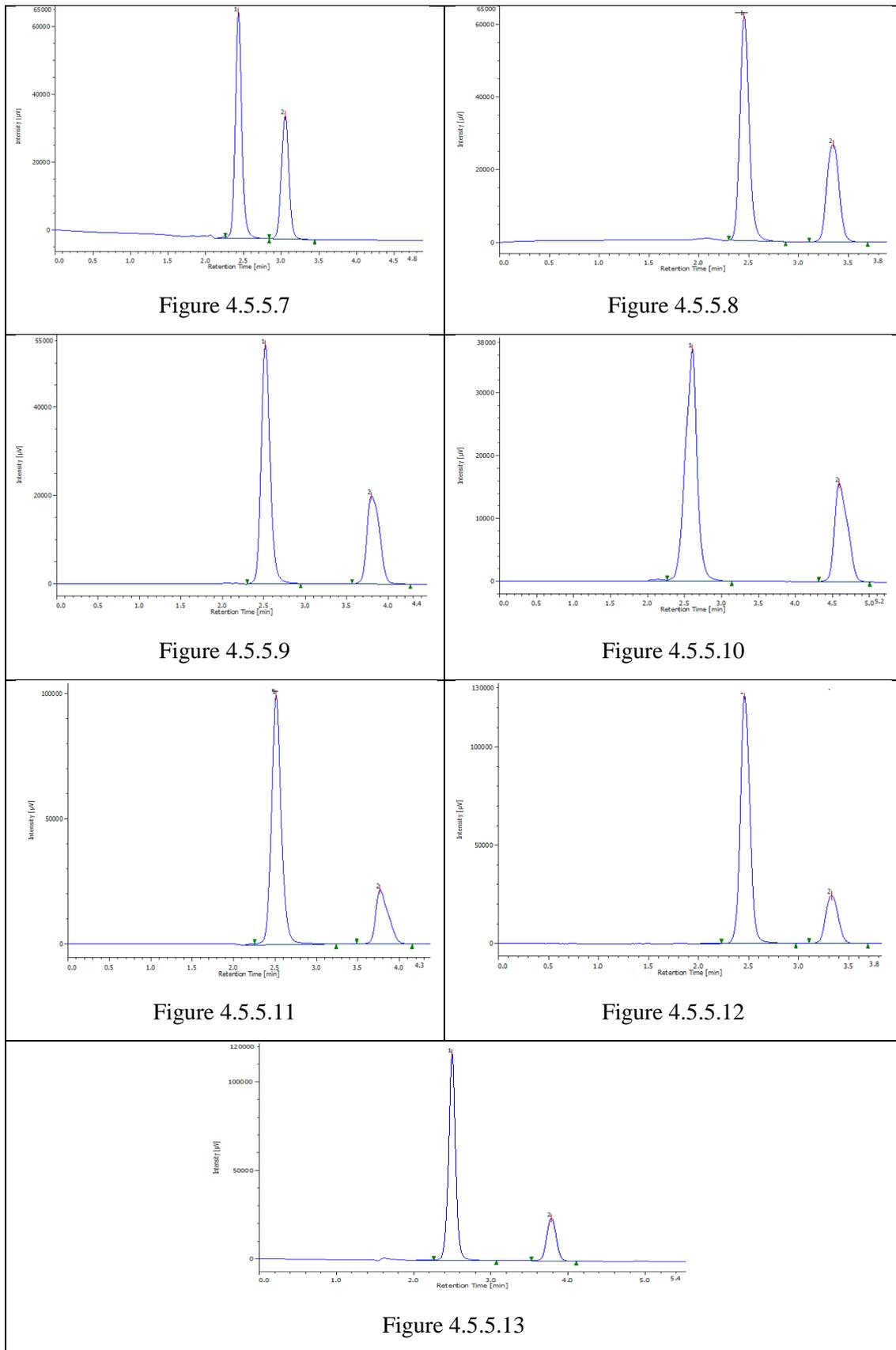


Figure 4.5.5: Representative chromatograms of exploratory trials for optimization of mobile phase composition for CZ and Imp A

It was noticed that when the pH of the buffer was maintained at moderately high to very low (3.5, 4.5, 5.5 and 6.5) incomplete separations, peak merging, partial elution or early elution (peaks eluted in dead volume) was observed. However at higher pH 2.5 (highly acidic) separations were satisfactory. Imp A peak were well resolved from drug peak as seen in Fig 4.5.5.5 and run time of less than 5 min.

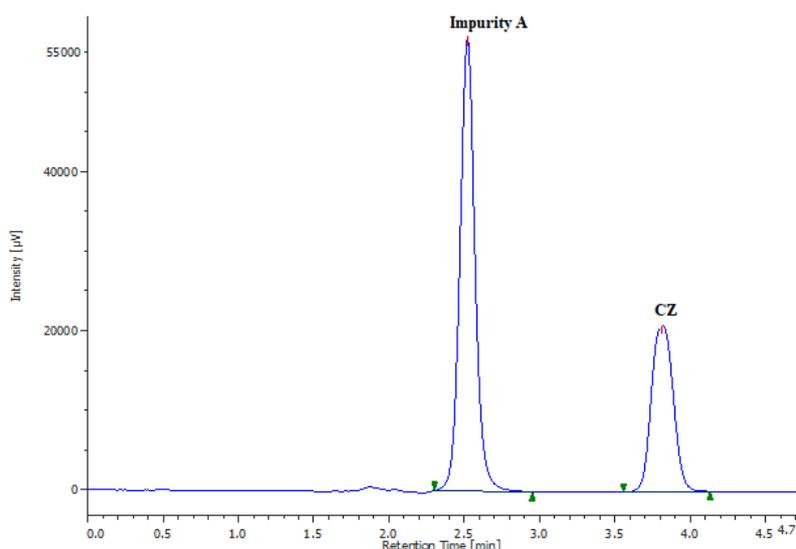


Figure 4.5.5.5: Optimized chromatogram of CZ and Imp A

Chromatographic conditions were optimized from discrete trials on Waters Sunfire C-18 column. Best fit mobile phase for separation of CZ from Imp A was selected based on satisfactory resolution of peaks on smooth baseline. Final optimized chromatographic condition is presented in Table 4.5.5.2.

Table 4.5.5.2: Optimized chromatographic conditions for separation of CZ and Imp A

Mobile phase	Acetonitrile: 10mM ammonium dihydrogen ortho phosphate buffer (pH adjusted to 2.5 by o-phosphoric acid) in the ratio of 50:50 % v/v.
Column	Waters -Sunfire ODS C18 (250 x 4.6 mm, 5 µm) column
Column temperature	Ambient
Flow rate	1 ml/min
Wavelength	260 nm
Injector loop size	10 µL

4.6. FORCED DEGRADATION STUDIES

The stress studies were conducted for the drug CZ by subjecting to hydrolysis (HCl, NaOH and neutral), oxidation, elevated temperature and photolytic stress conditions. Sequences of samples were prepared for each condition as per routine protocols (Table 4.6). Sample subjected to stress were acquired and analyzed by the proposed method.

Table 4.6: Protocol for stress degradation of CZ

Samples	Hydrolysis			Oxidative Deg.	Thermal Deg.	Photo Deg.
	Acid	Alkaline	Neutral			
Blank stored under normal condition	√	√	√	√	-----	-----
Blank subjected to stress condition	√	√	√	√	-----	-----
Drug / drug solution stored under normal condition	√	√	√	√	√	√
Drug / drug solution subjected to stress condition*	√	√	√	√	√	√

* To get desired degradation initially degradation was carried out at room temperature and if necessary samples were subjected to higher temperature or strength of stress reagent was increased.

4.6.1. Hydrolytic degradation

Hydrolytic stress studies were performed with HCl, NaOH and water to simulate acidic, alkaline and neutral conditions respectively. The acid and base degradation was executed using HCl and NaOH, while neutral degradation was conducted using water. The strength of HCl and NaOH was varied to obtain significant degradation. Initially strength of N/10 was used for study. Strength was then varied (to lower or higher) depending upon their ability to produce degradation.

The studies were performed at room temperature followed by heating the solution of drug with acid, base and water at higher temperature of 70 °C on water bath equipped with thermostat for extended time intervals, if negligible degradation was seen at RT. The drug solution treated with acid and base was neutralized with base and acid respectively by the same strength and made to the mark with mobile phase. These solutions were then analyzed by the proposed method.

4.6.1.1. Acid degradation

Acid degradation was conducted using Hydrochloric acid. Samples were prepared by taking 0.1 ml of stock solution of drug (1000 µg/ml) and 1 ml of N/10 HCl in 10 ml V.F and kept at RT for extended time interval of around 6 hr. The samples were cooled and then neutralized by 1 ml of same strength of NaOH and volume made up to the mark with mobile phase. The solutions were then analyzed by the proposed method. Observation of hydrolytic study of CZ with N/10 HCl at different time interval is presented in Table 4.6.1.1 and representative chromatogram as Fig 4.6.1.1.

Table 4.6.1.1: Degradation study of CZ with HCl

Concentration of HCl	Temperature	Time	% of active drug after degradation	RRT of additional peak formed
0.1N	RT	2 hr	64.71	1) 0.65 (DP I)

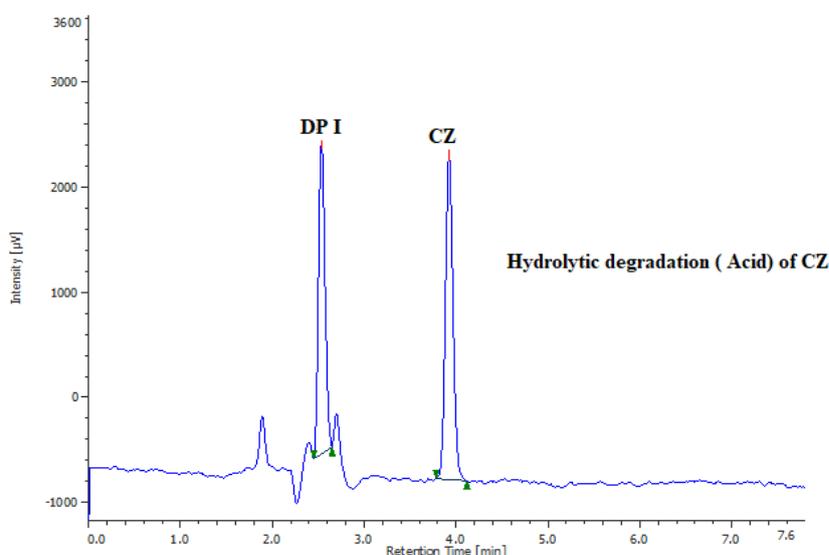


Figure 4.6.1.1: Chromatogram of CZ (10 µg/ml) treated with 0.1N HCl for 2 hr at RT

Peak name	CH	R _t (min)	RRT	Area(µV-sec)	NTP	Symmetry
CZ	1	3.92	1.00	17776	11024	1.07
DP I	1	2.53	0.65	14718	5802	1.19

4.6.1.2. Base degradation

Base degradation was conducted using sodium hydroxide. Samples were prepared by taking 0.1 ml of stock solution of drug (1000 µg/ml) and 1 ml of N/1000 NaOH in 10 ml V.F and kept at RT for extended time interval of around 30 min. The samples were neutralized by 1 ml of same strength of HCl and volume up to the mark was made with mobile phase. The solutions were then analyzed by the proposed method. Observation of hydrolytic study of CZ with N/1000 NaOH at different time interval is presented in Table 4.6.1.2 and representative chromatogram as Fig 4.6.1.2.

Table 4.6.1.2: Degradation study of CZ with NaOH

Concentration of NaOH	Temperature	Time	% of active drug after degradation	RRT of additional peak formed
0.001N	RT	5 min	88.43	1) 0.66 (DP I)

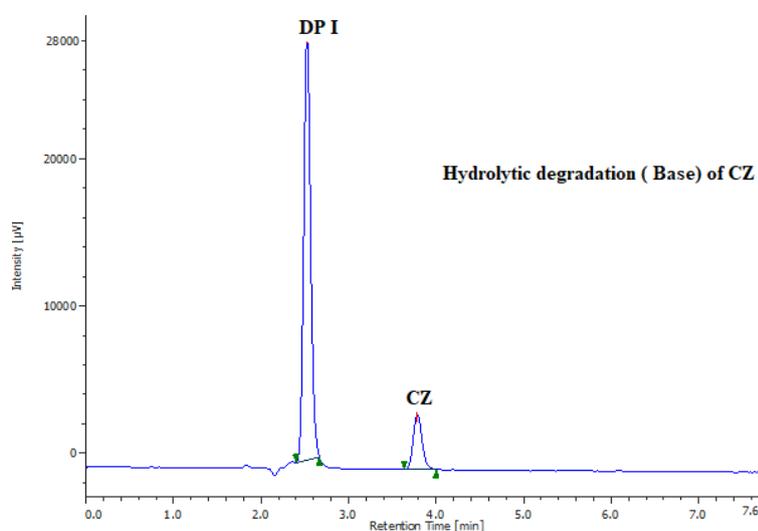


Figure 4.6.1.2: Chromatogram of CZ (10 µg/ml) treated with 0.001N NaOH for 5 min at RT

Peak name	CH	R _t (min)	RRT	Area(µV-sec)	NTP	Symmetry
CZ	1	3.78	1.00	26295	6749	1.17
DP I	1	2.52	0.66	158067	4959	1.12

4.6.1.3. Neutral degradation

Neutral degradation was conducted using distilled water. Samples were prepared by taking 0.1 ml of stock solution of drug (1000 µg/ml) and 1 ml of distilled water in 10 ml V.F and kept at RT for 6 hr and at higher temperature of 70 °C for extended time interval of 30 min. The samples were cooled and volume up to the mark was made with mobile phase. The solutions were analyzed by the proposed method. Observation of hydrolytic study of CZ with water at different temperature and time interval is presented in Table 4.6.1.3 and representative chromatogram as Fig 4.6.1.3.

Table 4.6.1.3: Degradation study of CZ with water

Temperature	Time	% of active drug after degradation	% degradation	RRT of additional peak formed
RT	6 hr	96.48	-	-
70 °C	10 min	32.58	33.76	1) 0.66 (DP I)

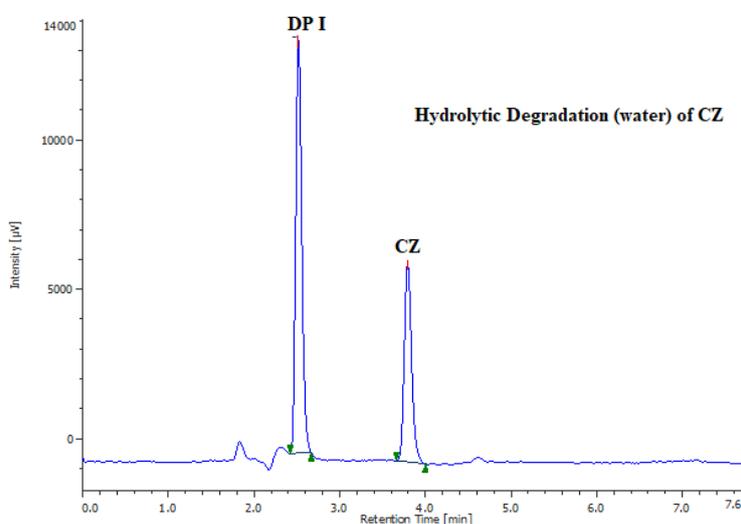


Figure 4.6.1.3: Chromatogram of CZ (10 µg/ml) treated with water for 10 min at 70 °C

Peak name	CH	R _t (min)	RRT	Area(µV-sec)	NTP	Symmetry
CZ	1	3.79	1.00	39821	9635	1.21
DP I	1	2.51	0.66	68696	5923	1.23

4.6.2. Oxidative degradation

Oxidative degradation was conducted using hydrogen peroxide (H_2O_2). CZ was found to be highly unstable in higher concentrations of hydrogen peroxide, hence various concentrations and volume of hydrogen peroxide was tried to obtain optimum results. Samples were prepared by taking 0.1 ml of stock solution of drug (1000 $\mu\text{g/ml}$) and 0.1 ml of hydrogen peroxide (0.1 %) in 10 ml volumetric flask at RT and kept aside for one day. The samples were diluted up to the mark by mobile phase and subjected for analysis by the proposed method.

Observation of oxidative study of CZ with 0.1 % v/v H_2O_2 is presented in Table 4.6.2 and representative chromatogram as Fig 4.6.2.

Table 4.6.2: Degradation study of CZ with H_2O_2

Temperature	Time	% of active drug after degradation	RRT of additional peak formed
RT	1 day	80.55	1) 0.66 (DP I)

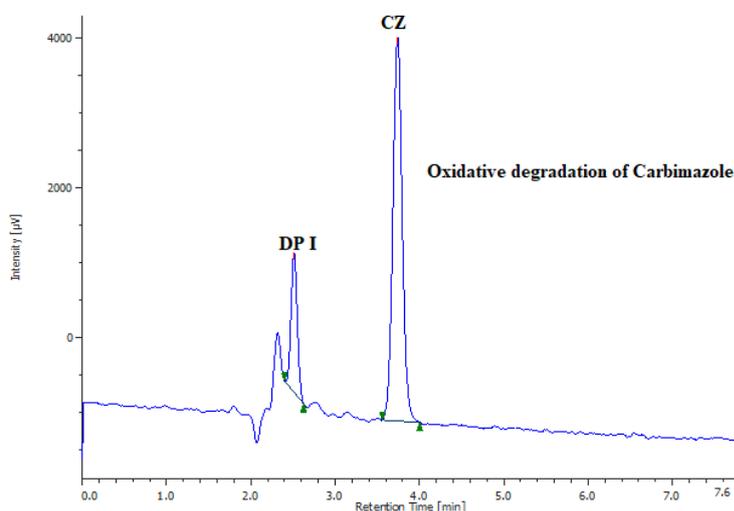


Figure 4.6.2: Chromatogram of CZ (10 $\mu\text{g/ml}$) treated with 0.1 % H_2O_2 for 1 day at RT

Peak name	CH	R_t (min)	RRT	Area($\mu\text{V}\cdot\text{sec}$)	NTP	Symmetry
CZ	1	3.74	1.00	39717	5595	1.03
DP I	1	2.50	0.66	9593	5409	1.12

4.6.3. Thermal degradation

CZ sample 10 mg each was taken in two 10 ml V.F and closed with stopper. One flask was exposed to dry heat in hot air oven for specified temperature (80 °C) and time interval (6 hr) and another was kept as control. After sufficient exposure two separate solutions were prepared by weighing appropriate amount of sample exposed to thermal stress and control to produce concentration of 10 µg/ml. The samples were diluted to the mark with mobile phase and injected separately for analysis by the proposed method.

Observation of thermal study of CZ in hot air oven (80 °C) is presented in Table 4.6.3 and representative chromatogram as Fig 4.6.3.

Table 4.6.3: Degradation study of CZ in hot air oven

State	Temperature	Time	% of active drug after degradation	RRT of additional peak formed
Solution	70 °C	10 min	32.58	0.66
Solid	80 °C	6 hr	73.58	0.66

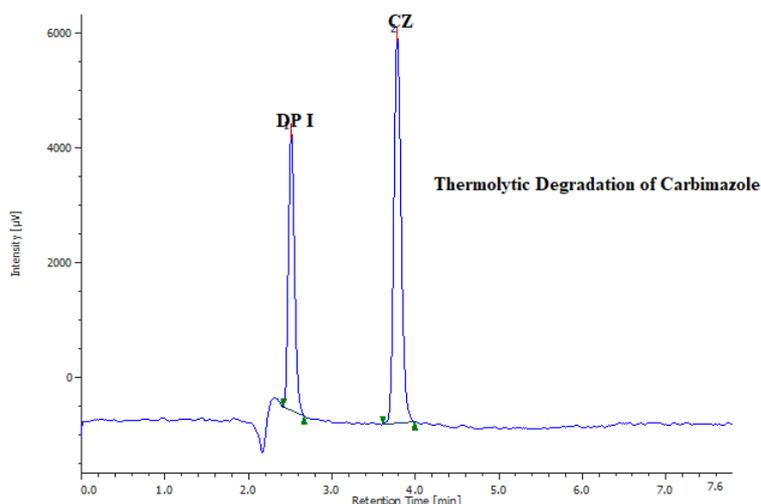


Figure 4.6.3: Chromatogram of CZ (10 µg/ml) in oven for 6 hr at 80 °C

Peak name	CH	R _t (min)	RRT	Area(µV-sec)	NTP	Symmetry
CZ	1	3.78	1.00	41278	9093	1.14
DPI	1	2.50	0.66	17020	5754	1.21

4.6.4. Photo degradation

Drug in sufficient amount was taken in 10 ml volumetric flask and was exposed to direct sunlight for a specified time period (7 days). Another flask containing drug protected from light was kept as control. After exposure, two separate solutions test and control were prepared by weighing appropriate amount of sample exposed to stress and control to produce concentration of 10 $\mu\text{g/ml}$. The samples were diluted to the mark with mobile phase and injected separately for analysis by the proposed method.

Observation of photo light study of CZ after direct exposure to sunlight is presented in Table 4.6.4 and representative chromatogram as Fig 4.6.4.

Table 4.6.4: Degradation study of CZ with direct exposure to sunlight

Temperature	Time	% of active drug after degradation
Sunlight	7 days	89.35

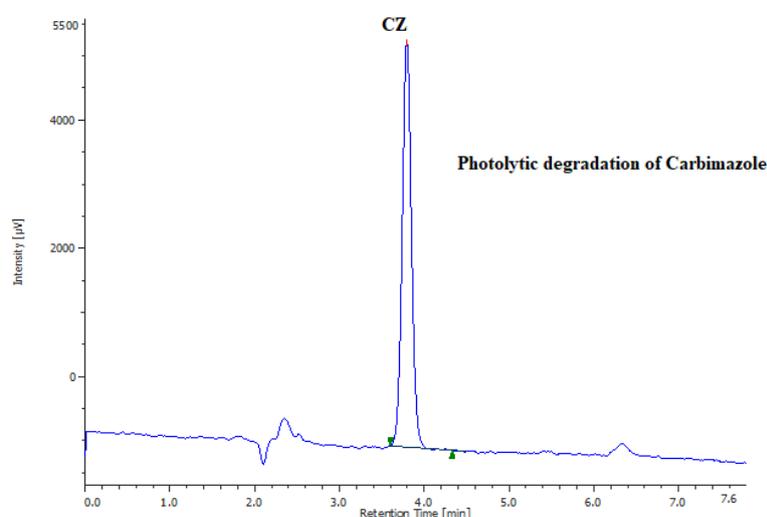


Figure 4.6.4: Chromatogram of CZ (10 $\mu\text{g/ml}$) exposed to direct sunlight for 7 days

Peak name	CH	R _t (min)	Area($\mu\text{V}\cdot\text{sec}$)	NTP	Symmetry
CZ	1	3.79	48052	5952	1.05
Control	1	3.80	48284	9463	1.15

4.7. VALIDATION OF DEVELOPED STABILITY INDICATING ANALYTICAL METHOD

Validation ensures confirmation of a method that it is intended to do. According to USP, SIAM is grouped under category II (Analytical methods for determination of impurities in bulk drugs or for determination of degradation compounds in finished pharmaceutical products). Various parameters recognized by ICH for method validation (Table: 4.7).

Table 4.7: Validation parameters and acceptance criteria

Sr.No.	Validation parameters	Acceptance criteria
1	Specificity	Peak (s) of degradation products and analyte should be pure and well separated from one other.
2	Linearity	Correlation coefficient not less than 0.999
3	Accuracy (across the specified range)	Recovery (%) between 98 % to 102 %
4	Precision	
	4.1) Intra-day precision	RSD (%) of replicate injections not more than 2.0
	4.2) Inter-day precision	
5	Robustness	
6	System suitability test	1) Resolution \leq 2.0 2) % RSD of replicate injections \geq 2.0 3) Theoretical plate number \leq 2000 4) Asymmetry of peak should not be more than 2.0

4.7.1. System suitability parameters

Results of the study for system suitability parameters when proposed method was applied for analysis of CZ, Imp A and DP I is presented in Table 4.7.1 and found to meet the acceptance criteria (Table 4.7).

Table 4.7.1: System suitability testing parameters of the proposed RP HPLC method

Sr.no	Components	RRT	Resolution	Peak asymmetry	Theoretical plate
1	CZ	1.00	NA	1.05	7791
2	Imp A	0.66	5.77	1.07	4693
3	DP I	0.66	7.70	1.40	5884

4.7.2. Specificity and Selectivity

Results of specificity and selectivity study on CZ along with Imp A and DP I are presented in Table 4.7.2 and are in agreement with acceptance criteria defined in Table 4.7.

Table 4.7.2: Selectivity of the HPLC method

Peaks	Peak (RRT)
CZ	1.00
Imp A	0.66
DP I	0.66

4.7.3. Linearity and Range

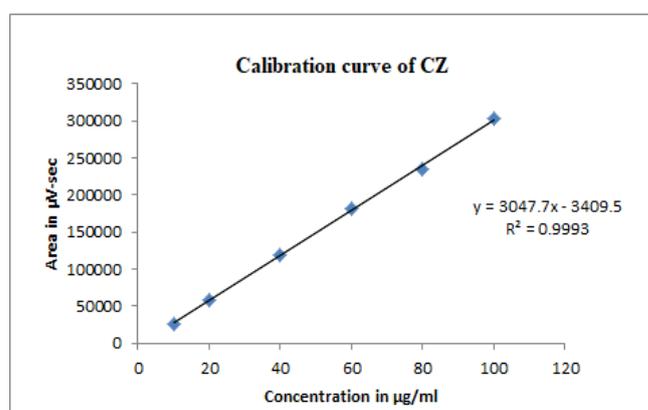
Determination of linearity range was undertaken with standard stock solution of CZ and Imp A with appropriate concentrations as mentioned under section 4.11.6.2. Results of the study are tabulated (Table 4.7.3.1) and linearity graphs presented as Fig 4.7.3.1 and Fig 4.7.3.3.

Table 4.7.3.1: Linearity Range

Compound	Linearity range ($\mu\text{g/ml}$)	R ²	Reference
CZ	10.66-100	0.9993	Fig 4.7.3.1
Imp A	1.71-10	0.9981	Fig 4.7.3.3

Table: 4.7.3.2: Linearity data of CZ

Sr.No	Conc. ($\mu\text{g/ml}$)	Peak Area				RSD (%)
		Injection 1	Injection 2	Injection 3	Mean	
1	10	25842	26332	25777	25984	1.16
2	20	58225	57974	57936	58045	0.27
3	40	121226	118621	118273	119373	1.35
4	60	180028	184532	182039	182199	1.23
5	80	233398	236897	234332	235148	0.77
6	100	298244	305647	306820	303570	1.53

**Figure 4.7.3.1: Linearity graph of CZ**

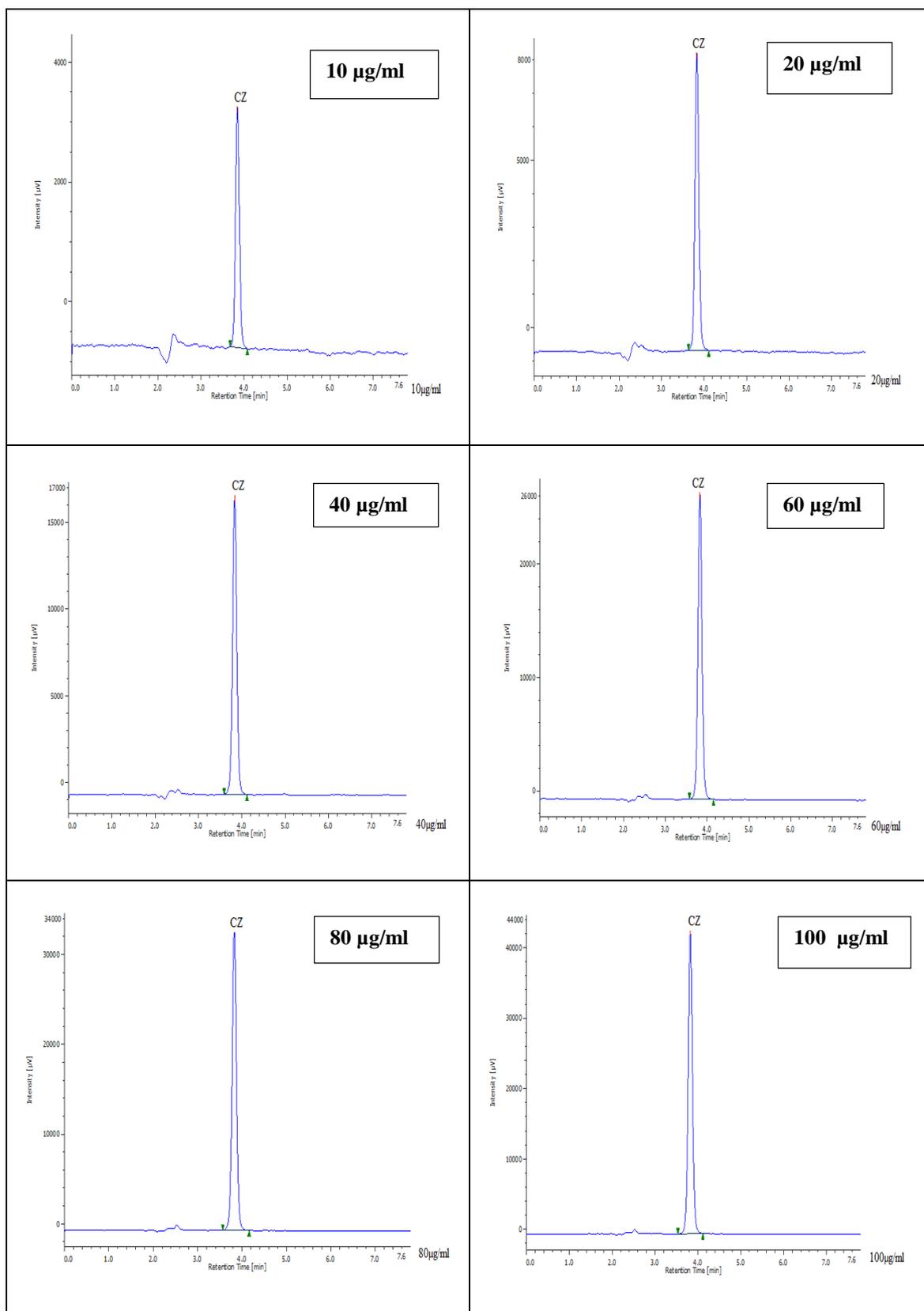


Figure 4.7.3.2: Representative chromatograms of CZ (Conc. 10, 20, 40, 60, 80, 100 µg/ml) (Linearity study)

Table 4.7.3.3: Linearity data of Imp A

Sr.No	Conc. (µg/ml)	Peak Area				RSD (%)
		Injection 1	Injection 2	Injection 3	Mean	
1	1	59293	59762	59642	59566	0.40
2	2	118018	116331	116863	117071	0.73
3	4	214345	218876	217692	216971	1.08
4	6	342684	342973	345926	321111	0.52
5	8	411811	418565	411531	413969	0.96
6	10	495940	499207	496002	497050	0.37

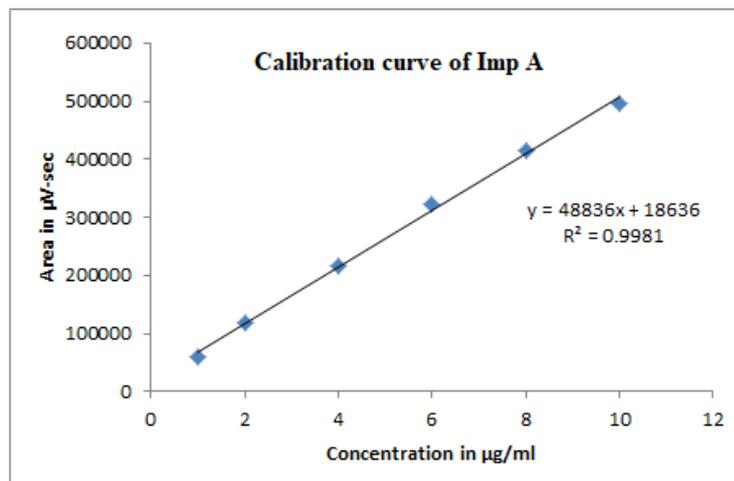
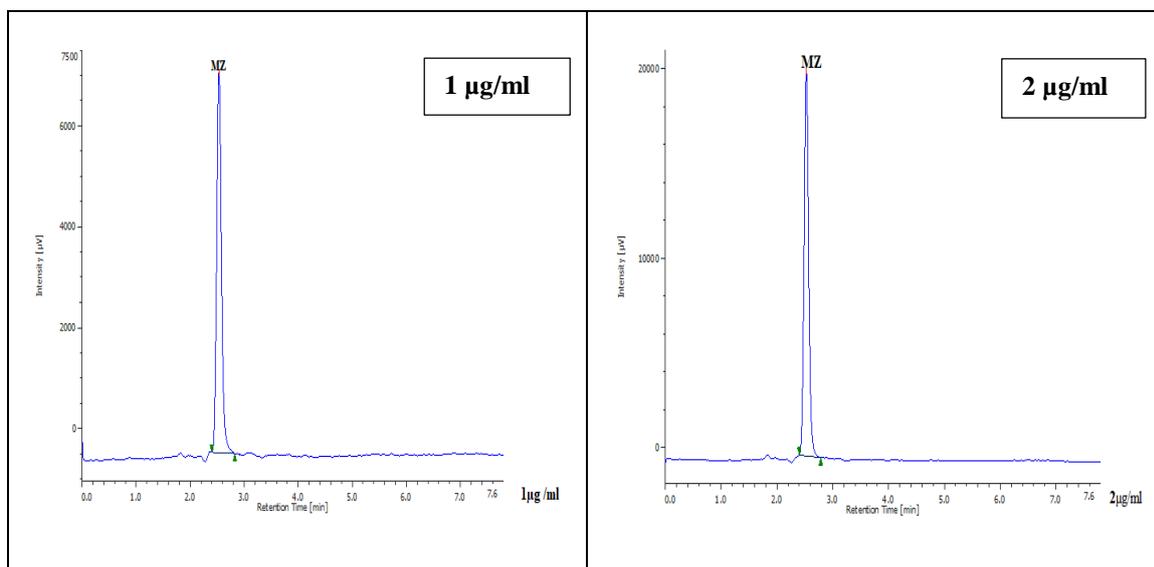


Figure 4.7.3.3: Linearity graph of Imp A



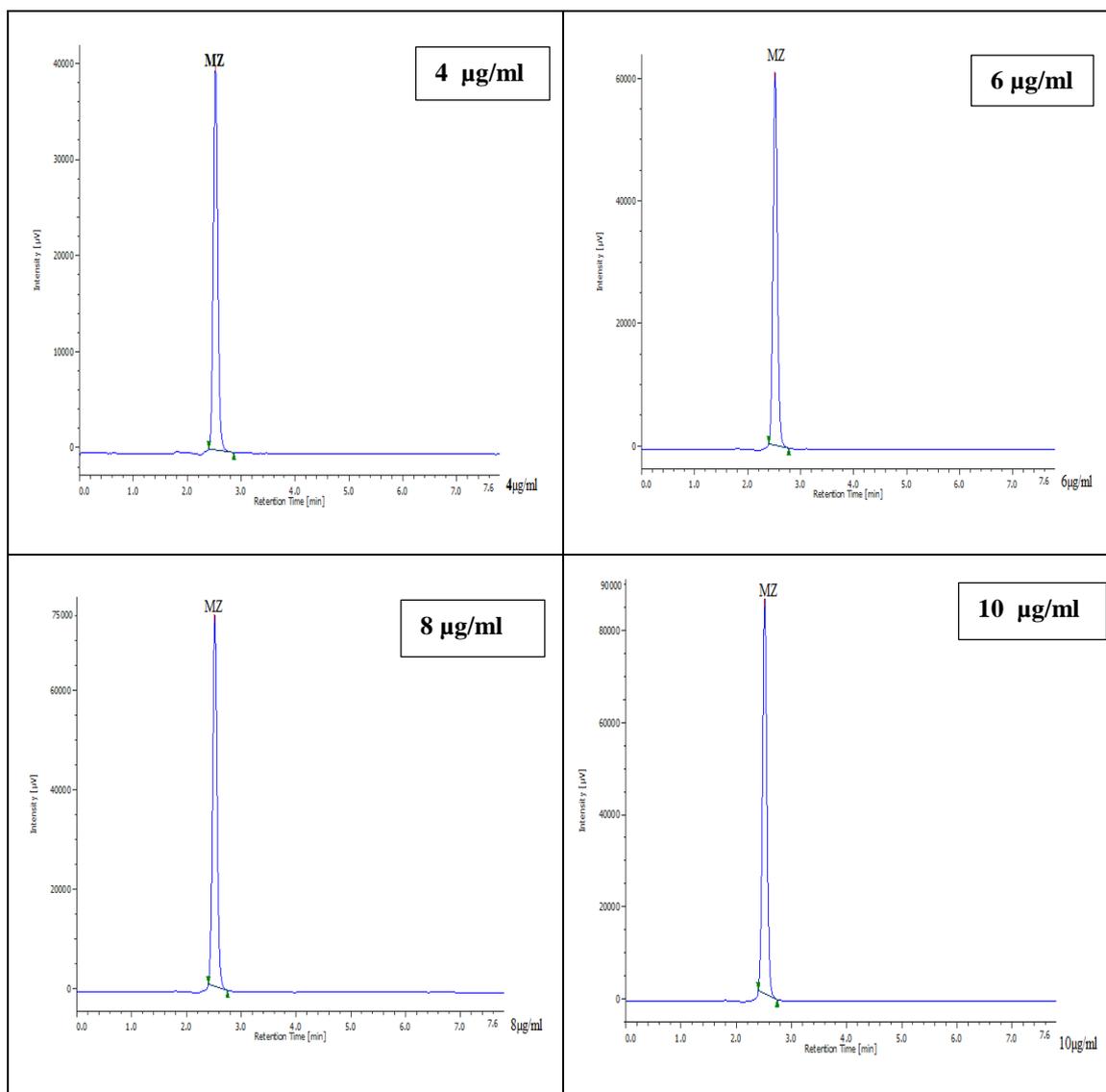


Figure 4.7.3.4: Representative chromatogram of Imp A (Conc., 1, 2, 4, 6, 8, 10 µg/ml) (Linearity study)

4.7.4. Recovery / Accuracy

Accuracy of the proposed method was calculated by recovery studies using **standard addition method**. The percentage recovery studies of CZ and Imp A was performed in triplicate at 3 different levels ranging from 80 % -120 %, by spiking standard samples as described under procedure at section 4.11.6.3. Result of recovery study is presented in Table 4.7.4.

Table 4.7.4: Recovery studies (n=3)

Component	Amount of sample (µg/ml) (Test Conc.)	Average peak area of sample	Level of spiking (%)	Amount of standard spiked (µg/ml)	Average Peak area* after standard addition	Peak area of recovered standard	Amount Recovered (µg/ml)	% Recovery
CZ	40	132121	80	32	228800	96679	30.99	98.85
			100	40	269969	137847	39.26	99.16
			120	48	311374	179253	48.67	101.41
Imp A	4	225179	80	3.2	424460	199281	3.106	98.08
			100	4	458981	233802	3.96	99.01
			120	4.8	524975	299796	4.83	100.64

(*Average of three readings)

4.7.5. Precision and sensitivity

Intraday repeatability of the method was estimated by analyzing three concentrations of CZ (40, 60 and 80 µg/ml), and three concentration of Imp A (4, 6 and 8 µg/ml). Interday precision was estimated by assaying the three chosen sets of same concentration of CZ and Imp A in triplicates on two successive days using the procedure stated under section 4.11.6.4 and 4.11.6.5. Results of the study are presented in Table 4.7.5.1 and Table 4.7.5.2.

Table 4.7.5.1: Intra and Interday precision data (n=3)

Compound	Concentration (µg/ml)	Intra-day		Inter-day	
		mean peak area ± SD	% RSD	mean peak area ± SD	% RSD
CZ	40	116714 ± 710	0.60	119373 ± 1613	1.35
	60	186330 ± 831	0.44	182199 ± 2256	1.23
	80	235351 ± 2667	1.13	235147 ± 1811	0.77
Imp A	4	246202 ± 1765	0.71	216971 ± 2349	1.08
	6	363107 ± 4707	1.29	343861 ± 1794	0.52
	8	447615 ± 4174	0.93	413969 ± 3982	0.96

Table 4.7.5.2: Sensitivity of the method (LOD and LOQ)

Parameters	CZ	Imp A
LOD (µg/ml)	3.52	0.51
LOQ (µg/ml)	10.66	1.71

4.7.6. Robustness

Robustness of an analytical method is the measurement of the methods ability to remain unchanged by minute but intended alterations in the methods variables and provide reliability during normal use. The robustness of the method was studied by making deliberate changes in flow rate and composition of mobile phase ratio.

4.7.6.1. Variation in the flow rate (± 0.2 ml/min of the optimized flow rate)

The optimized flow rate for the developed method was set at 1 ml/min. For robustness study, the flow rate was varied to 0.8 ml/min and 1.2 ml/min. The solutions were then injected into the chromatograph equilibrated with the flow rate of 0.8 ml/min and 1.2 ml/min. The results obtained upon the flow rate variation are displayed in Table 4.7.6.1 with representative chromatogram (Fig 4.7.6.1.1 and Fig 4.7.6.1.2).

Table 4.7.6.1: Results of flow rate variation

	CZ	Imp A
Flow rate	Average area (n=3)	Average area (n=3)
0.8 ml	185401	327464
1 ml	182200	321111
1.2 ml	182639	315316
% RSD	0.95 %	1.89 %

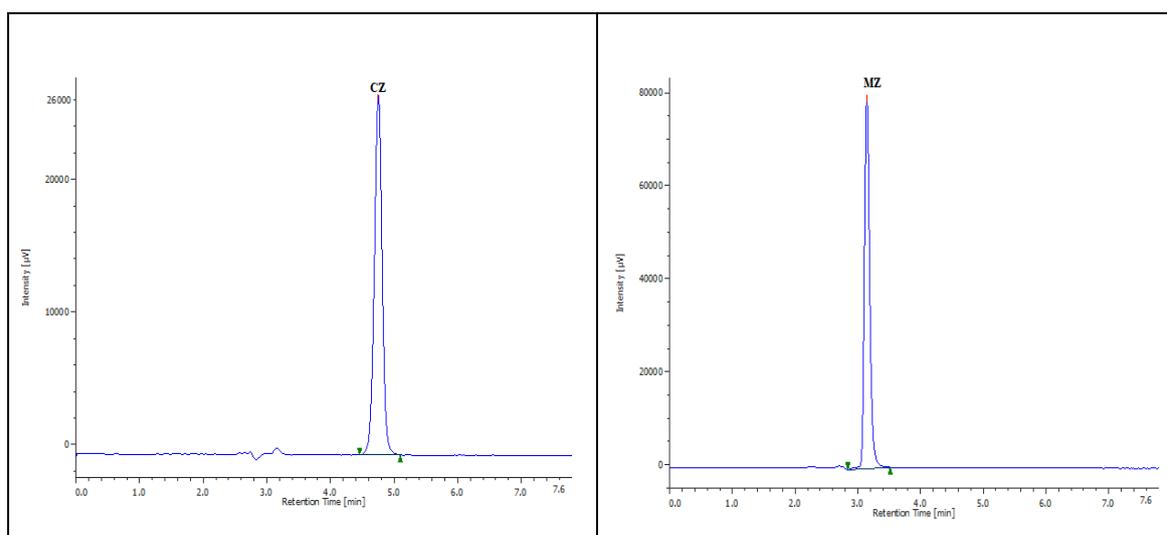


Figure 4.7.6.1.1: Chromatogram with flow rate 0.8 ml/min (optimized 1.0 ml/min)

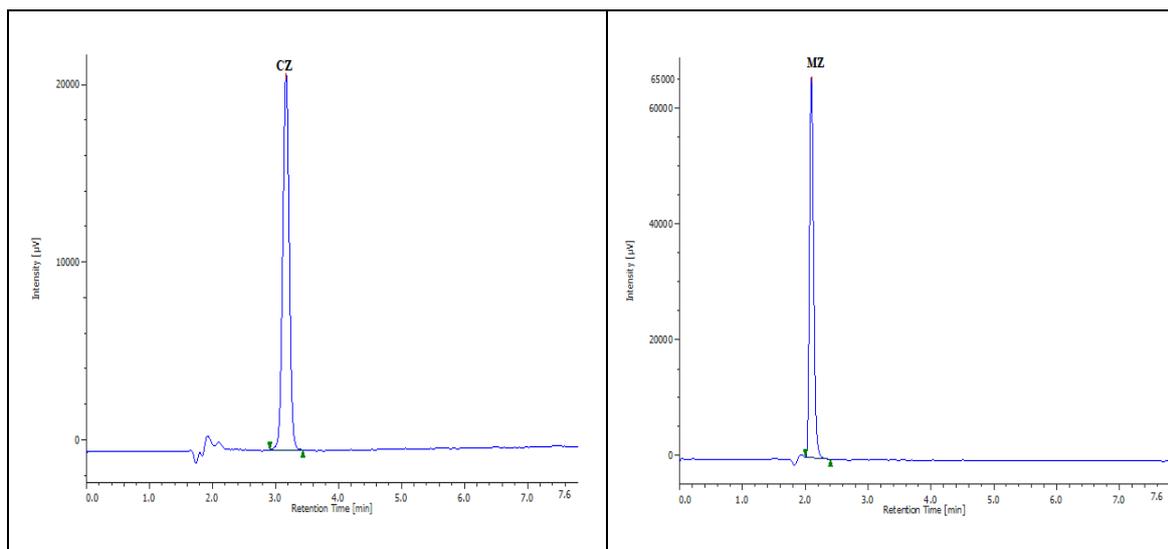


Figure 4.7.6.1.2: Chromatogram with flow rate 1.2 ml/min (optimized 1.0 ml/min)

4.7.6.2. Variation in the mobile phase composition (± 2 % of the optimized ratio)

The optimized isocratic elution method involved use of acetonitrile: ammonium dihydrogen ortho phosphate buffer (pH adjusted to 2.5 with OPA acid,) in ratio of 50:50 % v/v. For robustness study, organic phase ratio was varied to 48:52 % v/v (- 2 % of the optimized ratio) and 52:48 % v/v (+2 % of the optimized ratio). The results obtained wherein organic phase ratio of mobile phase was varied are presented in Table 4.7.6.2 with representative chromatograms (Fig 4.7.6.2.1 an Fig 4.7.6.2.2).

Table 4.7.6.2: Results of mobile phase variation

	CZ	Imp A
Composition	Average area (n=3)	Average area (n=3)
48:52 %v/v	177429	326581
50:50 %v/v	182220	321111
52:48 %v/v	181831	327778
% RSD	1.47 %	1.09 %

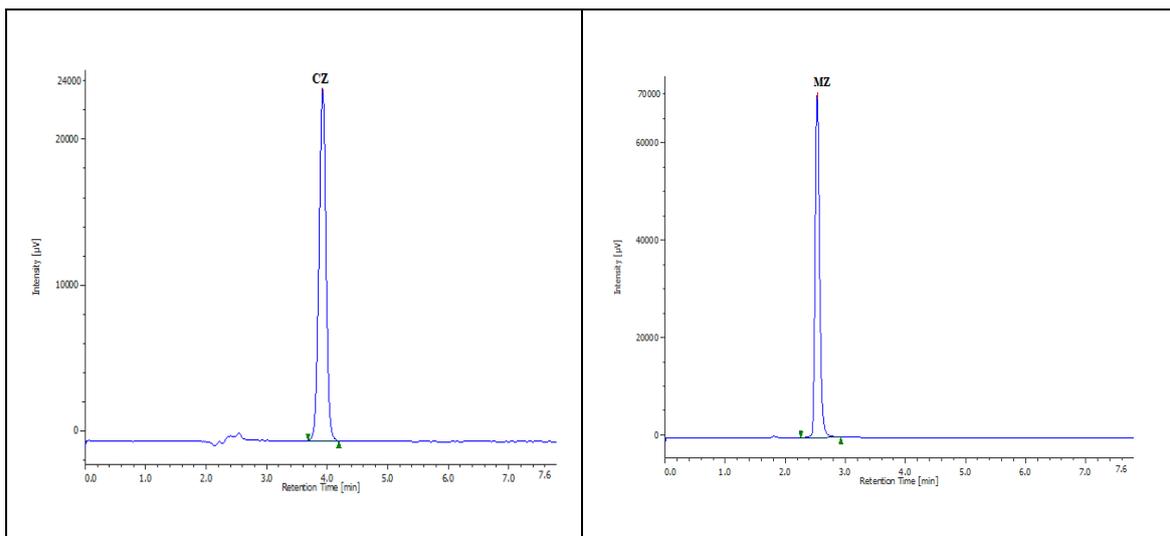


Figure 4.7.6.2.1: Chromatogram with organic phase ratio altered to 48:52 % v/v (optimized 50:50 % v/v)

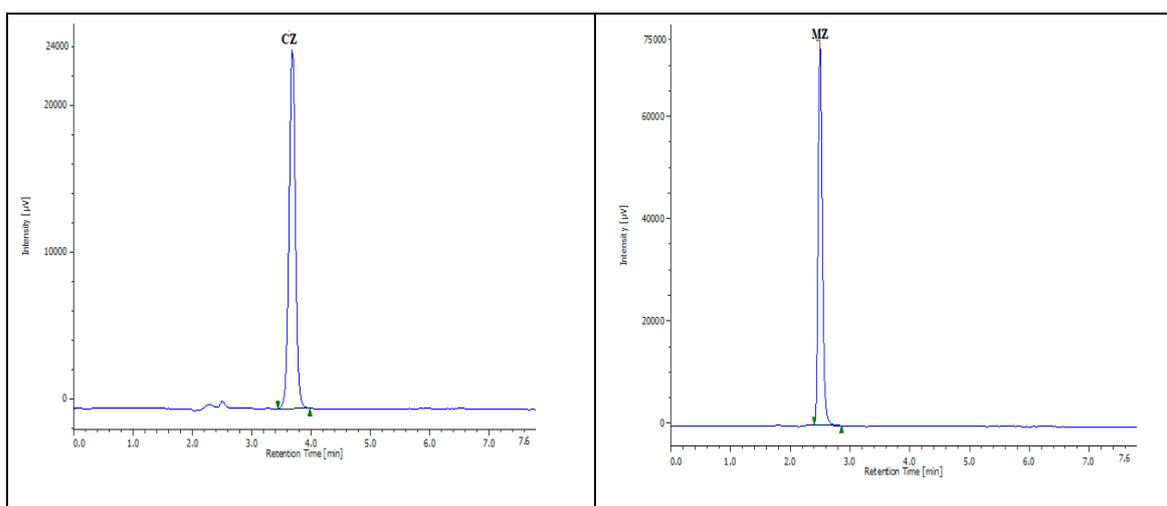


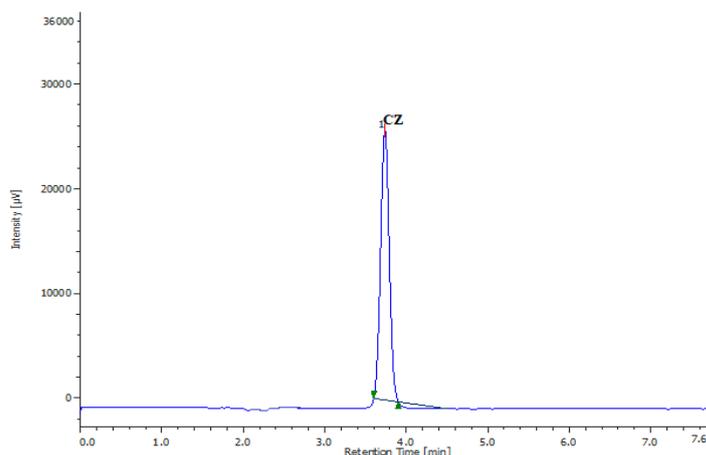
Figure 4.7.6.2.2: Chromatogram with organic phase ratio altered to 52:48 % v/v (optimized 50:50 % v/v)

4.8. ANALYSIS OF MARKETED PRODUCT

The validated new HPLC method was extended for determination of CZ in tablet dosage form of Neo-Mercazole[®] 5 (Mfg. Dheer Healthcare Pvt Ltd) as per the procedure under section 4.11.7. The assay result obtained showed purity of 98.56 %. Results of study are summarized in Table 4.8. Also no detectable peaks were found at the RRT of Imp A, conforming its absence in detectable concentration (Fig 4.8).

Table 4.8: Result of CZ in marketed product (Neo-Mercazole® 5)

Sr. no	Conc. of sample solution (µg/ml)	Peak area	Mean peak Area	% assay
1	40	188904	189259	98.56
2		189261		
3		189612		

**Figure 4.8: Chromatogram of CZ sample (Neo-Mercazole® 5)**

Thus proposed new RP HPLC is validated and demonstrated to be stability indicating analytical method for the determination of CZ, and can be a useful alternate for the sensitive determination of the drug as API and in formulations (Tablets) in presence of its possible specified impurity.

4.9. CHARACTERIZATION OF DEGRADATION PRODUCT BY LCMS

The LCMS spectra of CZ and its DP I were recorded by using positive mode of electro spray ionization (ESI). The output of mass spectrometer was validated before injecting the stress samples of Carbimazole. For LCMS studies the buffer of the developed method was replaced with acetate buffer with pH 2.5 which is compatible with LCMS as compared to phosphate buffer. Since the DP I obtained in the stressed samples (hydrolytic, oxidative and thermal) had the same RRT values, the hydrolytic stressed sample in water was used for LCMS studies.

Following figures depicts DAD spectra (Fig.4.9.1) and TIC (Fig.4.9.2) of the stress sample of CZ.

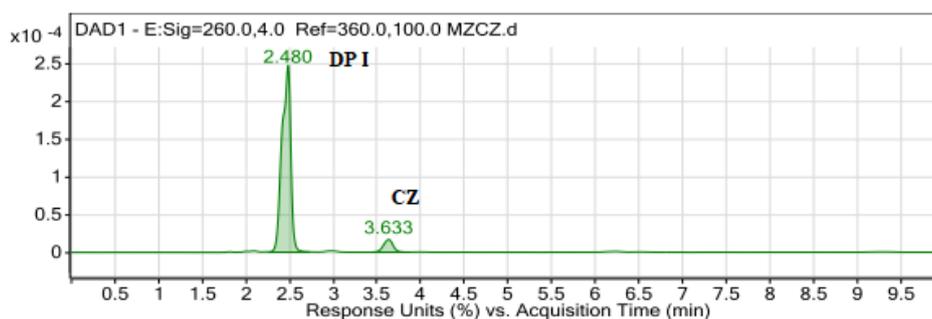


Figure 4.9.1: DAD spectra of stress sample of CZ

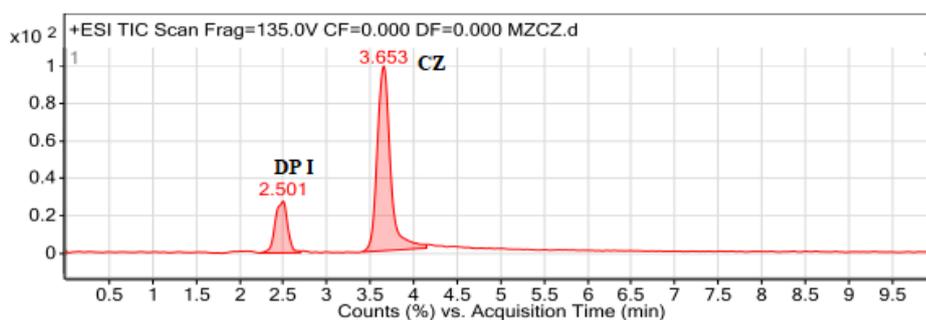


Figure 4.9.2: TIC of stress sample of CZ

The mass spectra of degradant (DP I) generated under hydrolytic stress was depicted as follows (Fig 4.9.3). The molecular weight of DP I (RRT = 0.66) was found to be 114.17 which is seen in the other stressed samples as well. The molecular ion peak of which appears at m/z value of 115.0.

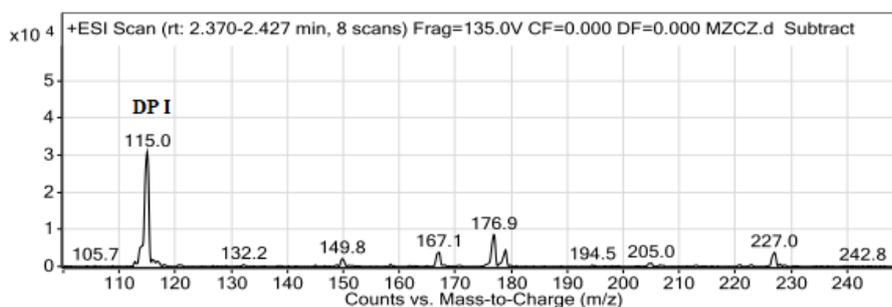


Figure 4.9.3: Mass spectra of DP I recorded in ESI positive mode

Similarly Mass spectra of CZ were generated as shown in Fig 4.9.4. The molecular weight of CZ is 186.2, the molecular ion peak of which is seen at m/z value of 187. It also seen that at the applied ionization frequency the drug is ionized to give a molecular ion peak at m/z value 115 resembling DP I.

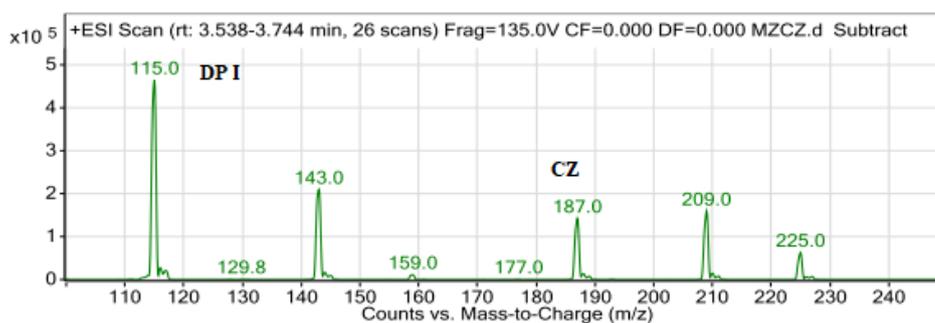
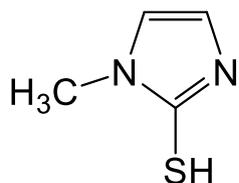


Figure 4.9.4: Mass spectra of CZ recorded in ESI positive mode

The degradation product (DP I) generated under hydrolysis, oxidative and thermal conditions is having molecular weight of 114.17 amu is thiamazole. This impurity is known and mentioned in BP as one of the related substance. There is need to isolate this degradation product in pure form for further structural elucidation by NMR and mass spectrometry.

Degradation product I (DP I)



Chemical Name: 1-methyl-1H-imidazole-2-thiol (thiamazole)

Molecular Formula: C₇H₁₀N₂O₂S

Molecular weight: 114.17 g/mol

4.10. DEGRADATION PATHWAY OF CARBIMAZOLE (CZ)

Degradation pathway of CZ based on the LCMS studies can be summarized as below.

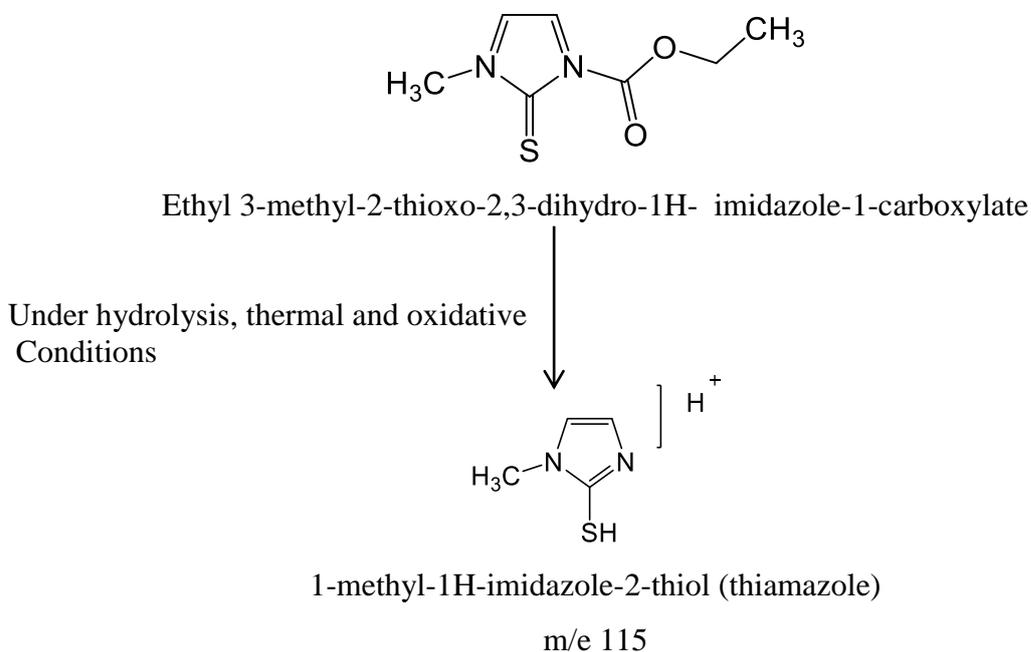


Figure 4.10: Predicted degradation pathway of CZ

4.11. METHODOLOGY FOR DETERMINATION OF CARBIMAZOLE AND ITS IMPURITY

4.11.1. Instrumentation

Instrument	Source
HPLC System	Jasco LC-4000 series quaternary pump system (PU-4180), an on-line degasser, an auto-sampler (AS-4050), a column temperature controller (CO-4061) and a diode array detector (MD-4010). Software: Jasco Chrom NAV software
Column	Waters -Sunfire ODS C18 (4.6 x 250mm,5 μ m) column.
Sonicator	Citizen Ultrasonic cleaner
pH meter	Digital pH Meter, Labtronics, LT-10
Electronic balance	Wensar Digital Electronic Balance MAB 220
HPLC water purification system	Bio-age Water purification system
Hot air oven	Universal Hot air Owen
Constant temperature water bath	Tempo
LCMS system	Agilent Technologies 6460 Triple quadrupole LC/MS, Fragmentor voltage 135kV

4.11.2. Chemicals and Reagents

- 1) Acetonitrile (HPLC grade), Rankem, India.
- 2) Water (HPLC grade), obtained from Bio age water purification system.
- 3) Ammonium dihydrogen Orthophosphate (AR grade), S.D. Fine-Chem Ltd., Mumbai, India.
- 4) Ortho phosphoric acid (AR grade), Qualigens fine chemicals, Mahape, India.
- 5) Hydrochloric acid (AR grade), Qualigens fine chemicals, Mahape, India.
- 6) Sodium Hydroxide (AR grade), Qualigens fine chemicals, Mumbai, India.
- 7) Hydrogen peroxide, S.D. Fine-Chem Ltd., Mumbai, India.

4.11.3. Working standard

Carbimazole (Abbott India Ltd, Goa), Imp A (Sigma Aldrich, USA)

4.11.4. Solution Preparation

4.11.4.1. Preparation of Stock Solution of Drug

CZ (100 mg) was weighed and transferred into 100 ml V.F., dissolve in 50 ml of acetonitrile and volume was made up to the mark to get the solution concentration 1000 µg/ml. Further dilutions were made from stock as per the requirements.

4.11.4.2. Preparation of Stock solution of Imp A

An amount of 100 mg of Imp A was weighed and transferred into 100 ml V.F., 75 ml of acetonitrile was added, mixed well and sonicated for 10 minutes. Final volume was made up to the mark and mixed well to get the solution concentration of 1000 µg/ml. Further dilutions were made from the stock solution as per the requirements.

4.11.4.3. Preparation of working standard solution of Imp A

1ml of stock solution of Imp A was transferred into a 10 ml V.F. Acetonitrile was added in the flask to the mark to get concentration of 100 µg/ml.

4.11.4.4. Preparation of buffer – 10 mM Ammonium dihydrogen orthophosphate (pH = 2.5)

Ammonium dihydrogen ortho phosphate (1.15gm) was dissolved in 1000 ml of HPLC grade water and pH was adjuster to 2.5 with o-phosphoric acid.

The solution was filtered through 0.45 μ membrane filter.

4.11.4.5. Preparation of 1N sodium hydroxide solution

Sodium hydroxide flakes (0.4 gm) were dissolved in 50 ml of water in 100 ml V.F., and volume up to the mark was made with water to get 1N sodium hydroxide solution. From the prepared 1N solution desired strength of 0.001N NaOH was prepared by dilution.

4.11.4.6. Preparation of 0.1N hydrochloric acid solution

Hydrochloric acid (0.1N) was prepared by diluting 0.85 ml of concentrated hydrochloric acid solution to 100 ml with water in 100 ml V.F. From the prepared 1N solution desired strength of 0.001N HCl was prepared by dilution.

4.11.4.7. Preparation of 0.1 % hydrogen peroxide solution

Hydrogen peroxide (10 %) was prepared by appropriately diluting 30 % of hydrogen peroxide with water in 100 ml V.F. From 10 % hydrogen peroxide 1ml was taken and diluted with water in 100 ml V.F to get the required concentration of 0.1 %.

4.11.4.8. Diluent

Mixture of acetonitrile and ammonium dihydrogen orthophosphate buffer (pH adjusted to 2.5 with o-phosphoric acid) in the ratio 50:50 % v/v was used as diluent for dilution of samples.

4.11.5. Stress studies

The forced degradation studies were carried out to achieve significant percent degradation of the drug. The drug was exposed to hydrolytic, oxidative, thermal and photolytic stress. The results were obtained by comparing four samples which were obtained for every stress conditions except thermal and photolytic stress viz., the blank stored under normal condition (blank untreated), the blank subjected to stress in same manner as that of drug solution (blank treated), zero time sample containing the drug (standard untreated) and the drug solution subjected to stress treatment. Whereas for thermal and photolytic stress conditions only two samples were generated one is sample exposed to stress condition and other is control.

4.11.5.1. Hydrolytic degradation

The hydrolytic degradations were carried out in acidic, alkaline and neutral conditions. Samples were prepared by taking 0.1 ml of stock solution of drug (1000 µg/ml) and 1ml of hydrolytic agent (HCl / NaOH / water) in 10 ml V.F.

Initially 0.1N strength of HCl and NaOH was used for study, which showed complete degradation of drug. The study was carried out at room temperature and at 6 hr time interval. The strength of hydrolytic agent was decreased and also the time interval was varied to get significant degradation of drug. The strength of base which caused significant degradation was 0.001N at RT and 5 min of time interval. After required exposure samples were neutralized by using equal strength of acid or alkali which ever was required. Hydrolytic degradation in neutral medium was conducted in water. Finally volume was made up to the mark by using diluent and subjected for HPLC analysis by proposed method.

4.11.5.2. Oxidative degradation

Oxidative degradation was carried out using H₂O₂. Initially samples were prepared by using 0.1 ml of stock solution of drug (1000 µg/ml) and 1 ml of hydrogen peroxide (10 %) in 10 ml V.F at RT. The concentration of H₂O₂ and the study time interval was varied to get significant degradation of drug. Hydrogen peroxide at concentration of 0.1 % and quantity of 0.1 ml was sufficient to degrade the drug. Further the samples were processed by diluting up to the mark using diluent and subjected for HPLC analysis by proposed method.

4.11.5.3. Thermal degradation

Drug sample 10 mg each was taken in two 10 ml V.F and stoppered. One flask was exposed to dry heat in hot air oven for specified temperature (80 °C) and time interval (6 hr) and another was kept as control. After exposure two separate solutions were prepared by weighing appropriate amount of sample exposed to thermal stress and control to produce concentration of 10 µg/ml. The samples were diluted up to the mark with the help of diluent and subjected to analysis by proposed HPLC method.

4.11.5.4. Photo degradation

Drug in sufficient amount was taken in 10 ml V.F and was exposed to direct sunlight for a specified time period (7 days). Another flask containing drug was kept as control. After required exposure two separate solutions were prepared by weighing appropriate amount of sample exposed to stress and control to produce concentration of 10 µg/ml. The samples were diluted up to the mark with the help of diluent and injected and analyzed by proposed HPLC method.

4.11.6. Validation studies

Validation of optimized stability indicating method was carried out with respect to parameters recommended under ICH guideline Q2 (R1).

4.11.6.1. Specificity and Selectivity

Establishment of resolution factor between the drug peak, pharmacopoeial impurity peaks and other obtained degradation peak was explored to determine the specificity of the method. Method Specificity was assessed by comparing relative retention time (RRT) of a drug, impurity and degradants obtained from stressed samples of CZ.

4.11.6.2. Linearity and Range

To establish linearity and range stock solution of drug (1000 µg/ml) was further diluted with the help of diluent to get the drug concentration range of 10-100 µg/ml. The samples were analyzed in triplicate. The impurity were diluted from the stock solutions to get a concentration range of 1-10 µg/ml. The samples were analyzed in triplicate by the proposed method.

4.11.6.3. Accuracy

Accuracy of the proposed method was determined by recovery studies using standard addition method. The percentage recovery studies of CZ and CZ-Imp A was carried out in triplicate at 3 different levels of 80 %, 100 %, 120 %, by spiking standard samples. Standard drug solutions of concentration 32 µg/ml (80 %), 40 µg/ml (100 %) and 48 µg/ml (120 %) were prepared by transferring 0.32 ml, 0.4 ml and 0.48 ml respectively from standard tock solution into a series of 10 ml V.F each containing sample solution.

The volume up to the mark was made with diluent. The solutions prepared were injected (n=3) into the chromatograph and the concentration were determined from the peak areas obtained from the chromatograms. Similarly study was carried out for imp A by preparing standard solution of concentration 0.32 µg/ml (80 %), 0.4 µg/ml (100 %) and 0.48 µg/ml (120 %), from working standard of Imp A.

4.11.6.4. Precision

Intraday repeatability of the method was evaluated by analyzing three concentrations of CZ (40, 60 and 80 µg/ml), and three concentration of CZ-Imp A (4, 6 and 8 µg/ml) prepared from respective stock solutions. . Interday precision was evaluated by assaying the chosen concentrations of CZ and CZ-Imp A, in triplicate on two successive days using the same procedure stated under chromatographic conditions. % RSD was calculated.

4.11.6.5. Sensitivity

The sensitivity of the developed method was determined by calculating LOD and LOQ for CZ and its impurity. LOD and LOQ were calculated for CZ and its impurity based on the Standard deviation of the Response and the Slope as mentioned below.

$$\text{LOD} = 3.3 \times \text{Standard deviation of the response} / \text{Slope of the calibration curve}$$

$$\text{LOQ} = 10 \times \text{Standard deviation of the response} / \text{Slope of the calibration curve}$$

4.11.6.6. Robustness

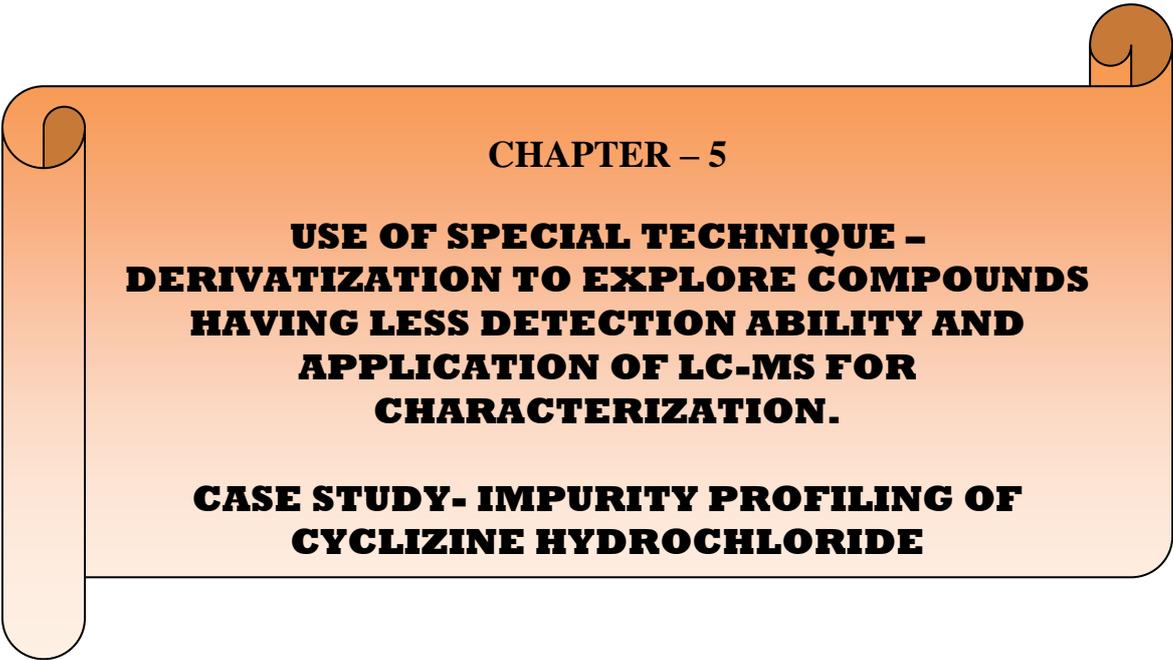
Robustness of the developed method was determined by making deliberate change in method parameters like flow rate, and composition of mobile phase ratio. Triplet injections of sample containing pharmacopoeial impurity and drug were made. The values of percent relative standard deviation (% RSD) of peak area was recorded.

Required aliquots were taken from stock solution of CZ and working standard solution of imp A in 10ml V.F. Volume was made up to the mark using diluent to get concentration of solution containing 50 µg/ml of CZ and 5 µg/ml of Imp A. To study the effect of variation in flow rate on the method, ± 0.2 ml of the optimized flow rate (1 ml) was studied on the method. The prepared solution was analyzed in triplicate by the proposed method by altering the flow rate of the proposed method to 0.8 ml (- 0.2 ml) and 1.2 ml (+ 0.2ml).

To analyze the effect of change in composition of mobile phase ratio on the proposed method, the composition of organic phase in the mobile phase was altered by $\pm 2\%$ of the optimized ratio (50:50 % v/v). Hence the mobile phase used for study was ACN: buffer at 48:52 % v/v (- 2 %) and 52:48 % v/v (+ 2 %). The prepared solution was analyzed in triplicate by the proposed method, carrying out the necessary changes in the mobile phase composition.

4.11.7. Analysis of Marketed product

For analysis of tablets, 10 tablets were weighed individually and their average weight determined. Tablets were then crushed to fine powder and powder equivalent to 50 mg was transferred to 50 ml V.F and dissolved in 40 ml acetonitrile with vigorous shaking for 15 minutes. The solution was sonicated for 10 min. Finally the volume up to the mark was made with acetonitrile. The solution was then filtered through Whatman filter paper (#1). From this sample solution, 4 ml was transferred into 100 ml V.F and diluent was added up to the mark to obtain a concentration of 40 $\mu\text{g}/\text{ml}$. The solution prepared were injected (n=3) into the chromatograph to determine the peak area. From the peak area of the standard solution and the tablet sample solution, the percentage content of CZ in the marketed tablets was calculated.



CHAPTER – 5

**USE OF SPECIAL TECHNIQUE –
DERIVATIZATION TO EXPLORE COMPOUNDS
HAVING LESS DETECTION ABILITY AND
APPLICATION OF LC-MS FOR
CHARACTERIZATION.**

**CASE STUDY- IMPURITY PROFILING OF
CYCLIZINE HYDROCHLORIDE**

5. USE OF SPECIAL TECHNIQUE-DERIVATIZATION TO EXPLORE COMPOUNDS HAVING LESS DETECTION ABILITY AND APPLICATION OF LCMS FOR CHARACTERIZATION.

CASE STUDY- IMPURITY PROFILING OF CYCLIZINE HYDROCHLORIDE

5.1. INTRODUCTION

A RP HPLC method was developed for estimation of Cyclizine HCl (CY) in presence of its pharmacopoeial impurities, and to confirm the developed method's stability indicating power by testing the stressed samples of CY.

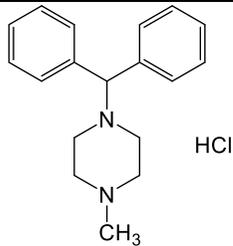
The following objectives were set to carry out the research activity:

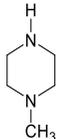
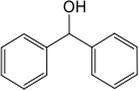
- 1) To identify the sources of pharmacopoeial impurities which could be processes related for CY API or its degradants.
- 2) To develop and optimize a RP HPLC method for estimating CY as API in presence of its pharmacopoeial impurities.
- 3) To perform stress induced studies for CY in accordance with ICH guidelines.
- 4) To validate the optimized method for detection and estimation of CY in presence of its pharmacopoeial impurities and degradation products.

5.2. PROFILE

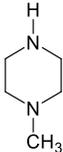
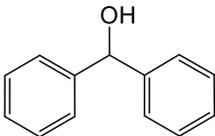
5.2.1. Drug profile

Cyclizine Hydrochloride is official in IP⁴⁴, BP⁴⁵, USP⁴⁶ and EP⁴⁷

General Name	Cyclizine Hydrochloride
Chemical Structure	
Chemical Name	1-(Diphenylmethyl)-4-methylpiperazine hydrochloride
Molecular Formula	C ₁₈ H ₂₂ N ₂ , HCl
Molecular Weight	302.8 g/mol
Melting Point	105 - 108 °C
Description	White or almost white, crystalline powder
Solubility	Slightly soluble in water and ethanol (96 per cent)
pKa	8.51
Drug Category	Histamine H ₁ receptor antagonist ; antihistamine

<p>Clinical Pharmacology</p>	<p>Cyclizine acts by blocking the histamine receptors in the vomiting center and thus reduce its activity. Used for the control of postoperative and drug-induced vomiting and in motion sickness. Cyclizine also possesses anti-cholinergic properties as well; hence the muscarinic receptors are similarly blocked¹⁰⁵.</p>
<p>Pharmacokinetics</p>	<p>Cyclizine is absorbed from the gastrointestinal tract and has an onset of action within 2 hours. The duration of action is reported to be about 4 hours. Cyclizine is metabolised in the liver to the relatively inactive metabolite, norcyclizine. Both Cyclizine and norcyclizine have plasma elimination half-lives of 20 hours. Less than 1 % of the total oral dose is eliminated in the urine in 24 hours¹⁰⁶.</p>
<p>Toxicity</p>	<p>Overdose may include either CNS depression (sedation, reduced mental alertness, apnea, and CV collapse) or CNS stimulation (insomnia, hallucinations, tremors, or seizures). Anticholinergic symptoms, such as dry mouth, flushed skin, fixed and dilated pupils, and GI symptoms, are common, especially in children¹⁰⁷. Acute CY intoxication result in death¹⁰⁸.</p>
<p>Impurity Specified impurities A, B</p>	<p>A</p> <div style="text-align: center;">  <p>1-Methylpiperazine</p> </div> <p>B</p> <div style="text-align: center;">  <p>Diphenylmethanol (benzhydrol)</p> </div>

5.2.2. Impurity Profile

Impurity	A	B
Chemical Structure		
Chemical Name	1-Methylpiperazine	Diphenylmethanol (benzhydrol)
Molecular Formula	C ₅ H ₁₂ N ₂	C ₁₃ H ₁₂ O
Molecular Weight	100.16 g/mol	184.23 g/mol
Boiling point/ Melting Point	138 °C	69 °C
pKa	13.55 ± 0.20 by Marvin Sketch (Ver 19.9.0, 2019, www.chemaxon.com)	a) 4.86 and b) 9.33 by Marvin Sketch (Ver 19.9.0, 2019, www.chemaxon.com)
Description	Clear liquid , light yellow	Off white powder
Solubility	Soluble in water.	Soluble in water, chloroform, alcohol, ether, and methanol.
Toxicity	Symptoms of overexposure may be headache, dizziness, tiredness, nausea and vomiting. Ingestion causes severe swelling, severe damage to the delicate tissue and danger of perforation. Symptoms of allergic reaction may include rash, itching, swelling, trouble breathing, tingling of the hands and feet, dizziness, lightheadedness, chest pain, muscle pain or flushing. Poison by intraperitoneal route ¹⁰⁹⁻¹¹¹	Diphenylmethanol is an irritant to the eyes, skin and respiratory system ¹¹²⁻¹¹⁴ .

5.3. LITERATURE SURVEY

Literature survey cited very few analytical methods for the estimation of CY in pharmaceutical formulations or biological fluids either individually or with other drugs by HPLC¹¹⁵⁻¹¹⁸. Limited literature is found on stability indicating HPLC method for CY. Following table summarizes the chromatographic conditions used in HPLC methods reported in literature for CY.

Table 5.3: Summary of reported chromatographic conditions used for determination of CY by HPLC

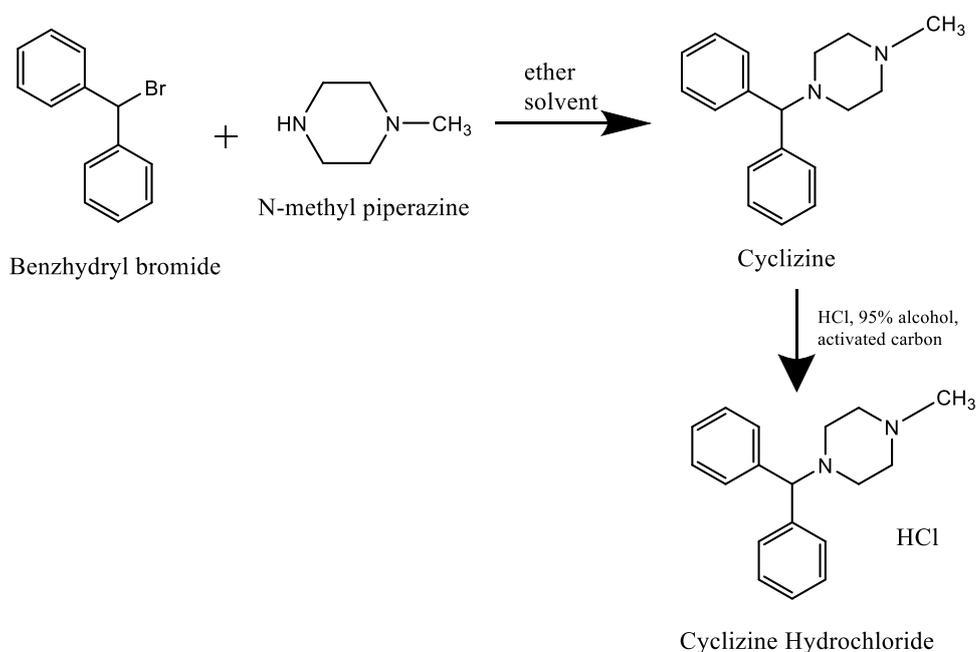
Sr.No.	Column type	Mobile phase composition	Flow rate	Detector used	Rt of drug	References
1	Determination of ergotamine tartarate and Cyclizine hydrochloride in pharmaceutical tablets by reverse phase HPLC					
	C18 (5 μ m)	0.01 M ammonium acetate in acetonitrile: water: triethylamine (35:64:1 % v/v/v) solution, the pH adjusted to 3.7 with glacial acetic acid	1.5 ml/min	UV (254 nm)	3.4 min	115
2	HPLC Determination of Cyclizine Hydrochloride, Caffeine and Vitamin B6 in compound Cyclizine Hydrochloride tablets					
	μ Bondapak C18 (5 μ m)	Acetonitrile :1.5 % diethylamine (phosphoric acid adjust pH 4.5) (30:70 % v/v)	1.5 ml/min	UV (225 nm)	---	116

5.4. LOCATING SOURCE OF IMPURITIES IN CYCLIZINE HCl

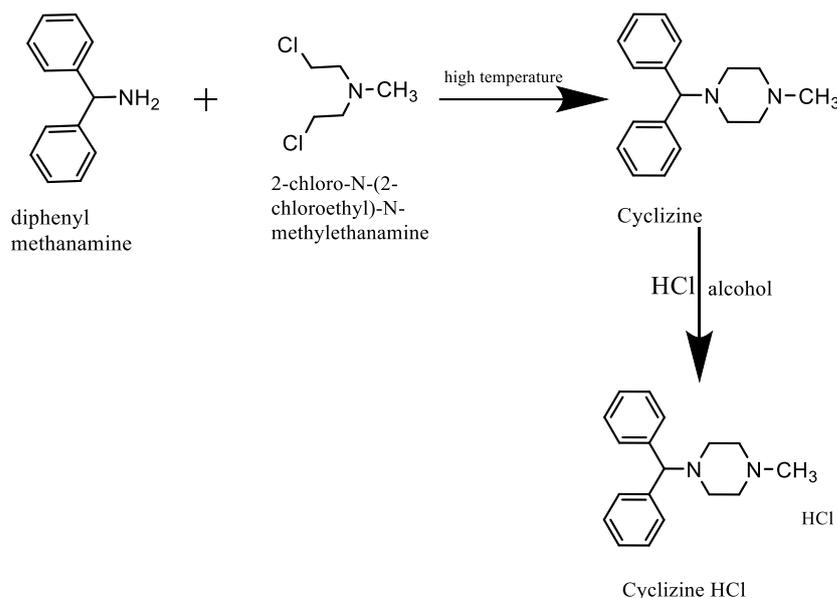
The presence of impurities in drug results from different sources and could be steps involved in the synthetic process or during preparation of pharmaceutical dosage forms. The impurities are specific to the synthetic route of the manufacturing process.

Synthetic schemes of CY from the available literature were surveyed for identifying the possible impurities which can remain with the final product during its chemical synthesis.

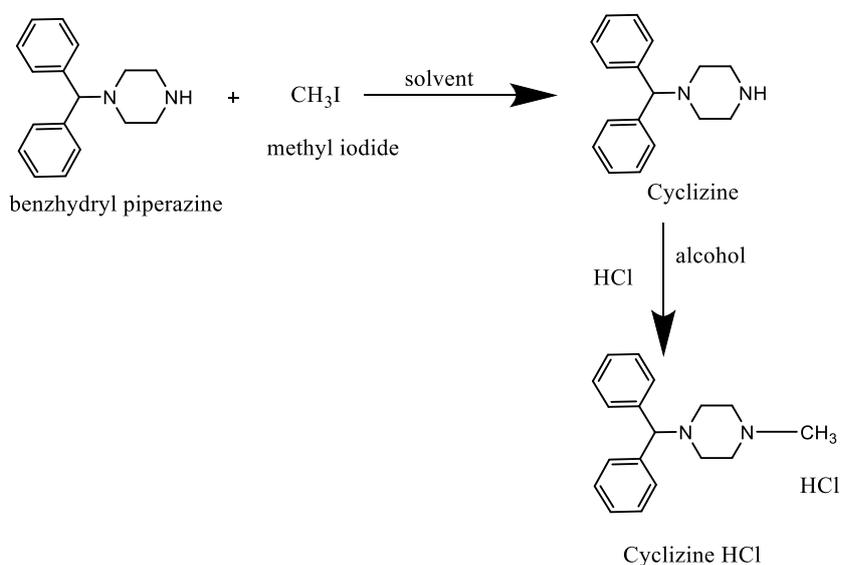
- A) One of the methods for CY synthesis involves condensation of benzhydryl bromide with N-methyl piperazine in the presence of ether solvent to give Cyclizine which is treated with hydrochloric acid to form CY. Benzhydryl bromide is prepared from diphenylcarbinol (benzhydrol) by Grignard synthesis¹¹⁹.



- B) Another method for CY synthesis involves reaction of diphenyl methanamine with 2-Chloro-N-(2-chloroethyl)-N-methylethanamine at high temperature to give Cyclizine¹¹⁹.



C) Synthesis of CY can also be carried out by methylating benzhydryl piperazine using methyl iodide to give Cyclizine free base, which is treated with carbon then the filtrate acidified with hydrochloric acid to form Cyclizine hydrochloride¹¹⁹.



From the above synthetic schemes it is observed that 1- Methyl piperazine and benzhydryl bromide (synthesized from benzhydrol) are used for the synthesis of CY. The trace amount of these compounds in the final API is listed as specified impurity A and B respectively in the monograph of CY.

5.5. DEVELOPMENT AND OPTIMIZATION OF RP HPLC METHOD FOR ESTIMATION OF CYCLIZINE HCl IN PRESENCE OF ITS PHARMACOPOEIAL SPECIFIED IMPURITIES

5.5.1. Selection of chromatographic method

The principle of separation in RP-HPLC is based on analytes' partition coefficient between polar mobile phase and hydrophobic (nonpolar) stationary phase in RPC. BP list gas chromatography method for related substances determination⁴⁵. In the present study RP HPLC was selected for separation and estimation of CY and of its Pharmacopoeial specified impurities.

5.5.2. Selection of stationary phase

Non polar C18 column was considered for the study.

5.5.3. Selection of wavelength for analysis

As Imp A lacks a distinct chromophore in its chemical structure, it was proposed to derivatize with a derivatization agent 4-Chloro-7-nitrobenzofurazan (NDB-Cl) (1 ml of 0.1 %) ¹²⁰. The derivatized Imp A exhibited absorbance in UV range. The overlain spectrum of CY, Imp A and derivatized Imp B was recorded in acetonitrile and shown in Fig. 5.5.3. Solutions of concentration, 10 µg/ml of CY and 1µg/ml of each impurity (A and B) were used for study. For the study one specific wavelength was selected where the absorptivity of Imp A and Imp B was comparatively higher than for CY for enhancing sensitivity. Wavelength of 225 nm was selected for the study and also found mentioned in literature¹¹⁶.

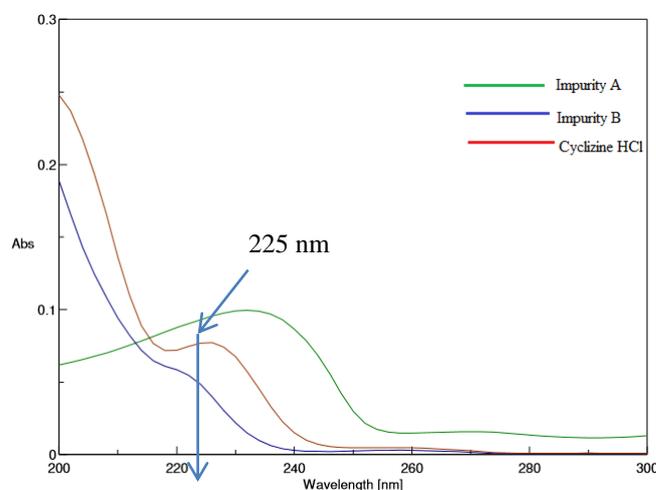


Figure 5.5.3: UV overlain spectrum of CY and impurities (A and B)

5.5.4. Selection and optimization of mobile phase for separation of CY and its impurities

A specific mobile phase to provide complete separation of CY from its specified pharmacopoeial impurities, and also monitor its degradation products under varied stress conditions was required to be developed. Based on the pKa values of CY (pKa 8.51), exploratory trials were carried out on mobile phase comprising of acetonitrile and 10 mM ammonium dihydrogen orthophosphate buffer at pH 6.5. Observations are recorded and presented in Table 5.5.4.1.

Table 5.5.4.1: Exploratory trials for optimization of mobile phase composition on Sunfire C-18 column (250 x 4.6 mm, 5 μ m) for CY and impurities (A and B)

Trial no	Mobile Phase composition (ACN and 10 mM potassium dihydrogen o-phosphate buffer) in % v/v	pH of mobile phase	Remark	Figure no
1	90:10	6.5	Resolution was about 3 between peaks	5.5.4.1.1
2	80:20		All 3 peaks were well resolved	5.5.4.1.2
3	70:30		Resolution of peak 2 was close to 2	5.5.4.1.3

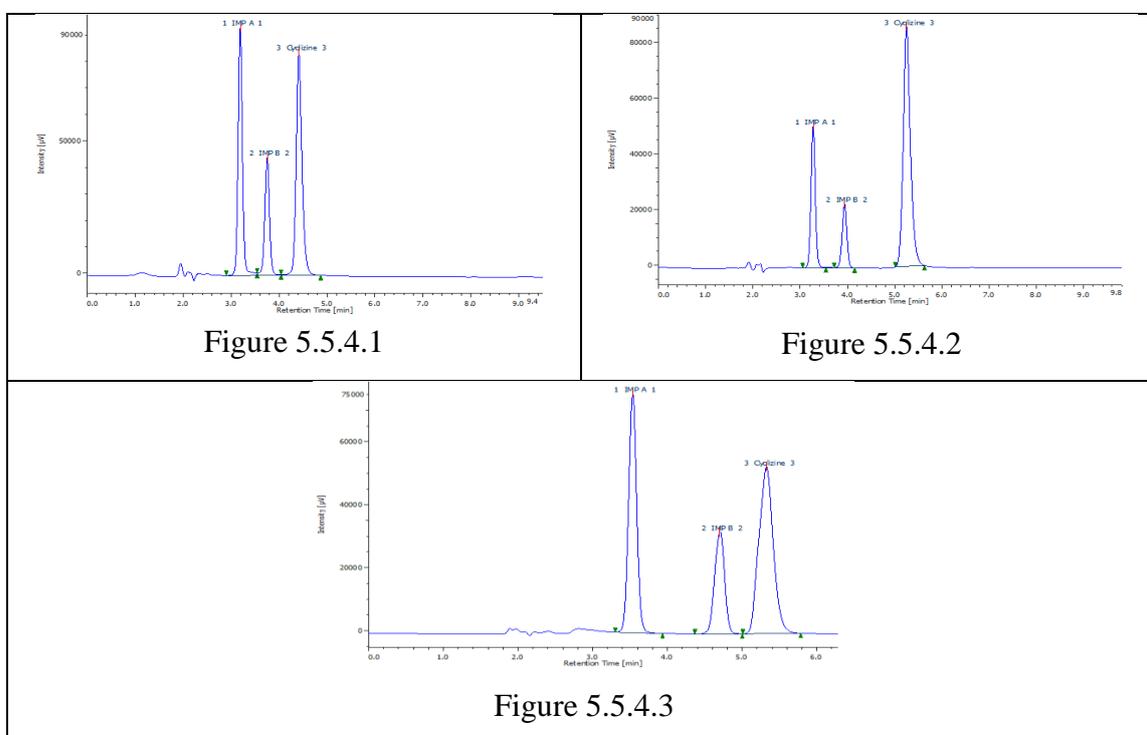


Figure 5.5.4: Representative chromatograms of exploratory trials for optimization of mobile phase composition for CY and impurities (A and B)

Separation was optimum with best resolution when organic to aqueous phase ratio of 80:20 % v/v was used as shown in Fig 5.5.4.2.

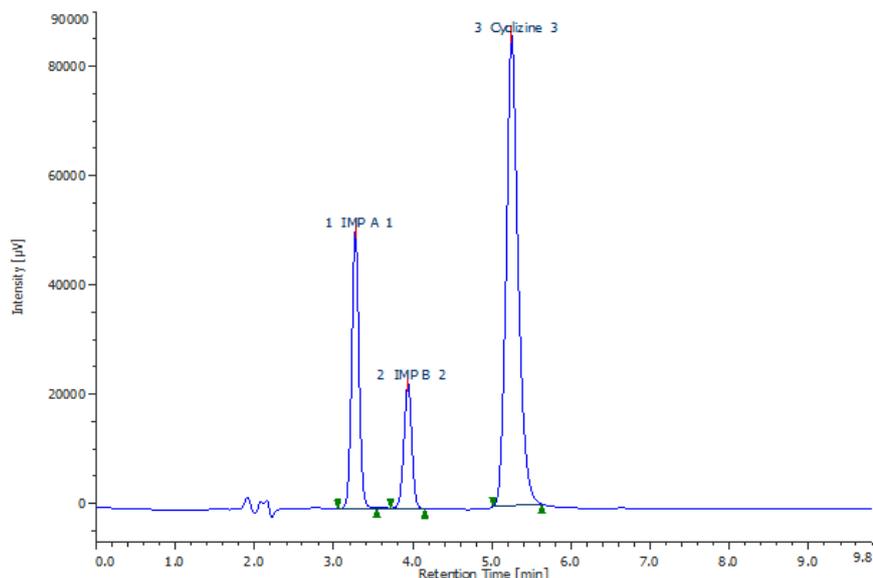


Figure 5.5.4.2: Optimized chromatogram of CY and impurities (A and B)

Chromatographic conditions were optimized from exploratory trial on Waters Sunfire C-18 column. Best fit mobile phase for separation of CY from Imp A and Imp B was selected based on good resolution of peaks with smooth baseline. Final optimized chromatographic conditions are shown in Table 5.5.4.2.

Table 5.5.4.2: Optimized chromatographic conditions for separation of CY and impurities (A and B)

Mobile phase	Acetonitrile: 10 mM Ammonium dihydrogen ortho phosphate buffer (pH adjusted to 6.5 by dilute ammonia solution) in the ratio of 80:20 % v/v.
Column	Waters -Sunfire ODS C18 (250 x 4.6 mm ,5 µm)
Column temperature	Ambient
Flow rate	1 ml/min
Wavelength	225 nm
Injector loop size	10 µL

5.6. FORCED DEGRADATION STUDIES

The forced degradation studies were conducted for the drug. CY was exposed to hydrolysis (HCl, NaOH and neutral), oxidation, elevated temperature and photolytic stress. Series of samples were prepared for each condition as per routine protocols (Table 5.6). Sample subjected to stress was obtained and analyzed by the proposed method.

Table 5.6: Protocol for stress degradation of CY

Samples	Hydrolysis			Oxidative Deg.	Thermal Deg.	Photo Deg.
	Acid	Alkaline	Neutral			
Blank stored under normal condition	√	√	√	√	-----	-----
Blank subjected to stress condition	√	√	√	√	-----	-----
Drug / drug solution stored under normal condition	√	√	√	√	√	√
Drug / drug solution subjected to stress condition*	√	√	√	√	√	√

* To get desired degradation, initially degradation was carried out at room temperature and if necessary samples were subjected to higher temperature or strength of stress reagent was increased.

5.6.1. Hydrolytic degradation

Hydrolytic studies were carried with HCl, NaOH and water to simulate acidic, alkaline and neutral conditions respectively. The acid and base degradation was carried out using hydrochloric acid and sodium hydroxide initially with strength of N/10 and followed by 1N if no degradation was seen at lower strength. The studies were performed initially at room temperature followed by heating the solution of drug with acid, base and water at 70 °C on water bath equipped with thermostat for extended time intervals, if no degradation was seen at RT.

The drug solution treated with acid and base was neutralized using base and acid respectively of the same strength and diluted with the diluent up to the mark. These solutions were then subjected to analysis by the proposed method.

5.6.1.1. Acid degradation

Aliquot volume of 0.1ml of stock solution of drug (1000 µg/ml) was transferred into 10 ml V F. To this 1 ml of N/10 HCl was added and kept at RT and at higher temperature of 70 °C for extended time interval around 6 hr. Further the study was conducted using 1ml of 1N HCl under the RT and 70 °C. The samples were cooled and then neutralized by 1 ml of same strength of NaOH and volume made up to the mark with mobile phase. The solutions were then analyzed by the proposed method.

Observation of hydrolytic study of CY with varied concentration of HCl at different temperature and time interval is presented in Table 5.6.1.1 and representative chromatogram as Fig 5.6.1.1.

Table 5.6.1.1: Degradation study of CY with HCl

Concentration of HCl	Temperature	Time	% of active drug after degradation	RRT of additional peak formed
0.1N	RT	6 hr	98.76	-
0.1N	70 °C	6 hr	94.48	-
1N	RT	4 hr	83.86	1) 0.72 (DP I)

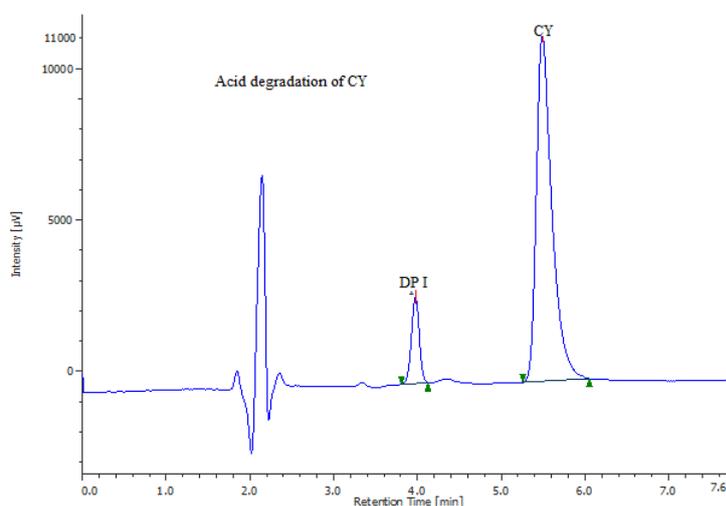


Figure 5.6.1.1: Chromatogram of CY (10 µg/ml) treated with 1N HCl for 4 hr at RT

Peak name	CH	R _t (min)	RRT	Area(µV-sec)	NTP	Symmetry
CY	1	5.45	-	143647	4370	1.58
DP I	1	3.97	0.72	27697	8281	0.96

5.6.1.2. Base degradation

Aliquot volume of 0.1ml of stock solution of drug (1000 µg/ml) was transferred into multiple sets of 10 ml V.F. To one set 1 ml of N/10 NaOH was used and kept at RT. For the other, higher temperature of 70 °C was provided for extended time interval of around 6 hr. Further the study was conducted using 1 ml of 1N NaOH at RT and 70 °C. The samples were cooled and neutralized with 1 ml of HCl of same strength and volume made up to the mark, with diluent. The solutions were then analyzed by the proposed method. Results of hydrolytic study of CY with varied concentration of NaOH at different temperature and time interval is presented in Table 5.6.1.2 and representative chromatogram as Fig 5.6.1.2.

Table 5.6.1.2: Degradation study of CY with NaOH

Concentration of NaOH	Temperature	Time	% of active drug after degradation
0.1N	RT	6 hr	96.45
0.1N	70 °C	6 hr	95.62
1N	RT	6 hr	96.21
1N	70 °C	6 hr	93.07

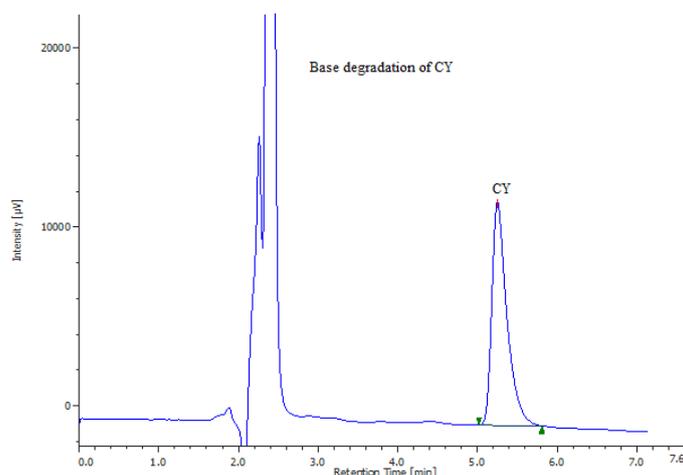


Figure 5.6.1.2: Chromatogram of CY (10 µg/ml) treated with 1N NaOH for 6 hr at 70 °C

Peak name	CH	R _t (min)	Area(µV-sec)	NTP	Symmetry
CY	1	5.38	174013	4138	1.61
Control	1	5.25	186976	4985	1.57

5.6.1.3. Neutral degradation

Aliquots volume 0.1 ml of stock solution of drug (1000 µg/ml) was transferred in two sets of 10 ml V.F. To one of the set 1 ml of distilled water was added and kept at RT. For the second set 1ml of water was added and kept at higher temperature of 70 °C for extended time interval of around 6 hr. The samples were cooled and volume was made up to the mark with mobile phase. The solutions were then analyzed by the proposed method.

Results of hydrolytic study of CY under neutral conditions at different temperature and time interval is presented in Table 5.6.1.3 and representative chromatogram as Fig 5.6.1.3.

Table 5.6.1.3: Degradation study of CY with water

Temperature	Time	% of active drug after degradation
RT	6 hr	98.65
70 °C	6 hr	98.04

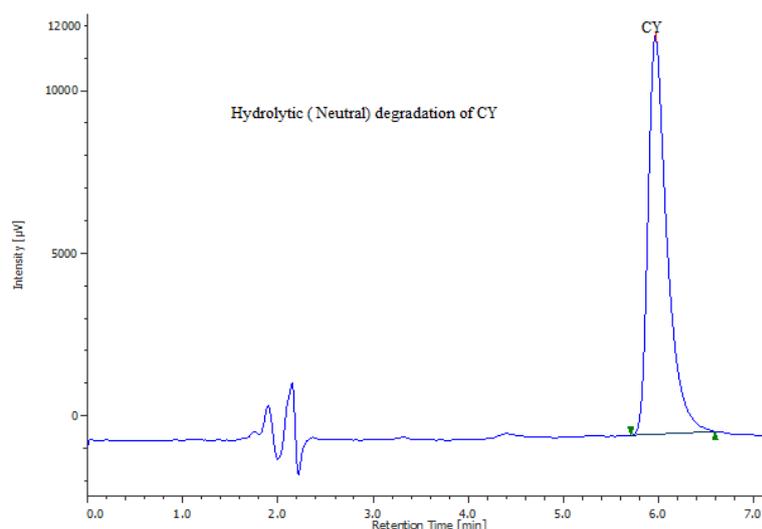


Figure 5.6.1.3: Chromatogram of CY (10 µg/ml) treated with water for 6 hr at 70 °C

Peak name	CH	R _t (min)	Area(µV-sec)	NTP	Symmetry
CY	1	5.96	168947	4890	1.57
Control	1	5.91	172320	4825	1.61

5.6.2. Oxidative degradation

Oxidative degradation was carried out by using hydrogen peroxide. Samples were prepared by using 0.1 ml of stock solution of drug (1000 µg/ml) and 1 ml of hydrogen peroxide (10 % v/v) in 10 ml V.F at RT for specified time interval (7 days) to obtain sufficient degradation. The results obtained from 2nd day of study till 7th day were found to be consistent. Hence after required exposure, samples were diluted up to the mark by mobile phase and subjected for analysis by the proposed method.

Results of oxidative study of CY with 10 % v/v H₂O₂ is presented in Table 5.6.2 and representative chromatogram as Fig 5.6.2.

Table 5.6.2: Degradation study of CY with H₂O₂

Temperature	Time	% of active drug after degradation	RRT of additional peak formed
RT	2 day	53.51	1) 0.54 (DP I) 2) 0.68 (DP II)

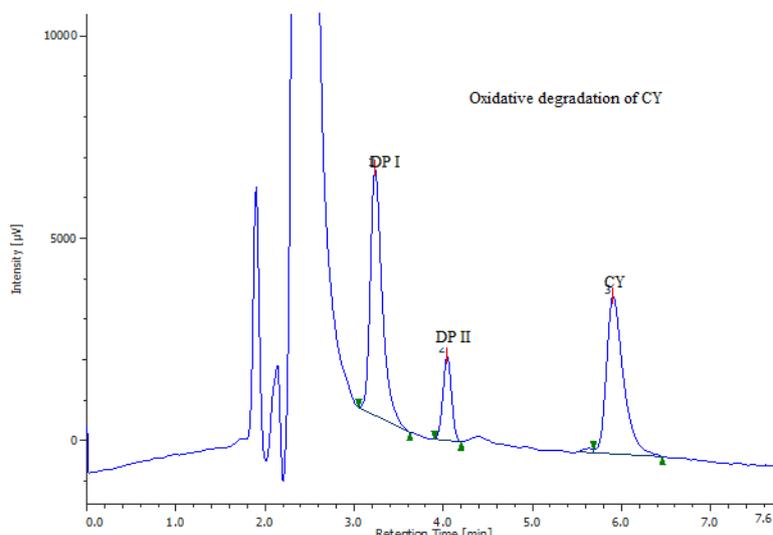


Figure 5.6.2: Chromatogram of CY (10 µg/ml) treated with 10 % H₂O₂ after 2 days at RT

Peak name	CH	R _t (min)	RRT	Area(µV-sec)	NTP	Symmetry
CY	1	5.90	-	51440	5148	1.54
DP I	1	3.23	0.54	56887	3157	1.49
DP II	1	4.04	0.68	14017	8294	1.02

5.6.3. Thermal degradation

Drug sample 10 mg each was taken in two 10 ml V.F and closed with stopper. One flask was exposed to dry heat by heating in hot air oven at specified temperature (80 °C) for specified time interval 4 days and second flask was kept as control. After required exposure, two separate sets of solutions were prepared by weighing appropriate amount of sample exposed to thermal stress and control to produce concentration of 10 µg/ml. The samples were diluted up to the mark with mobile phase and injected separately for analysis by the proposed method.

Results of thermal study of CY in hot air oven (80 °C) are presented in Table 5.6.3 and representative chromatogram as Fig 5.6.3.

Table 5.6.3: Degradation study of CY in hot air oven

State	Temperature	Time	% of active drug after degradation
Solution	70 °C	6 hr	98.04
Solid	80 °C	4 days	98.53

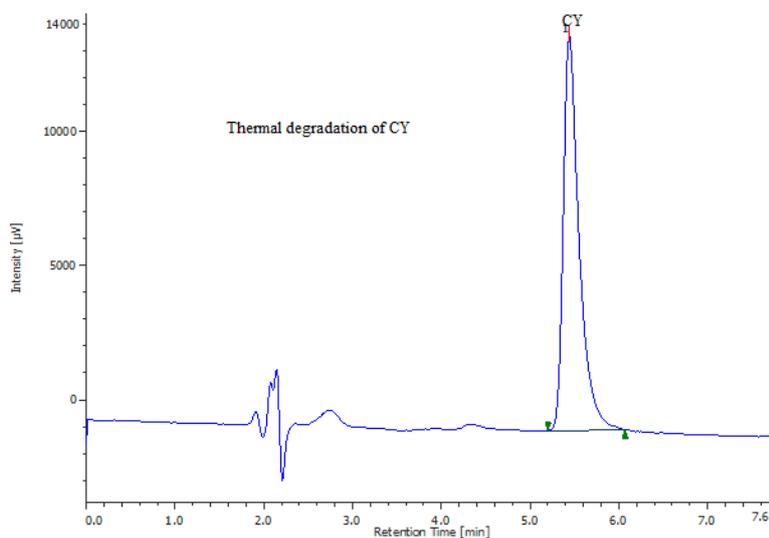


Figure 5.6.3: Chromatogram of CY (10 µg/ml) in oven for 4 days at 80 °C

Peak name	CH	R _t (min)	Area(µV-sec)	NTP	Symmetry
CY	1	5.66	169821	4038	1.94
Control	1	5.73	172840	4162	1.83

5.6.4. Photo degradation

Drug in sufficient amount was taken in 10 ml volumetric flask and exposed to direct sunlight for a specified time period (7 days). Another flask containing drug was kept as control. After required exposure two separate solutions were prepared by weighing appropriate amount of sample one exposed to light and second control to produce concentration of 10 µg/ml each. The samples were diluted up to the mark with mobile phase and injected separately for analysis by the proposed method.

Results of photo light study of CY after direct exposure to sunlight is presented in Table 5.6.4 and representative chromatogram as Fig 5.6.4.

Table 5.6.4: Degradation study of CY with direct exposure to sunlight

Temperature	Time	% of active drug after degradation
Sunlight	7 days	92.93

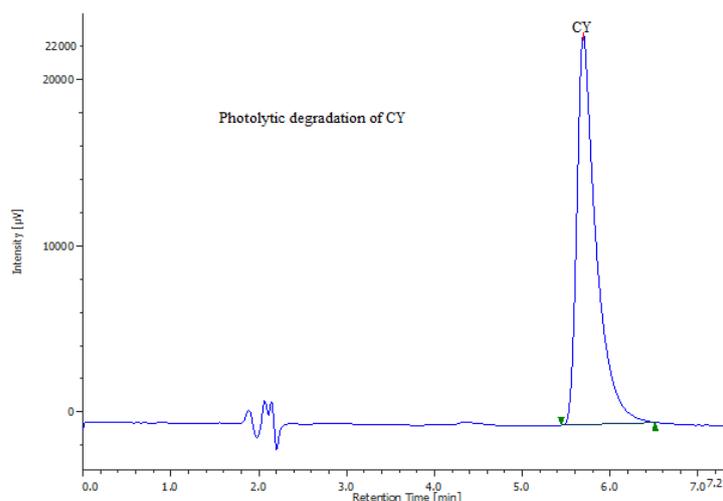


Figure 5.6.4: Chromatogram of CY acid (10 µg/ml) exposed to direct sunlight for 7 days

Peak name	CH	R _t (min)	Area(µV-sec)	NTP	Symmetry
CY	1	5.71	137291	6052	1.87
Control	1	5.51	147750	4001	1.10

5.7. VALIDATION OF DEVELOPED STABILITY INDICATING ANALYTICAL METHOD

Validation is an act of confirming that a method performs its intended application. According to USP SIAM is grouped under category II (Analytical methods for determination of impurities in bulk drugs or for determination of degradation compounds in finished pharmaceutical products). Parameters recognized by ICH for method validation (Table 5.7)

Table 5.7: Validation parameters and acceptance criteria

Sr.No.	Validation Parameters	Acceptance Criteria
1	Specificity	Peak (s) of degradation products and analyte should be pure and well separated from one other.
2	Linearity	Correlation coefficient not less than 0.999
3	Accuracy (across the specified range)	Recovery (%) between 98.0 to 102%
4	Precision	
	4.1) Intra-day precision	RSD (%) of replicate injections not more than 2.0
	4.2) Inter-day precision	
5	Robustness	
6	System Suitability Test	1) Resolution \leq 2.0 2) % RSD of replicate injections \leq 2.0 3) Theoretical plate number \leq 2000 4) Asymmetry of peak should not be more than 2.0

5.7.1. System suitability parameters

Results of the study for system suitability parameters when proposed method was applied for analysis of CY in presence of Imp A, B and DPs (DP I, DP II) is presented in Table 5.7.1. Values obtained meet the acceptance criteria Table 5.7.

Table 5.7.1: System suitability testing parameters of the proposed RP HPLC method

Sr.no	Components	RRT	Resolution	Peak asymmetry	Theoretical plate
1	CY	1.00	NA	1.23	6405
2	Imp A	0.62	3.80	0.99	6303
3	Imp B	0.75	5.91	0.99	7479
4	DP I (acidic condition)	0.72	6.17	1.50	4651
5	DP I (oxidative condition)	0.54	3.97	1.49	3157
6	DP II (oxidative condition)	0.68	5.87	0.96	8281

5.7.2. Specificity and Selectivity

Results of specificity and selectivity study undertaken on CY along with Imp A, Imp B and degradation products I and II are presented in Table 5.7.2 and are in agreement with acceptance criteria defined in Table 5.7.

Table 5.7.2: Selectivity of the HPLC method

Component	Peak (RRT)
CY	1.00
Imp A	0.62
Imp B	0.75
DP _{AcH} I (Acidic condition)	0.72
DP _o I (Oxidative condition)	0.54
DP _o II (Oxidative condition)	0.68

5.7.3. Linearity and Range

Study involving determination of linearity range was undertaken with working/standard solution of CY, Imp A and Imp B diluted to appropriate concentrations as mentioned under 5.11.6.2. Results of the study are tabulated (Table 5.7.3.1) and linearity graphs presented as (Fig 5.7.3.1 to Fig 5.7.3.3).

Table 5.7.3.1: Linearity Range

Compound	Linearity range (µg /ml)	R ²	Reference
CY	16.65-100	0.9997	Fig 5.7.3.1
Imp A	1.87-10	0.9988	Fig 5.7.3.3
Imp B	2.46-10	0.9994	Fig 5.7.3.5

Table 5.7.3.2: Linearity data of CY

Sr.no	Conc. (µg /ml)	Peak area				RSD (%)
		Injection 1	Injection 2	Injection 3	Average of 3 injection	
1	10	158692	162844	163141	161558	1.53
2	20	304610	311834	314489	310311	1.64
3	40	607227	618291	621192	615569	1.19
4	60	952061	961611	969058	960909	0.88
5	80	1275677	1274425	1263735	1275051	0.51
6	100	1586756	1589825	1586744	1587775	0.11

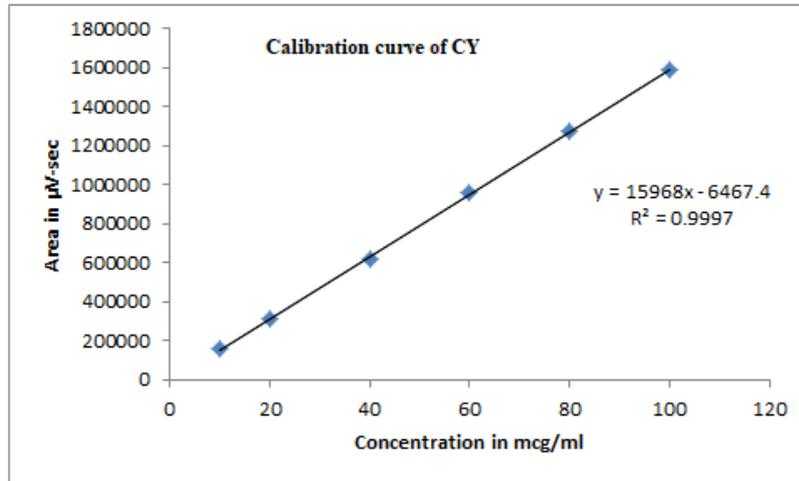
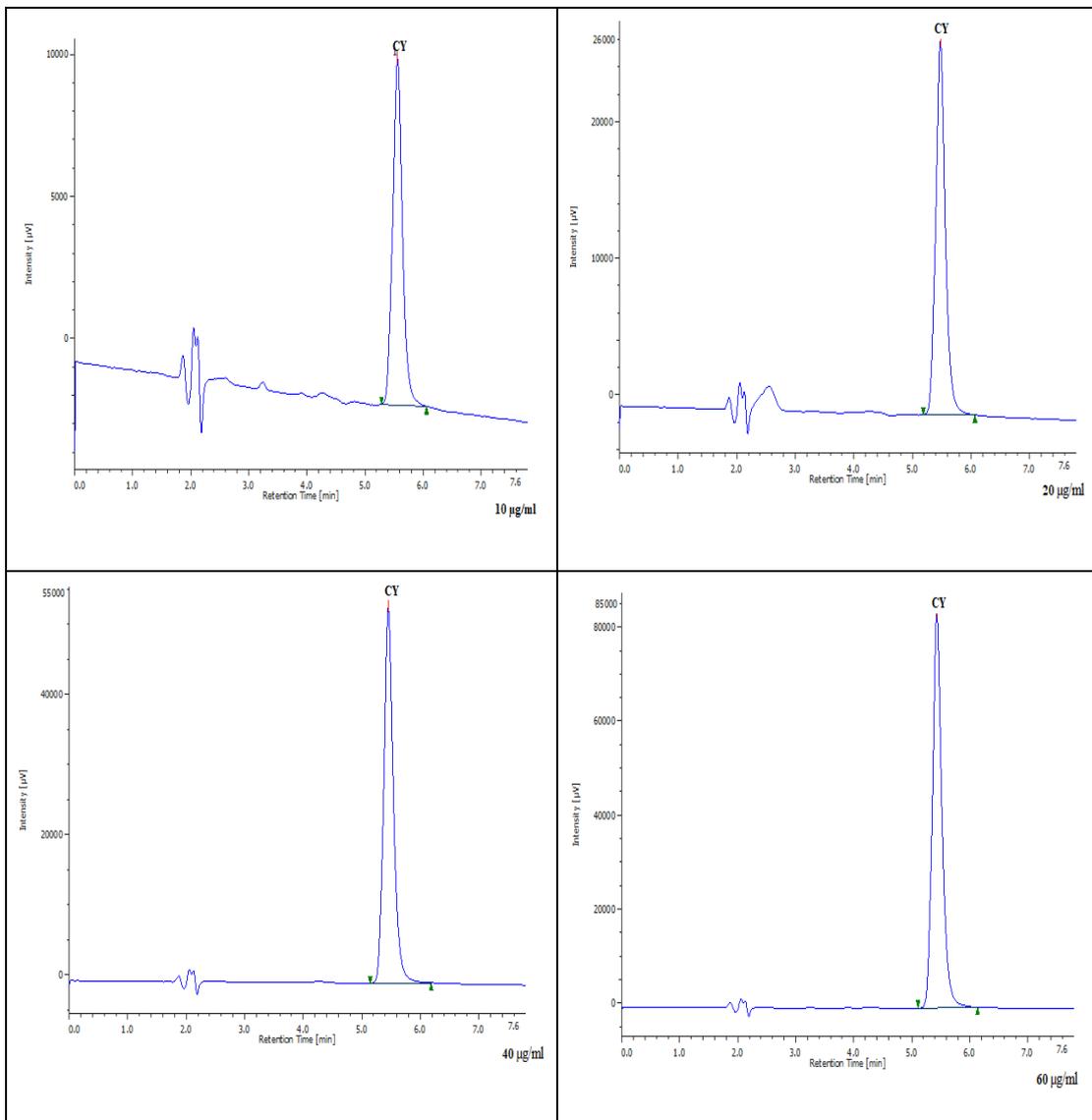


Figure 5.7.3.1: Linearity graph of CY



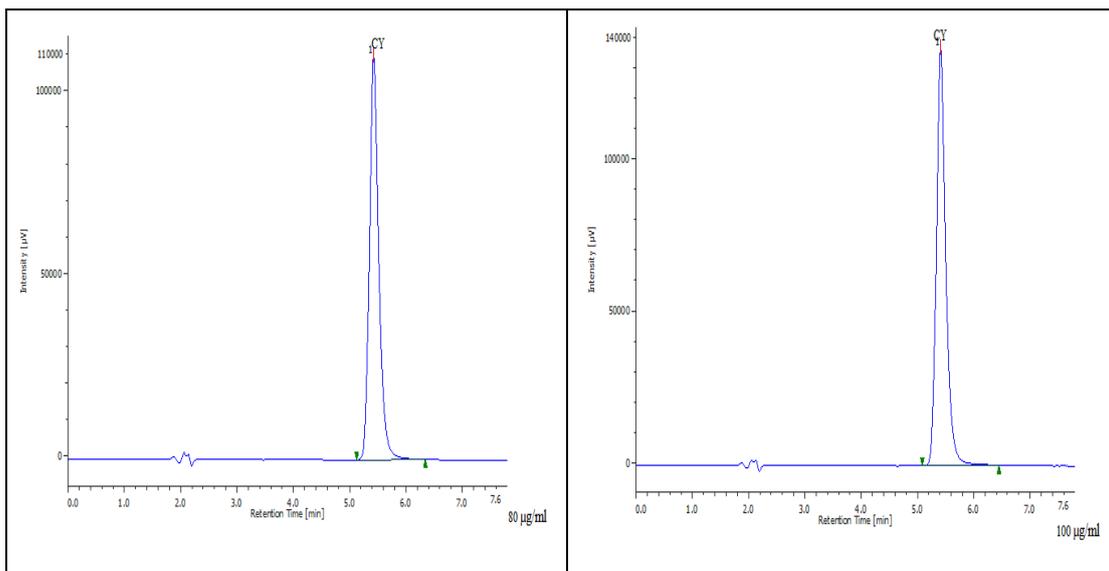


Figure 5.7.3.2: Representative chromatograms of CY (Conc. 10, 20, 40, 60, 80, 100 µg/ml) after first injection

Table 5.7.3.3: Linearity data of Imp A

Sr.No	Conc. (µg/ml)	Peak Area				RSD (%)
		Injection 1	Injection 2	Injection 3	Average of 3 injections	
1	1	112754	112293	112011	112352	0.33
2	2	161798	162639	161870	162102	0.28
3	4	304287	295826	299729	299947	1.41
4	6	436679	433936	437304	435972	0.41
5	8	554895	555764	554234	555329	0.13
6	10	667374	670929	674313	670871	0.51

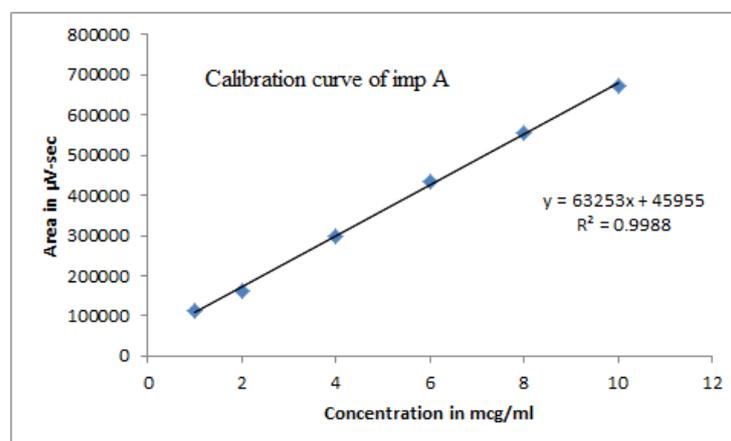


Figure 5.7.3.3: Linearity graph of Imp A

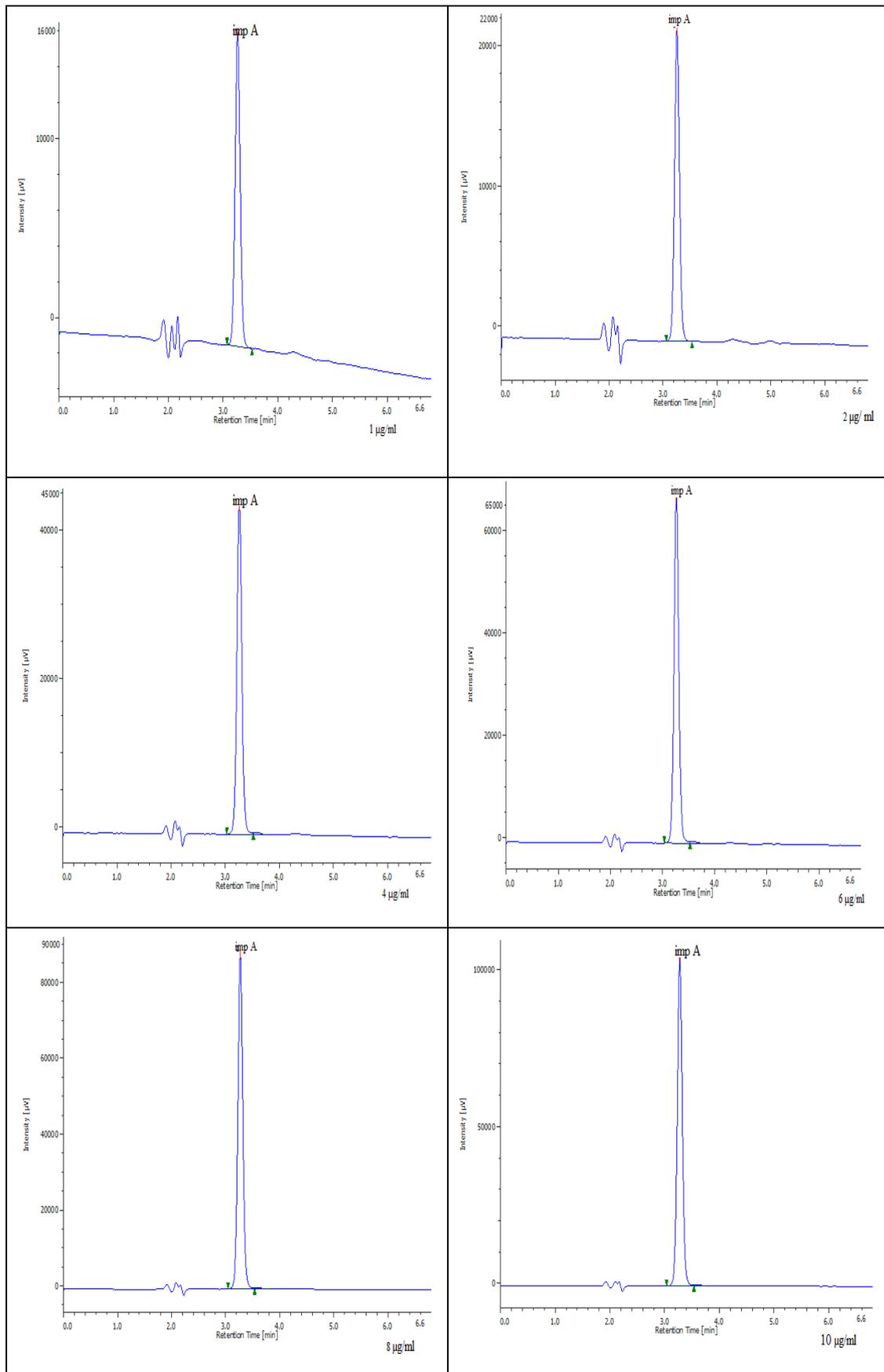


Figure 5.7.3.4: Representative chromatogram of Imp A (Conc., 1, 2, 4, 6, 8, 10 µg/ml) after first injection

Table 5.7.3.4: Linearity data of Imp B

Sr.No	Conc. ($\mu\text{g/ml}$)	Peak area				RSD (%)
		Injection 1	Injection 2	Injection 3	Average of 3 injections	
1	1	37569	37991	38014	37857	0.66
2	2	75343	75002	75293	75212	0.24
3	4	128209	127625	129769	128534	0.86
4	6	188982	189497	188820	189099	0.18
5	8	248531	246358	249465	247444	0.64
6	10	307150	307564	305709	306807	0.31

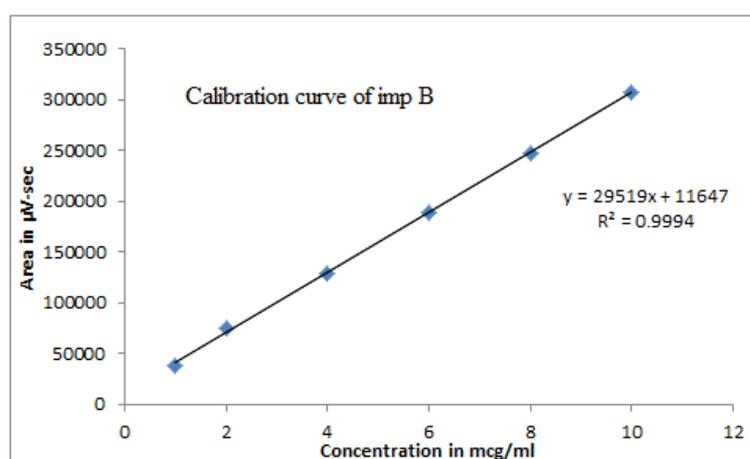
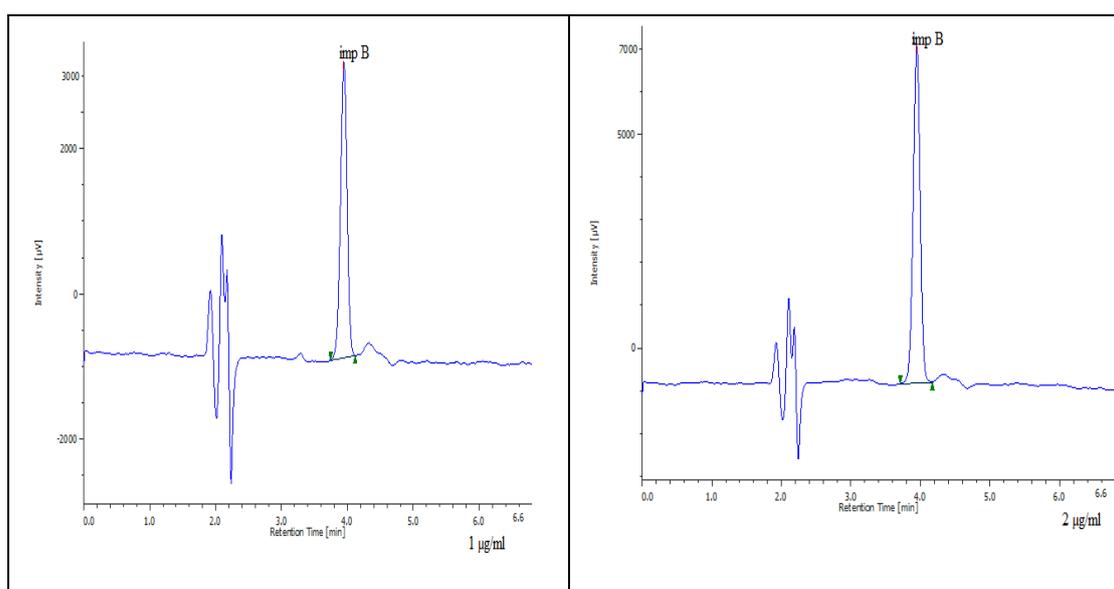


Figure 5.7.3.5: Linearity graph of Imp B



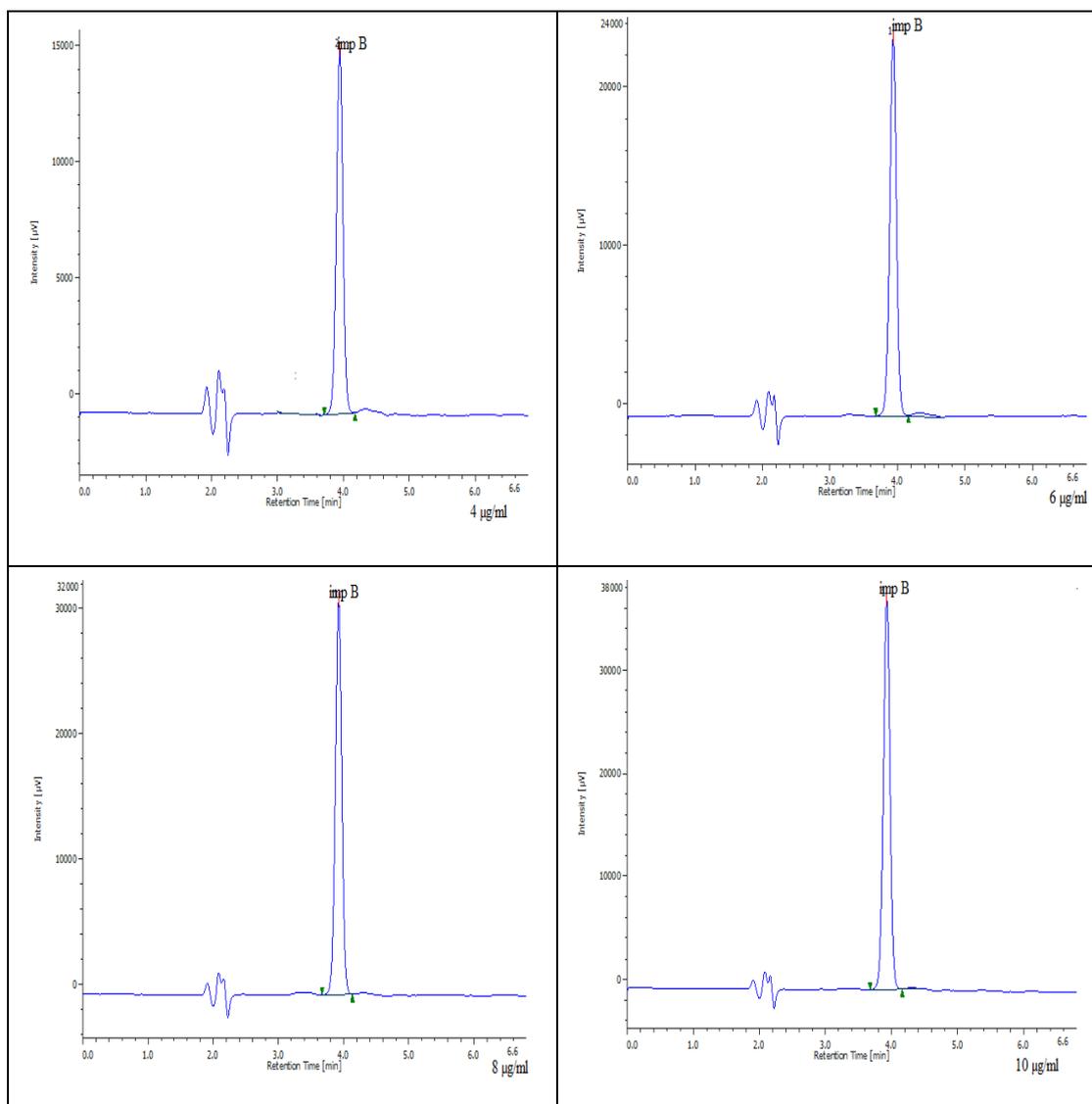


Figure 5.7.3.6: Representative chromatogram of Imp B (Conc, 1, 2, 4, 6, 8, 10 µg/ml) after first injection

5.7.4. Recovery / Accuracy

Accuracy of the proposed method was obtained by recovery studies (**standard addition method**). The percentage recovery studies of CY, Imp A and Imp B was carried out in triplicate at 3 different levels ranging from 80 % -120 %, by spiking standard samples as described under procedure at section 5.10.6.3. Result of recovery study is presented in Table 5.7.4.

Table 5.7.4: Recovery studies (n=3)

Component	Amount of sample (µg/ml)	Average peak area of sample	Level of spiking (%)	Amount of standard spiked (µg/ml)	Average Peak area* after standard addition	Peak area of recovered standard	Amount Recovered (µg/ml)	% Recovery
CY	40	615569	80	32	993531	377962	31.02	98.94
			100	40	1142932	527363	40.17	100.43
			120	48	1331583	716014	50.03	104.24
Imp A	4	268947	80	3.2	467065	198118	3.06	98.70
			100	4	509627	240680	3.88	99.13
			120	4.8	598286	329339	4.98	101.85
Imp B	4	115534	80	3.2	193967	78433	3.03	98.99
			100	4	221335	105801	4.00	100.24
			120	4.8	248017	132483	4.81	101.28

(*Average of three readings)

5.7.5. Precision and Sensitivity

Intraday repeatability of the method was evaluated by analyzing three concentrations of CY (40, 60 and 80 µg/ml), and three concentration of Imp A, and Imp B (4, 6 and 8 µg/ml). Interday precision was evaluated by assaying the chosen concentration of CY, Imp A, and Imp B in triplicates on two successive days using the procedure stated under section 5.10.6.4 and 5.10.6.5. Results of the study are presented in Table 5.7.5.1 and 5.7.5.2.

Table 5.7.5.1: Intra and Interday precision data (n=3)

Compound	Concentration (µg/ml)	Intra-day		Inter-day	
		mean peak area ± SD	% RSD	mean peak area ± SD	% RSD
CY	40	615569 ± 7369	1.19	603521 ± 8246	1.36
	60	960909 ± 8519	0.88	957358 ± 1500	0.15
	80	1275051 ± 6563	0.51	1256415 ± 4999	0.39
Imp A	4	299947 ± 4234	1.41	280486 ± 2435	0.86
	6	435972 ± 1791	0.41	443676 ± 5322	1.19
	8	555329 ± 767	0.13	560100 ± 7624	1.36
Imp B	4	128534 ± 1108	0.86	107707 ± 617	0.57
	6	189099 ± 353	0.18	168413 ± 1065	0.63
	8	247444 ± 1593	0.64	221799 ± 1038	0.46

Table 5.7.5.2: Sensitivity of the method (LOD and LOQ)

Parameters	CY	Imp A	Imp B
LOD($\mu\text{g/ml}$)	5.49	0.56	0.74
LOQ($\mu\text{g/ml}$)	16.65	1.87	2.46

5.7.6. Robustness

Robustness of an analytical method is the measurement of the methods ability to remain unchanged by minute but intended alterations in the methods variables and provide reliability during normal use. The robustness of the method was studied by making deliberate changes in flow rate, and composition of mobile phase ratio. The method was found to be robust as the % RSD of peak area were within acceptance limits.

5.7.6.1. Variation in the flow rate (± 0.2 ml/ min of the optimized flow rate)

The optimized flow rate was for the method was set at 1 ml/min. For robustness study, the flow rate was varied to 0.8 ml/min and 1.2 ml/min. The solutions were then injected into the chromatograph equilibrated with the flow rate of 0.8 ml/min and 1.2 ml/min. The results obtained upon the flow rate variation are displayed in following tables with representative chromatogram.

Table 5.7.6.1: Results of flow rate variation

	CY	Imp A	Imp B
Flow rate	Average area (n=3)	Average area (n=3)	Average area (n=3)
0.8 ml	994374	426789	182217
1 ml	960909	435973	189099
1.2 ml	980672	425946	187951
% RSD	1.71	1.29	1.97

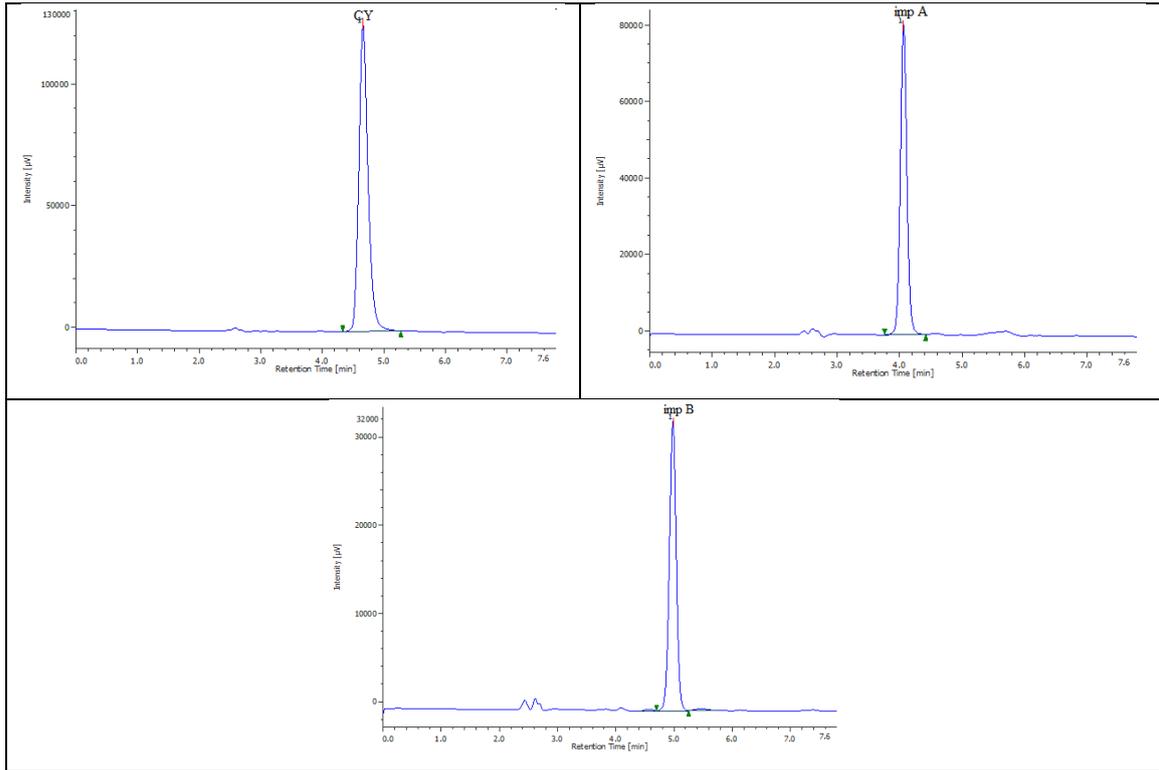


Figure 5.7.6.1.1: Chromatogram with flow rate 0.8 ml/min (optimized 1.0 ml/min)

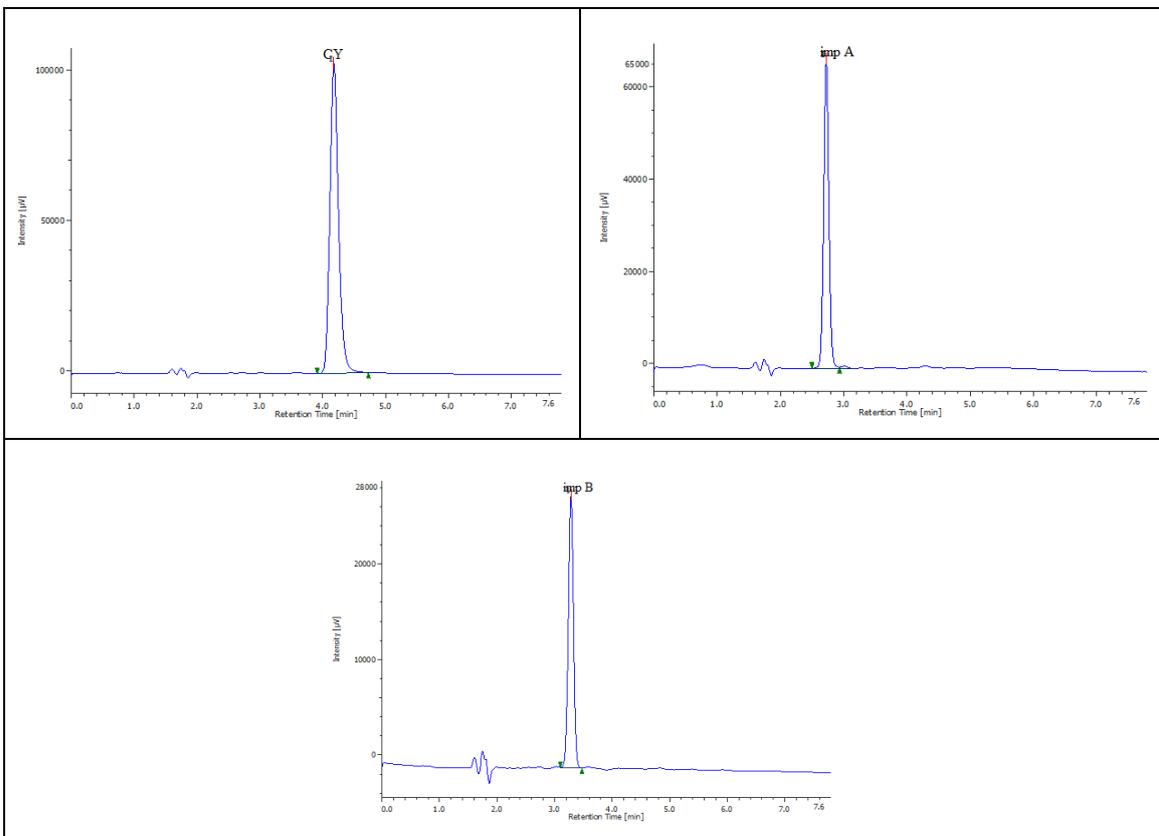


Figure 5.7.6.1.2: Chromatogram with flow rate 1.2 ml/min (optimized 1.0 ml/min)

5.7.6.2. Variation in the mobile phase composition ($\pm 2\%$ of the optimized ratio)

The optimized isocratic elution program was acetonitrile: ammonium dihydrogen Ortho Phosphate buffer (pH adjusted to 6.5 with dilute ammonia solution) in ratio of 80:20 % v/v. For robustness study, organic phase ratio was varied to 78:22 % v/v (- 2 % of the optimized ratio) and 82:18 % v/v (+ 2 % of the optimized ratio). The results obtained for robustness study wherein organic phase ratio of mobile phase was varied is presented in following table with representative chromatograms.

Table 5.7.6.2: Results of mobile phase variation

	CY	Imp A	Imp B
Composition	Average area (n=3)	Average area (n=3)	Average area (n=3)
78:22 % v/v	978942	444749	183264
80:20 % v/v	960909	435973	189099
82:18 % v/v	980844	444336	185603
% RSD	1.13	1.12	1.57

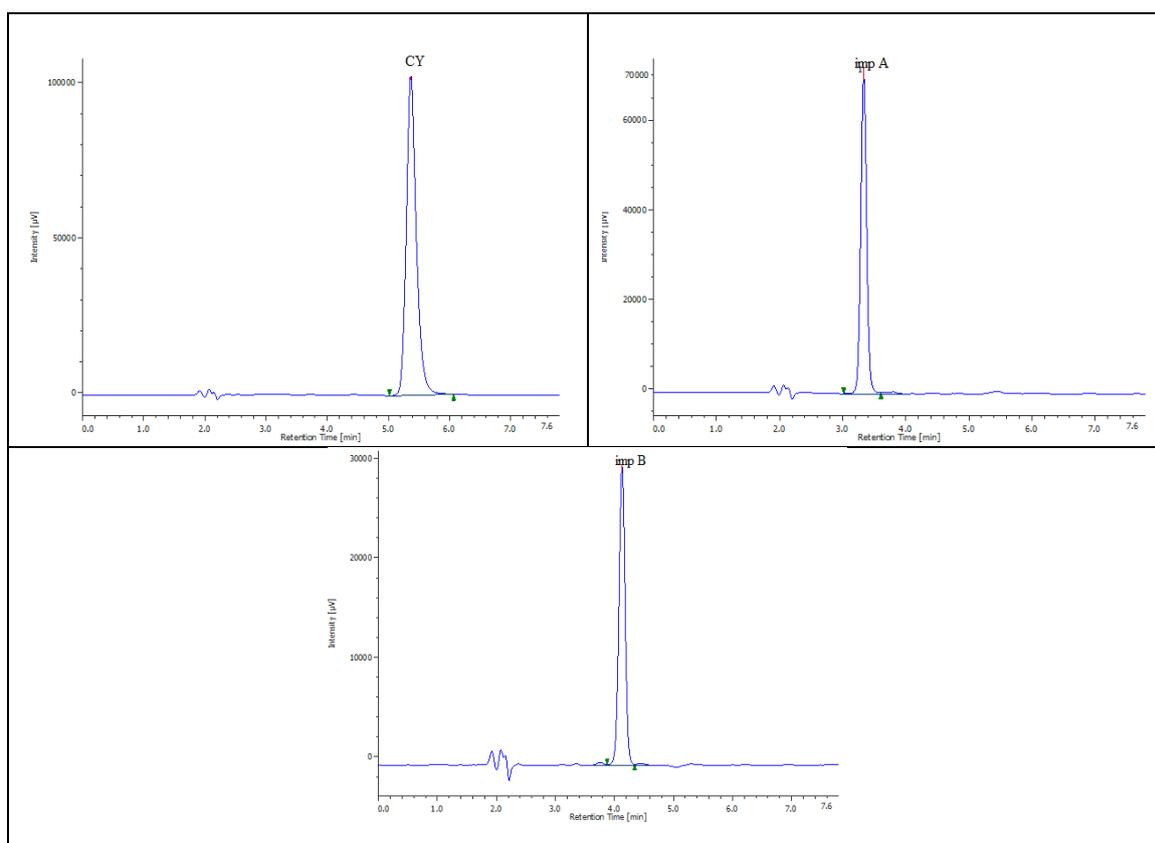


Figure 5.7.6.2.1: Chromatogram with organic phase ratio altered to 78:22 % v/v (optimized 80:20 % v/v)

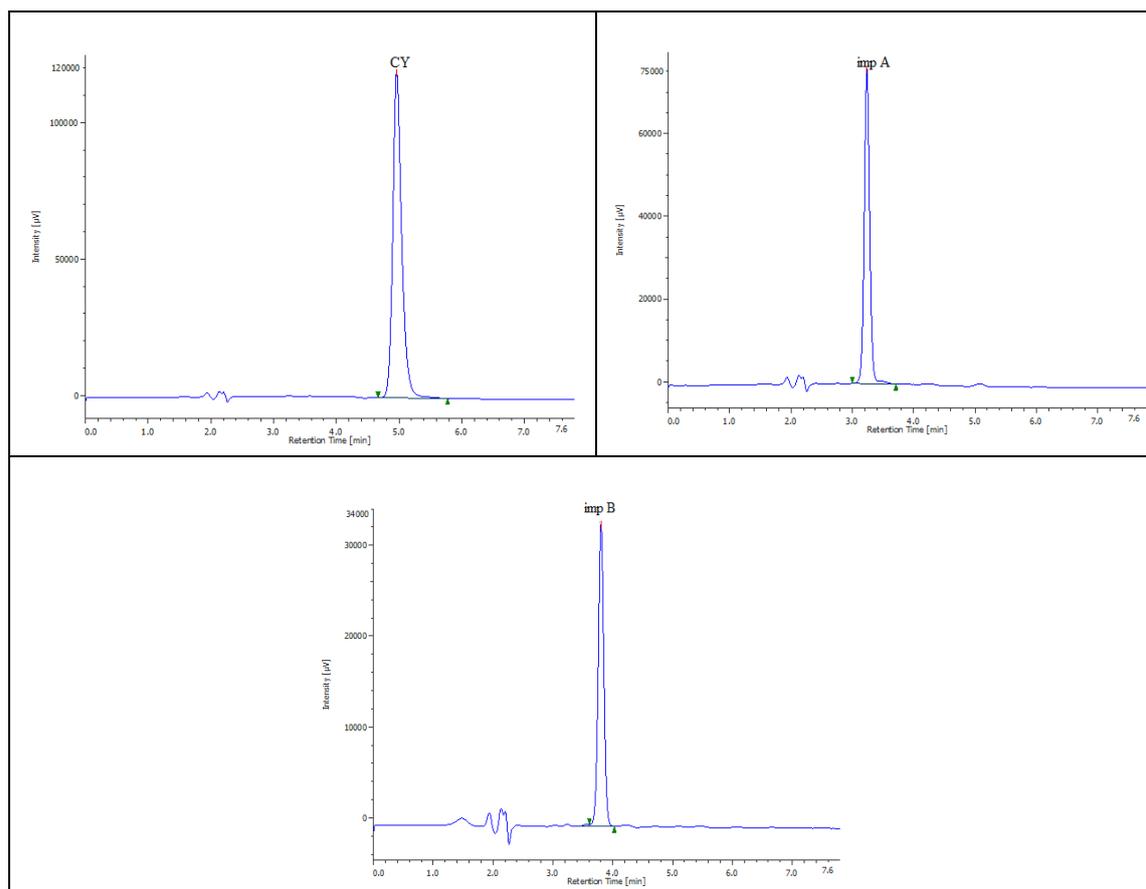


Figure 5.7.6.2.2: Chromatogram with organic phase ratio altered to 82:18 % v/v (optimized 80:20 % v/v)

5.8. ANALYSIS OF MARKETED PRODUCT

The proposed HPLC method was extended for analysis of CY in marketed formulation (Cyclizine HCl 50 mg tablets by AMDIPHARM) as per the procedure under section 5.11.7. The results were found to be satisfactory as observed for calculated purity of 99.18 % which was in accordance to label claim. Results are summarized in Table 5.8. Further no detectable peaks were found at the RRTs of Pharmacopoeial impurities (A and B) and degradation products, indicating their absence in detectable concentrations in the marketed dosage form (Fig 5.8).

Table 5.8: Result of CY in marketed product (Cyclizine HCl 50mg)

Sr. No	Conc. of sample solution ($\mu\text{g/ml}$)	Peak Area	Mean Peak Area	% Assay
1	50	592893	591893	99.18
2		590893		
3		591893		

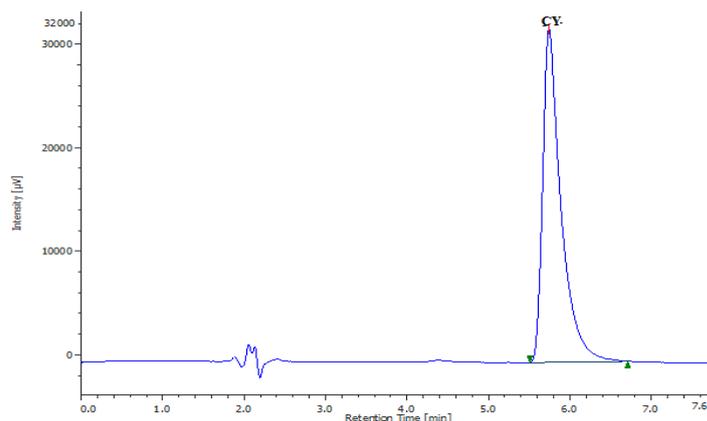


Figure 5.8: Chromatogram of CY sample (Cyclizine HCl 50 mg)

Thus a new RP HPLC validated stability indicating analytical method was developed as an alternative to official method (BP) for the estimation of CY in presence of its pharmacopoeial Imp A, Imp B and degradation products. Method was successfully applied for analysis of CY in marketed dosage form.

5.9. CHARACTERIZATION OF DEGRADATION PRODUCT BY LCMS

The LCMS spectra of CY and its DPs were recorded by using positive mode of electro spray ionization (ESI). The output of mass spectrometer was validated before injecting the stress samples of CY. For LCMS studies the buffer of the developed method was replaced with acetate buffer with pH 6.5 which is compatible with LCMS as compared to phosphate buffer.

Mass chromatograms for the degradants generated under acidic and oxidative stress conditions were recorded in positive ESI mode. It was observed that m/z value for single degradants generated under acid and oxidative stress, which resolves at RRT \sim 0.72 matches with the molecular weight of pharmacopoeial specified impurity B; diphenyl methanol (mol.wt. 184.23). The m/z value for the other degradant which resolves at RRT \sim 0.54 formed only under oxidative stress was unknown impurity, 4-benzhydryl-methylpiperazine 1-oxide (mol.wt. 282.38). The results are depicted in following figures. The mass spectra of degradant generated under acidic hydrolytic stress was depicted as follows (Fig 5.9.1). The molecular weight of degradant (RRT = 0.72) was found to be 184.23. The molecular ion peak of which appears at m/z value of 167.1.

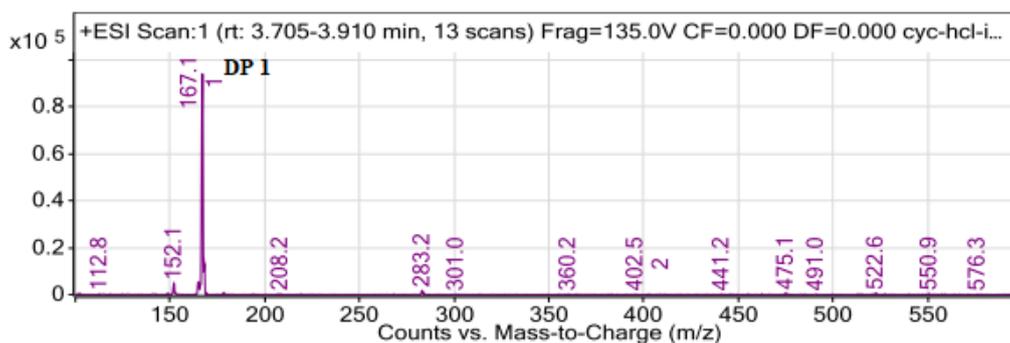


Figure 5.9.1: Mass spectra of degradant under acidic stress condition

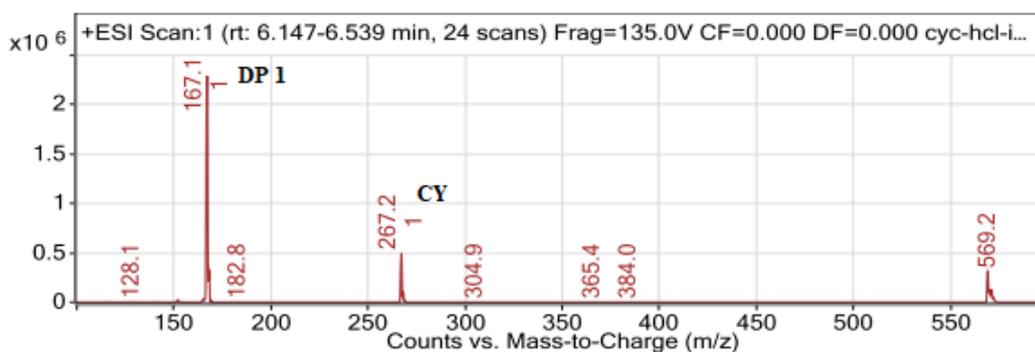


Figure 5.9.2: Mass spectra of CY under acidic stress condition

Similarly Mass spectra of CY in acidic stress condition were generated as shown in Fig 5.9.2. The molecular weight of CY is 266.381, the molecular ion peak of which is seen at m/z value of 267.2. It also seen that at the applied ionization frequency the drug is fragmented to give a molecular ion peak at m/z value 167.1.

The mass spectrum of degradant (RRT = 0.72) generated under oxidative stress is depicted in Fig 5.9.3. The molecular weight of degradant (RRT = 0.72) was found to be 184.23, the molecular ion peak of which appears at m/z value of 167.1. The degradant is the same which is formed under acidic condition. Whereas the molecular weight of degradant (RRT= 0.54) was found to be 282.38, the molecular ion peak of which appears at m/z value of 283.2 as shown in Fig 5.9.4.

Mass spectra of CY in oxidative stress condition were generated as shown in Fig 5.9.5. The molecular weight of CY is 266.381, the molecular ion peak of which is seen at m/z value of 267.2. It also seen that at the applied ionization frequency the drug is fragmented to give a molecular ion peak at m/z value 167.1.

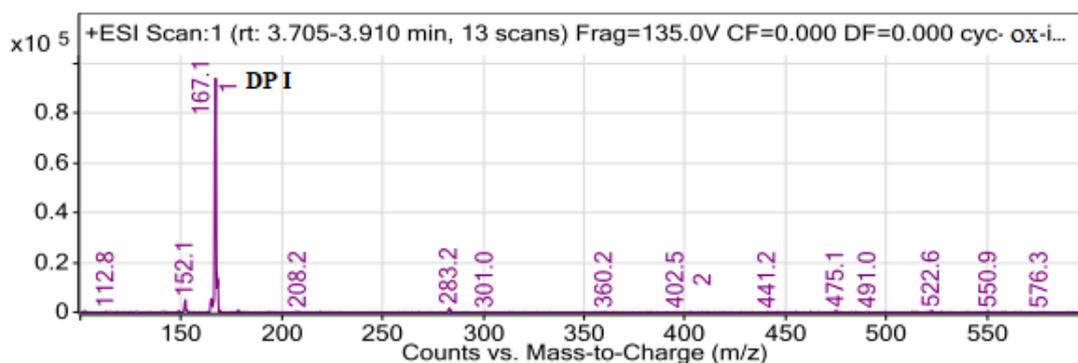


Figure 5.9.3: Mass spectra of degradant (DP I) under oxidative stress condition

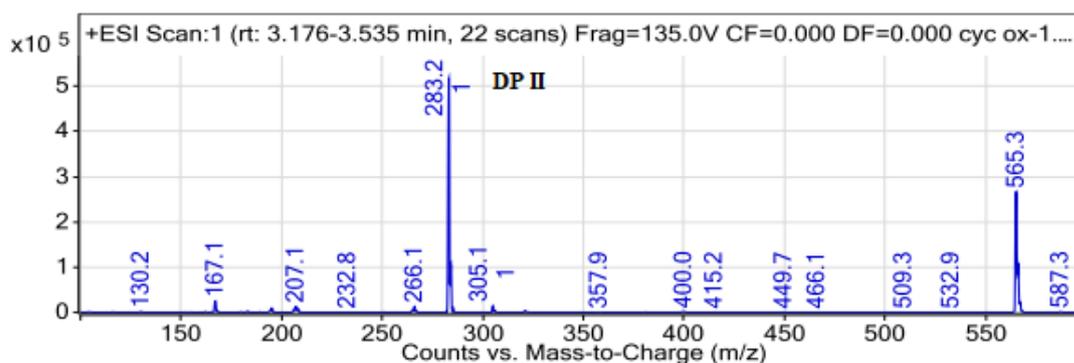


Figure 5.9.4: Mass spectra of degradant (DP II) under oxidative stress condition

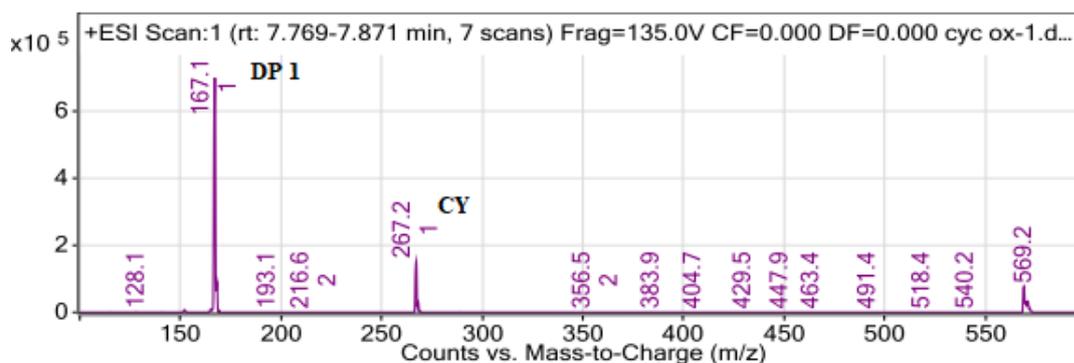
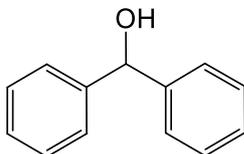


Figure 5.9.5: Mass spectra of CY under oxidative stress condition

Following structures of the two degradants were proposed from the recorded mass spectrometer data. The degradation product (RRT = 0.72) generated under acidic condition and oxidative condition is having molecular weight of 184.23 amu is diphenylmethanol which undergoes dehydration to give a fragment at m/e 167.1. This impurity is known and mentioned in BP as one of the related substance. Other degradation product (RRT= 0.54) which was generated only under peroxide stress is N-oxide of CY. It had molecular mass of 266.38 amu, which was confirmed by analyzing in the ESI mode.

There is need to isolate this degradation product in pure form for further structural elucidation by NMR and mass spectrometry.

Degradation product I (**DP_{AcH} I** and **DP_o II**) – under acidic and oxidative condition

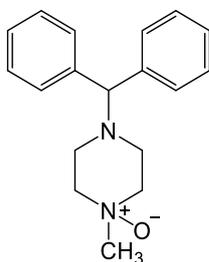


Chemical Name: Diphenylmethanol (benzhydrol)

Molecular Formula: C₁₃H₁₂O

Molecular weight: 184.23 g/mol

Degradation product II (**DP_o I**)- Under oxidative condition



Chemical Name: 4-benzhydryl-1-methylpiperazine 1-oxide

Molecular Formula: C₁₈H₂₂N₂O

Molecular weight: 282.38 g/mol

5.10. DEGRADATION PATHWAY OF CYCLIZINE HCl (CY)

Through stress studies following degradation pathway of CY is predicted.

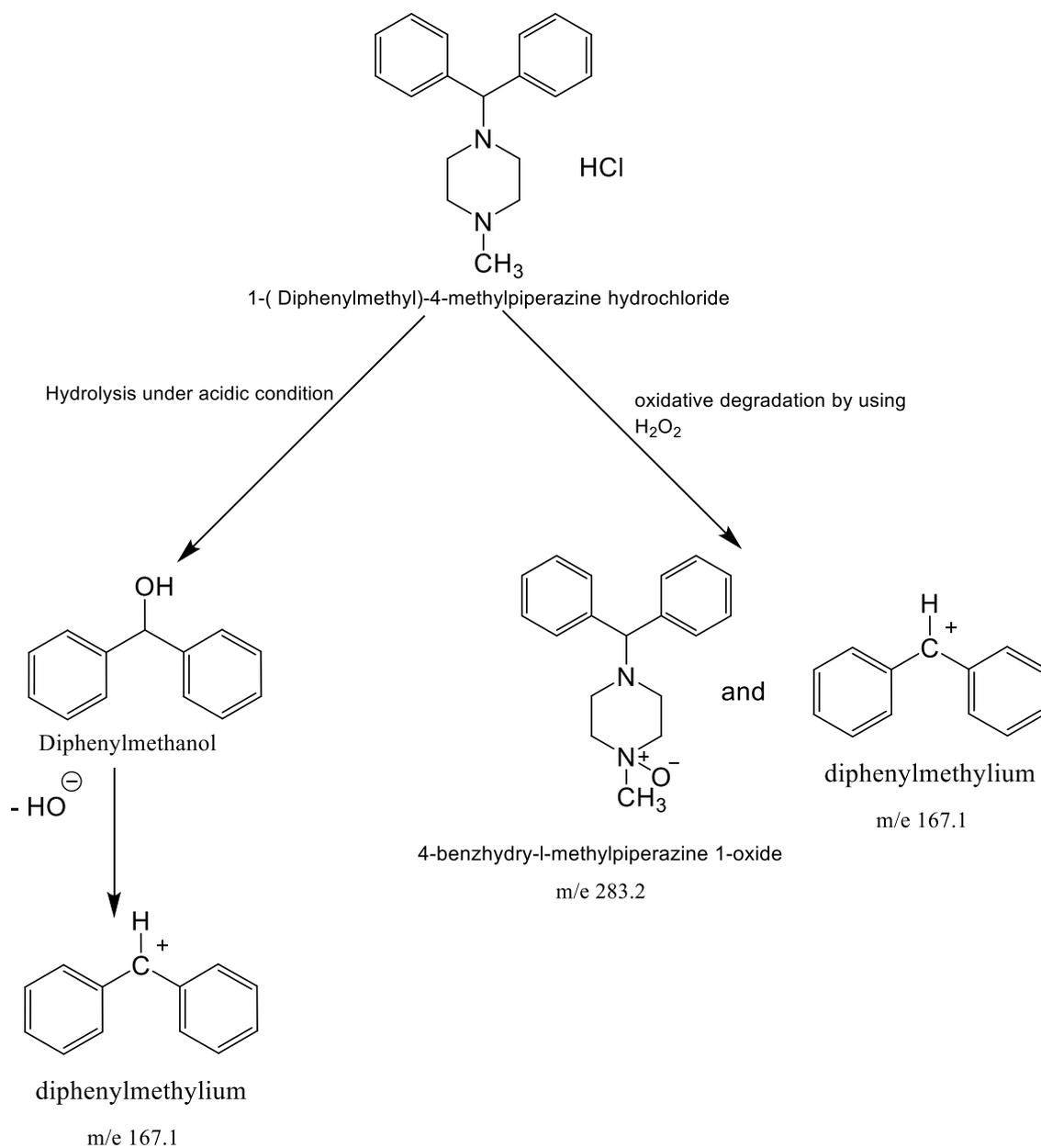


Figure 5.10: Predicted degradation pathway of CY

5.11. METHODOLOGY FOR DETERMINATION OF CYCLIZINE HCl AND ITS IMPURITIES

5.11.1. Instrumentation

Instrument	Source
HPLC System	Jasco LC-4000 series quaternary pump system (PU-4180), an on-line degasser, an auto-sampler (AS-4050), a column temperature controller (CO-4061) and a diode array detector (MD-4010). Software: Jasco Chrom NAV software
Column	Waters -Sunfire ODS C18 (4.6 x 250mm,5 μ m) column.
Sonicator	Citizon Ultrasonic cleaner
pH meter	Digital pH Meter, Labtronics, LT-10
Electronic balance	Wensar Digital Electronic Balance MAB 220
HPLC water purification system	Bio-age Water purification system
Hot air oven	Universal Hot air Owen
Constant temperature water bath	Tempo.

5.11.2. Chemicals and Reagents

- 1) Acetonitrile (HPLC grade), Rankem, India.
- 2) Water (HPLC grade), obtained from Bio age water purification system.
- 3) Ammonium dihydrogen ortho phosphate (AR grade), S.D. Fine-Chem Ltd., Mumbai, India.
- 4) Ammonia solution (25 % v/v) (AR grade), Qualigens fine chemicals, Mahape, India.
- 5) Hydrochloric acid (AR grade), Qualigens fine chemicals, Mahape, India.
- 6) Sodium Hydroxide (AR grade), Qualigens fine chemicals, Mumbai, India.
- 7) Hydrogen peroxide, S.D. Fine-Chem Ltd., Mumbai, India.

5.11.3. Working standard

5.11.3.1. Cyclizine HCl (Sigma laboratories Ltd, Tivim, Goa), 1-methylpiperazine (CY-Imp A), Diphenylmethanol (CY- Imp B) (Sigma Aldrich, USA).

5.11.4. Solution preparation

5.11.4.1. Preparation of stock solution of CY

CY (100 mg) was weighed and transferred into 100 ml V.F., dissolved in 50 ml of acetonitrile and volume made up to the mark to obtain solution concentration 1000 µg/ml.

Further dilutions were made from stock as per the requirements.

5.11.4.2. Preparation of stock solution of Imp B

An amount of 100 mg of CY- Imp B was weighed and transferred into 100 ml V.F. 75 ml of acetonitrile added, mixed well and sonicated for 10 min. Final volume was made up to the mark to obtain concentration of each impurity 1000 µg/ml.

5.11.4.3. Preparation of stock solution of derivatization agent for Imp A

An amount of 100 mg of NBD chloride (4-chloro-7-nitrobenzofuran) was transferred to 100 ml V.F., dissolved and diluted to mark with acetonitrile.

5.11.4.4. Preparation of stock solution of Imp A

An amount of 25 mg of CY-Imp A was weighed and transferred into 50 ml V.F., dissolved and dilute to volume with stock solution of derivatizing agent for Imp A to get a concentration of 1000 µg/ml.

5.11.4.5. Preparation of working standard solution of impurities

1ml of stock solution of each impurity was transferred into two different 10 ml V.F. Acetonitrile was added in the flasks to the mark to get concentration of 100 µg/ml of each impurity.

5.11.4.6. Preparation of buffer – 10 mM ammonium dihydrogen ortho phosphate (pH = 6.5)

Ammonium dihydrogen ortho phosphate (1.15 gm) was dissolved in 1000 ml of HPLC grade water and pH adjuster to 6.5 with dilute ammonia solution. The solution was filtered through 0.45 µ membrane filter.

5.11.4.7. Preparation of 1N sodium hydroxide solution

Sodium hydroxide flakes (4 gm) were dissolved in 50 ml of water in 100 ml V.F and volume made up to the mark with water to get 1N sodium hydroxide solution. Lower concentration strength was prepared after proper dilution.

5.11.4.8. Preparation of 1N hydrochloric acid solution

Hydrochloric acid (1N) was prepared by diluting 8.5 ml of Conc. HCl to 100 ml with water in V.F. Lower concentration strength was prepared after proper dilution.

5.11.4.9. Preparation of 10 % v/v hydrogen peroxide solution

Hydrogen peroxide (10 % v/v) was prepared by appropriately diluting 30 % v/v H₂O₂ to 100 ml with water in V.F.

5.11.4.10. Diluent

Mixture of ACN and ammonium dihydrogen orthophosphate buffer (pH 6.5) in the ratio 80:20 % v/v.

5.11.5. Stress studies

The forced degradation studies were carried out with objectives to achieve substantial degradation of the drug. The drug was exposed to hydrolytic, oxidative, thermal and photolytic stress. The results were obtained by comparing four samples which were generated for every stress condition except thermal and photolytic stress viz., the blank stored under normal condition (blank untreated), the blank subjected to stress in same manner as that of drug solution (blank treated), zero time sample containing the drug (standard untreated) and the drug solution subjected to stress treatment. For thermal and photolytic stress conditions, only two samples were generated one sample exposed to stress condition and other is control.

5.11.5.1. Hydrolytic degradation

The hydrolytic degradations were carried out in acidic, alkaline and neutral conditions. Samples were prepared by taking 0.1 ml of stock solution of drug (1000 µg/ml) and 1ml of hydrolytic agent (HCl / NaOH / water) in 10 ml volumetric flask.

Initially 0.1N strength of HCl and NaOH was used for study followed by 1N strength, if no degradation was seen in 0.1N strength. The study was carried out at room temperature. If required, the samples were heated on constant temperature water bath at 70 °C for specified time intervals. After required exposure samples were neutralized by using equal strength of acid or alkali which ever was required. Finally volume was made up to the mark by using diluent and subjected for HPLC analysis by proposed method.

5.11.5.2. Oxidative degradation

Oxidative degradation was carried out using H₂O₂. Samples were prepared by using 0.1 ml of stock solution of drug (1000 µg/ml) and 1 ml of hydrogen peroxide (10 %) in 10 ml V.F at RT for varied time intervals (7 days). Further the samples were processed by diluting up to the mark using diluent and subjected for HPLC analysis by proposed method.

5.11.5.3 Thermal degradation

Drug sample 10 mg each was taken in two 10 ml V.F and stoppered. One flask was exposed to dry heat in hot air oven for specified temperature (80 °C) and time interval (4 days) and another was kept as control. After exposure two separate solutions were prepared by weighing appropriate amount of sample exposed to thermal stress and control to produce concentration of 10 µg/ml. The samples were diluted up to the mark with the help of diluent and subjected to analysis by proposed HPLC method.

5.11.5.4. Photo degradation

Drug in sufficient amount was taken in 10 ml V.F and was exposed to direct sunlight for a specified time period (7 days). Another flask containing drug was kept as control. After required exposure two separate solutions were prepared by weighing appropriate amount of sample exposed to stress and control to produce concentration of 10 µg/ml. The samples were diluted up to the mark with the help of diluent and injected and analyzed by proposed HPLC method.

5.11.6. Validation studies

Validation of optimized stability indicating method was carried out with respect to parameters recommended under ICH guideline Q2 (R1).

5.11.6.1. Specificity and Selectivity

Establishment of resolution factor between the drug peak, pharmacopoeial impurity peaks and other obtained degradation peak was explored to determine the specificity of the method. Method Specificity was evaluated by comparing relative retention time (RRT) of CY, Imp A, Imp B and stressed samples of CY on the developed method. Chromatograms were recorded and RRT were calculated.

5.11.6.2. Linearity and Range

To establish linearity and range, stock solution of drug (1000 µg/ml) was further diluted with the help of diluent to get the drug concentration range of 10-100 µg/ml. The samples were analyzed in triplicate. For impurities, each impurity was diluted from standard stock solutions to get a concentration range of 1-10 µg/ml. The samples were analyzed in triplicate.

5.11.6.3. Accuracy

Accuracy of the proposed method was estimated by recovery studies using standard addition method. The % recovery of CY, CY-Imp A, and CY-Imp B was carried out in triplicate at 3 different levels of 80 %, 100 % and 120 % by spiking standard samples. Standard drug solutions of concentration 32 µg/ml (80 %), 40 µg/ml (100 %) and 48 µg/ml (120 %) were prepared by transferring 0.32 ml, 0.4 ml and 0.48 ml respectively from stock solution of drug into a series of 10 ml V.F each containing 0.4 ml of sample stock solution. The volume up to the mark was made with diluent. The solutions prepared were injected (n=3) into the chromatograph and the concentration were determined from the peak areas obtained from the chromatograms. Similarly study was carried out for individual impurity by preparing solutions of concentration 0.32 µg/ml (80 %), 0.4 µg/ml (100 %) and 0.48 µg/ml (120 %), from respective working standard of each impurity.

5.11.6.4. Precision

Intraday repeatability of the method was evaluated by analyzing three concentrations of CY (40, 60 and 80 µg/ml), and three concentration of CY-Imp A, and CY-Imp B (4, 6 and 8 µg/ml). Interday precision was evaluated by assaying the chosen sets of CY, CY-Imp A and CY-Imp B in triplicate on two successive days using the same procedure stated under chromatographic conditions. % RSD was calculated.

5.11.6.5. Sensitivity

The sensitivity of the developed method was determined by calculating LOD and LOQ of CY and its Pharmacopoeial impurities. LOD and LOQ were calculated based on the Standard deviation of the Response and the Slope as mentioned below.

$$\text{LOD} = 3.3 \times \text{Standard deviation of the response} / \text{Slope of the calibration curve}$$

$$\text{LOQ} = 10 \times \text{Standard deviation of the response} / \text{Slope of the calibration curve}$$

5.11.6.6. Robustness

Robustness of the developed method was determined by making three injections of mixture containing CY and Imp A and B by making deliberate change in method parameters like flow rate, and composition of mobile phase ratio. The values of percent relative standard deviation (% RSD) of peak area was recorded.

Required aliquots were taken from standard stock solution of CY and impurities in 10ml V.F. Volume was made up to the mark using diluent to get concentration of solution containing 60 µg/ml of CY and 6 µg/ml of impurities. To study the effect of variation in flow rate on the method, ± 0.2 ml of the optimized flow rate (1 ml) was studied on the method. The prepared solution was analyzed in triplicate by the proposed method by altering the flow rate of the proposed method to 0.8 ml (- 0.2 ml) and 1.2 ml (+ 0.2 ml).

To analyze the effect of change in composition of mobile phase ratio on the proposed method, the composition of organic phase in the mobile phase was altered by ± 2 % of the optimized ratio (50:50 % v/v). Hence the mobile phase used for study was ACN: buffer at 48:52 % v/v (- 2 %) and 52:48 % v/v (+ 2 %). The prepared solution was analyzed in triplicate by the proposed method, carrying out the necessary changes in the mobile phase composition.

5.11.7. Analysis of marketed product

For analysis of tablets, 10 tablets were weighed individually and their average weight determined. Tablets were then crushed to fine powder and powder equivalent to 50 mg was transferred to 50 ml V.F and dissolved in 40 ml acetonitrile with vigorous shaking for 15 minutes. The solution was sonicated for 10 min. Finally the volume up to the mark was made with acetonitrile. The solution was then filtered through Whatman filter paper (#1). 5 ml of the filtered solution was transferred into 100 ml V.F and the volume up to the mark was made with diluent to obtain a concentration of 50 µg/ ml. The solution prepared were injected (n=3) into the chromatograph to determine the peak area. From the peak area of the standard solution and the tablet sample solution, the percentage content of CY in the marketed tablets was calculated.

CHAPTER – 6

**HPLC METHOD DEVELOPMENT FOR
MULTICOMPONENT MIXTURE WITH
VARIABLE ACID DISSOCIATION
CONSTANTS.**

**CASE STUDY- TOLFENAMIC ACID
DETERMINATION IN PRESENCE OF IMP A
AND IMP B**

6. HPLC METHOD DEVELOPMENT FOR MULTICOMPONENT MIXTURE WITH VARIABLE ACID DISSOCIATION CONSTANTS.

CASE STUDY- TOLFENAMIC ACID DETERMINATION IN PRESENCE OF IMP A AND IMP B

6.1. INTRODUCTION

A RP HPLC method is developed for estimation of Tolfenamic acid (TA) in presence of its pharmacopoeial impurities, and to verify the developed method's stability indicating power by analyzing the stressed samples of TA.

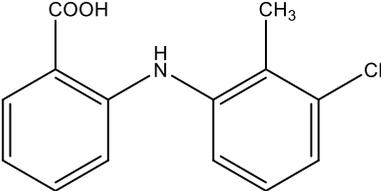
The following objectives were set to carry out the research activity:

- 1) To identify the pharmacopoeial impurities which are processes related for TA API.
- 2) To develop and optimize a RP HPLC method for estimating TA as API in presence of their pharmacopoeial impurities.
- 3) Stress induced studies for drug in accordance to ICH guidelines.
- 4) To validate the optimized method for detection and estimation of TA in presence of its pharmacopoeial impurities and degradation products.

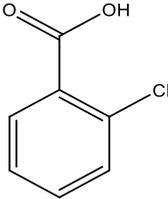
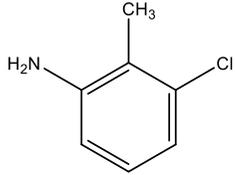
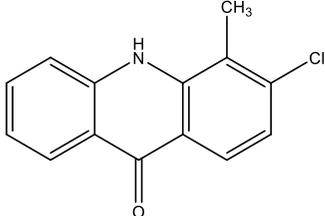
6.2. PROFILE

6.2.1. Drug profile

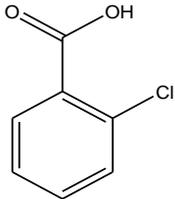
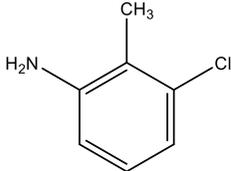
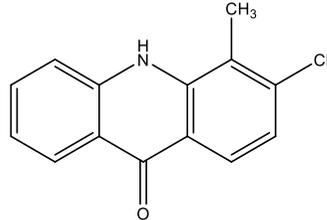
Tolfenamic acid is official in, BP⁴⁵, and EP⁴⁷

General Name	Tolfenamic acid
Chemical Structure	
Chemical Name	2-[(3-Chloro-2-methylphenyl)amino]benzoic acid
Molecular Formula	C ₁₄ H ₁₂ ClNO ₂
Molecular Weight	261.7 g/mol
Melting Point	About 213 °C
Description	White or slight yellow, crystalline powder,
Solubility	Practically insoluble in water, soluble in dimethylformamide, sparingly soluble in ethanol and in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.
pKa	5.11
Drug Category	Cyclooxygenase inhibitor; analgesic; anti-inflammatory

<p style="text-align: center;">Clinical Pharmacology</p>	<p>TA inhibits the biosynthesis of prostaglandins, also have inhibitory actions on the prostaglandin receptors. The mechanism of action of TA consists of the inhibition of COX-1 and COX-2 pathways to inhibit prostaglandin secretion, to exert its anti-inflammatory and pain-blocking action. Nonetheless, some literature currently indicates that TA inhibits leukotriene B4 chemotaxis of human polymorphonuclear leukocytes leading to an inhibition of even 25 % of the chemotactic response.</p> <p>This activity is a not ligand specific additional anti-inflammatory mechanism of Tolfenamic acid¹²¹.</p>
<p style="text-align: center;">Pharmacokinetics</p>	<p>TA is readily absorbed from the gastrointestinal tract and peak plasma concentrations occur about 60 to 90 minutes after an oral dose. TA is about 99 % bound to plasma proteins. The plasma half-life is about 2 hours. TA is metabolised in the liver; the metabolites and unchanged drug are conjugated with glucuronic add.</p> <p>About 90 % of an ingested dose is excreted in the urine and the remainder in the faeces. TA is distributed into breast milk¹²².</p>
<p style="text-align: center;">Toxicity</p>	<p>TA has a relatively low acute toxicity with LD 50 values in 200-1000 mg/kg. The metabolites of TA are reported to have an even less important toxicity. Some of the expected toxicity is related to the presence of gastrointestinal effects such as gut ulceration and renal papillitis¹²¹.</p>

Impurities A, B, C	A	 2-Chlorobenzoic acid
	B	 3-Chloro-2-methylaniline
	C	 3-Chloro-4-methyl-9-oxo-9,10-dihydroacridine

6.2.2. Impurity Profile

Impurity	A	B	C
Chemical Structure			
Chemical Name	2-Chlorobenzoic acid	3-Chloro-2-methylaniline	3-Chloro-4-methyl-9-oxo-9,10-dihydroacridine
Molecular Formula	C ₈ H ₁₁ N	C ₇ H ₈ ClN	C ₁₄ H ₁₀ ClNO
Molecular Weight	121.18 g/mol	141.6 g/mol	243.69 g/mol
Boiling point/ Melting Point	221.5 °C at 760 mm Hg	245.0 °C at 760 mm Hg	-
pKa	2.89	3.47	15.97 & -7.59
Description	White to off white Powder or powder with chunk(s)	Clear yellow to red or red- brown	NA
Solubility	Soluble in water, acetone benzene and methanol, freely soluble in alcohol, ether	Soluble in hot water, alcohol, insoluble in ether and benzene	NA
Toxicity	This compound may be harmful	Absorption into the body leads	NA

	<p>by inhalation, ingestion or skin absorption. It is irritating to eyes, skin, mucous membranes and the upper respiratory tract. When heated to decomposition this compound emits toxic fumes. No components of this product present at levels greater than or equal to 0.1 % is identified as probable, possible or confirmed human carcinogen by IARC.</p>	<p>to the formation of methemoglobin which in sufficient concentration causes cyanosis. Onset may be delayed 2 to 4 hours or longer. To the best of knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated¹²³⁻¹²⁵.</p>	
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6.3. LITERATURE SURVEY

Study on TA by various analytical methods has been reported in the literature. Few of them include spectrophotometry¹²⁶, spectrofluorimetry¹²⁷ and reversed phase high-performance liquid chromatography (RP-HPLC) methods individually and in combination¹²⁸⁻¹³⁶. A literature highlighted the separation of two pharmacopoeial impurities of TA i.e. Imp A; 2-Chlorobenzoic acid (CBA) and Imp B; 3-Chloro- 2-methylaniline (CMA) by HPLC and TLC densitometric method¹³⁶ without commenting on the stability indicating potential of the method. Whereas minimum literature till date is available where in stability indicating method for TA by RP HPLC in presence of its pharmacopoeial impurities has been reported. Following table summarizes few chromatographic conditions used in HPLC methods reported in literature.

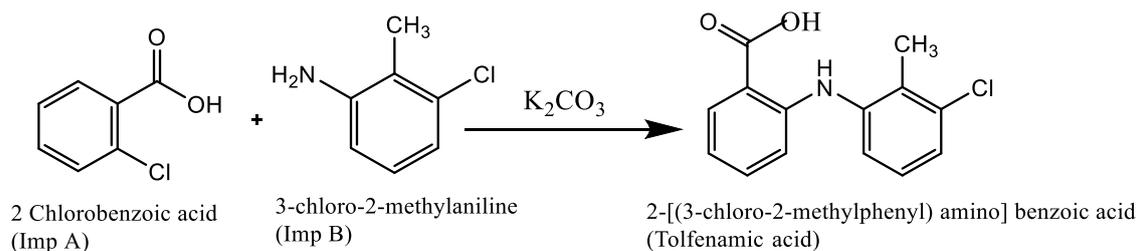
Table 6.3: Summary of reported chromatographic conditions used for determination of TA by HPLC

Sr.No.	Column Type	Mobile Phase Composition	Flow Rate	Detector Used	R _t of drug	References
1	BP method					
	C18 (5 μ m)	Glacial acetic acid: water: ethanol (2:350:650 % v/v/v)	0.8 ml/min	UV fixed wavelength (232 nm)	15 min	45
2	Determination of Tolfenamic acid in human plasma by HPLC					
	C18 (5 μ m)	Acetonitrile:10 mM phosphoric acid (60:40 % v/v) pH 2.6	1.1 ml/min	UV fixed wavelength (280 nm)	11.5 min	132
3	Rapid assay for the determination of Tolfenamic acid in pharmaceutical preparations and biological fluids by High performance liquid chromatography					
	C18 (10 μ m)	Acetate buffer (pH 4.6 and constant ionic strength 0.05): methanol(18:82 % v/v)	1.9 ml /min	UV fixed wavelength (282 nm)	2.62 min	133
4	Validated chromatographic methods for simultaneous determination of Tolfenamic acid and its major impurities					
	C18 (5 μ m)	0.05 M KH ₂ PO ₄ buffer (pH 3):acetonitrile (45:55 % v/v)	1 ml/min	UV fixed wavelength (230 nm)	5.8 min	136

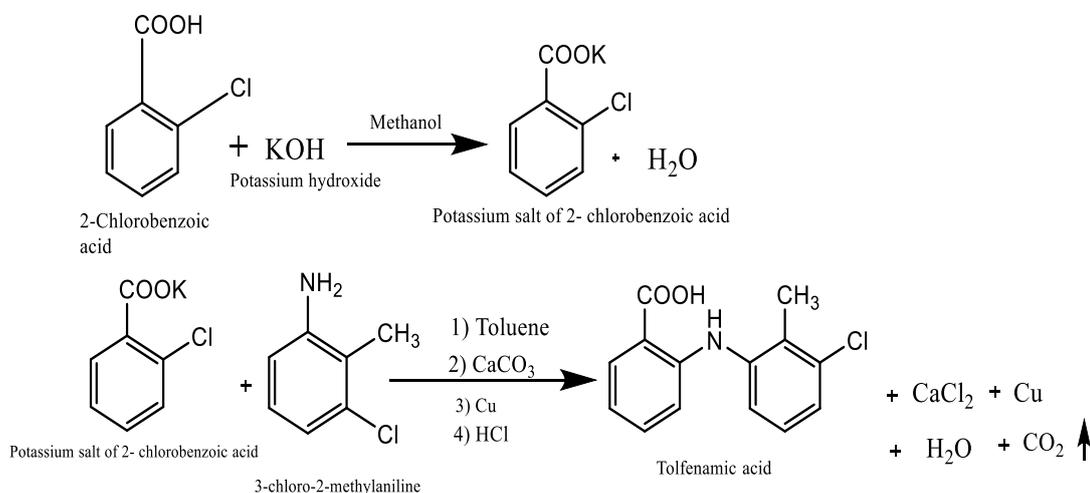
6.4. LOCATING SOURCE OF IMPURITIES IN TOLFENAMIC ACID

Synthetic schemes of Tolfenamic acid from the available literature were collected for identifying the possible impurities which can remain with the final product of TA API during its chemical synthesis.

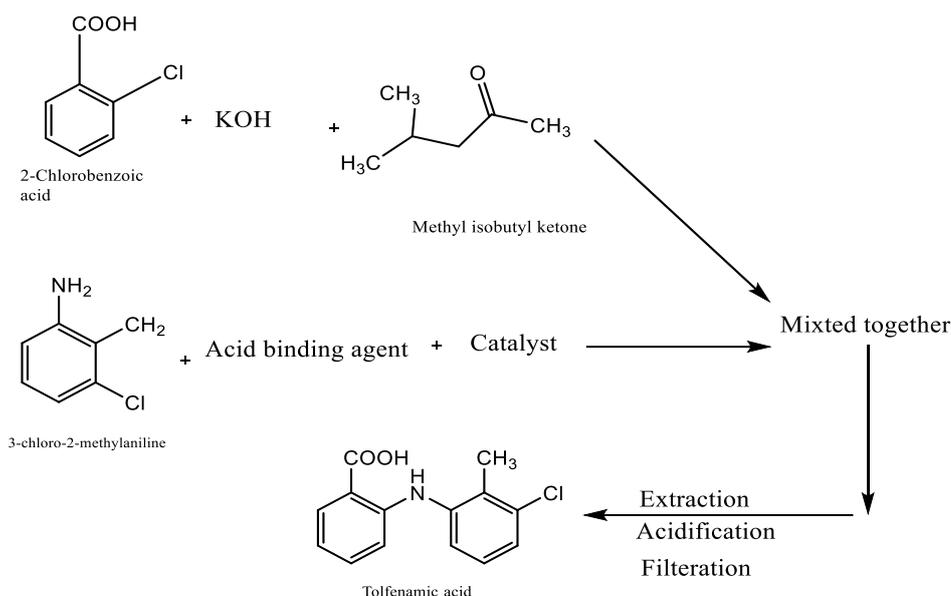
A) A general method involved reaction of 2-Chlorobenzoic acid and 3-Chloro-2-methylaniline in presence of potassium carbonate to give Tolfenamic acid¹³⁷.



B) In another method 2-Chlorobenzoic acid is reacted with potassium hydroxide in presence of methanol gives potassium salt of 2-Chlorobenzoic acid, which than treated with 3-Chloro-2-methylaniline in presence of various reagents to give crude drug of Tolfenamic acid which on further recrystallization gives pure Tolfenamic acid¹³⁸.



C) Another synthesis method involves adding 2-Chlorobenzoic acid and alkali metal hydroxide into methyl isobutyl ketone followed by heating and raising temperature and subsequent adding 3-Chloro-2-methylaniline, an acid-binding agent and a catalyst into mixture to obtain crude Tolfenamic acid (recrystallized to obtain pure drug)¹³⁸.



Cyclization of TA during its synthesis might result in formation of Imp C. From these synthetic schemes, it is observed that 2-Chlorobenzoic acid and 2-Chloro-3-methylaniline are used as raw materials for the synthesis of TA. The trace amounts of these compounds in the final product (TA) are considered as Imp A and Imp B respectively, in the BP monograph (of TA)⁴⁵. Hence it was inferred that Imp A and B listed in the monograph of TA are ‘process related impurity’ which might remain with TA during its chemical synthesis. These compounds as Imp A and Imp B were considered for developing a new RP HPLC method for separation and estimation of TA in their presence.

6.5. DEVELOPMENT AND OPTIMIZATION OF RP HPLC METHOD FOR ESTIMATION OF TOLFENAMIC ACID IN PRESENCE OF SELECTED PHARMACOPOEIAL IMPURITIES

6.5.1. Selection of Chromatographic method

The most popular technique of chromatography is reversed phase chromatography (RPC). Around 70 % of the separations involving HPLC are carried out using (RPC). The principle of separation is based on analytes’ partition coefficient between polar mobile phase and hydrophobic (nonpolar) stationary phase in RPC. Official methods and methods published in literature for TA are based on RPC separation. So RPC method was selected.

6.5.2. Selection of stationary phase

BP monograph⁴⁵ of Tolfenamic acid recommends C18 column for related substances estimation by RP HPLC. Literature revealed various studies for estimation of TA

individually, in combination and for stability studies of TA with C18 column. Non polar C18 column was considered for the study.

6.5.3. Selection of wavelength for analysis

The overlain spectrum involving TA and its two pharmacopoeial impurities (A and B) was recorded in acetonitrile as shown in Fig. 6.5.3. The solutions of concentration used 10 µg/ml of TA and 1µg/ml each impurity (A and B) for study. All the components showed different wavelength maximum. For the study one specific wavelength was selected where the absorptivity of Imp A and Imp B was comparatively higher than for TA, enhancing sensitivity. Wavelength of 205 nm was selected for the study.

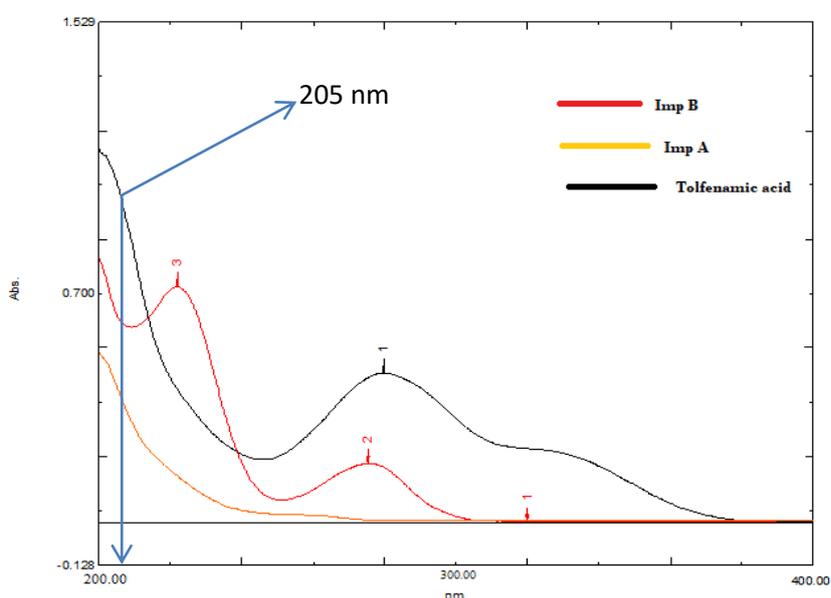


Figure 6.5.3: UV overlain spectrum of TA and impurities (A and B)

6.5.4. Selection and optimization of mobile phase for separation of TA and its impurities

A specific mobile phase that could provide complete separation of TA from its pharmacopoeial impurities, and also monitor degradation behavior under varied stress conditions was required to be developed. In BP monograph of TA ternary system comprising of glacial acetic acid, water and ethanol, the flow rate 0.8 ml/min, injection volume of 20 µl and detection wavelength of 232 nm, on C18 column (4.6 x 250 mm, 5 µm) is used.

Mobile phase composition comprising of acetonitrile and 10 mM ammonium dihydrogen orthophosphate buffer was varied over pH varying from 2.5 to 6.5 to obtain optimized

mobile phase composition and pH of the buffer. Observations are recorded and presented in Table 6.5.4.1.

Table 6.5.4.1: Exploratory trials for optimization of mobile phase composition on Sunfire C-18 column (250 mm x 4.6, 5 µm) for TA and impurities (A and B)

Trial no	Mobile phase composition (ACN and 10 mM potassium dihydrogen orthophosphate Buffer) in % v/v	pH of mobile phase	Remark	Figure no
1	90:10	6.5	All the peaks were well separated with Rt of less than 5 min	6.5.4.1.1
2	80:20		All the peaks were well separated with Rt of less than 5 min. Peak of Imp A was close to dead volume	6.5.4.1.2
3	70:30		All the peaks were well separated with Rt of less than 6 min. Peak of Imp A eluted in dead volume	6.5.4.1.3
1	90:10	5.5	No proper resolved peaks were seen with one peak seen in dead volume.	6.5.4.1.4
2	80:20		All the peaks were well separated, with retention time of less than 5 min. Imp A appeared close to dead volume.	6.5.4.1.5
3	70:30		All the peaks were well separated, with retention time of less than 6 min. Imp A appeared close to dead volume.	6.5.4.1.6
1	90:10	3.5	All the peaks were well separated, with less than 5 min. But Imp A appeared in dead volume	6.5.4.1.7
2	80:20		All the peaks were well separated, with less than 6 min, but Imp A appeared in dead volume.	6.5.4.1.8

3	70:30		All the peaks were well separated, with less than 10 min, but Imp A appeared in dead volume.	6.5.4.1.9
1	90:10	2.5	All the peaks were well separated; with final peak having retention time of less than 5 min. Impurity A appeared close to dead volume.	6.5.4.1.10
2	80:20		All the peaks were well separated, with final peak having retention time of less than 7 min.	6.5.4.1.11
3	70:30		All the peaks were well separated, with final peak having retention time of more than 10 min.	6.5.4.1.12

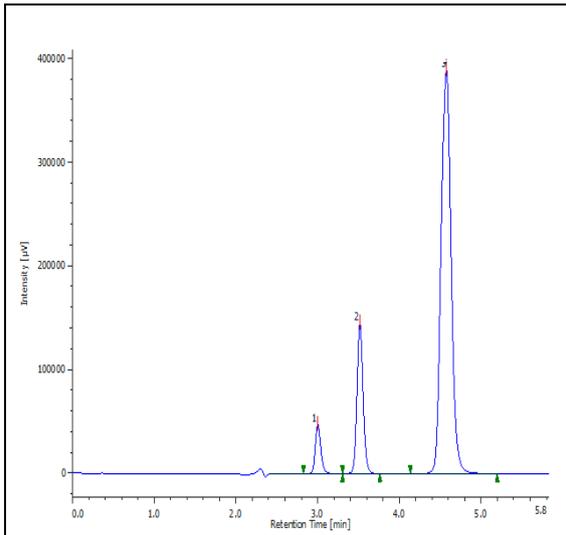


Figure 6.5.4.1

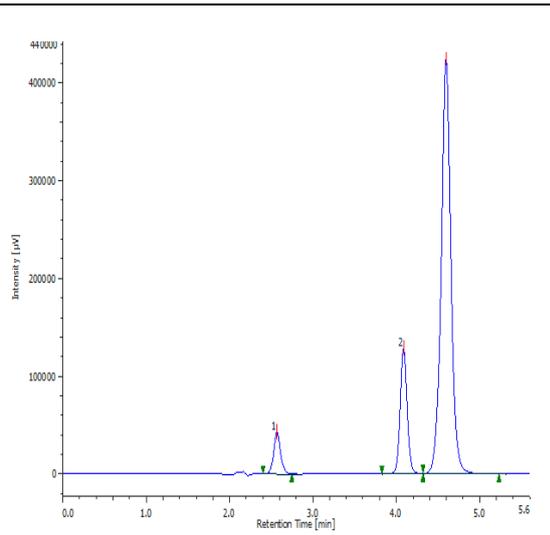


Figure 6.5.4.2

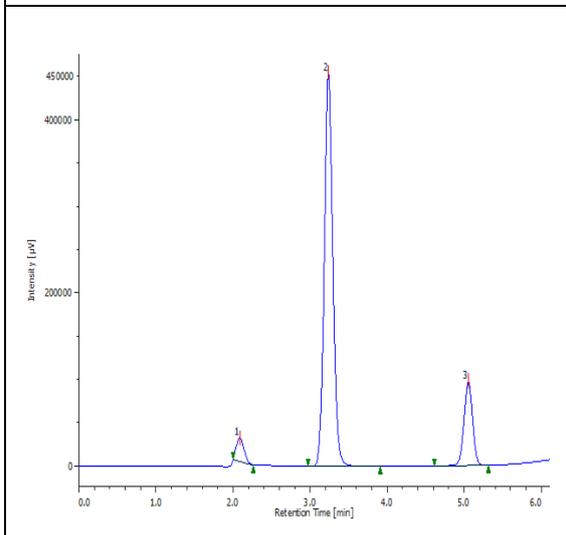


Figure 6.5.4.3

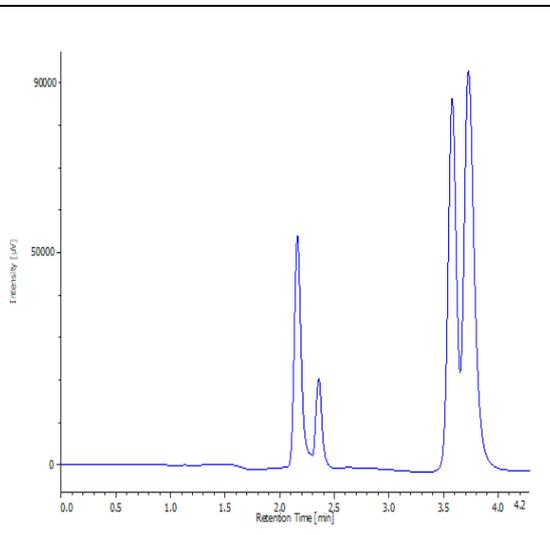


Figure 6.5.4.4

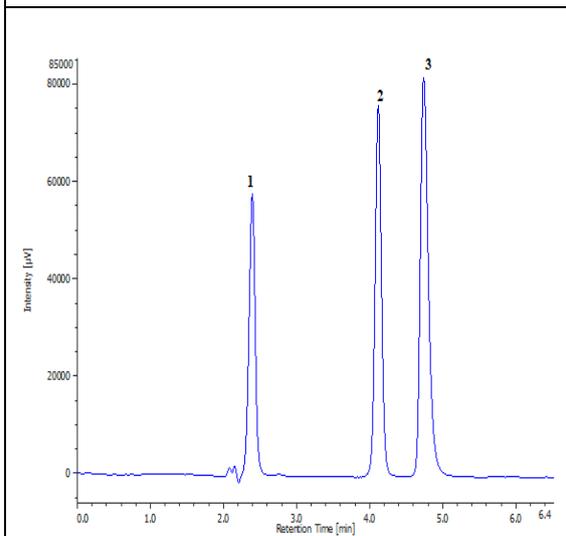


Figure 6.5.4.5

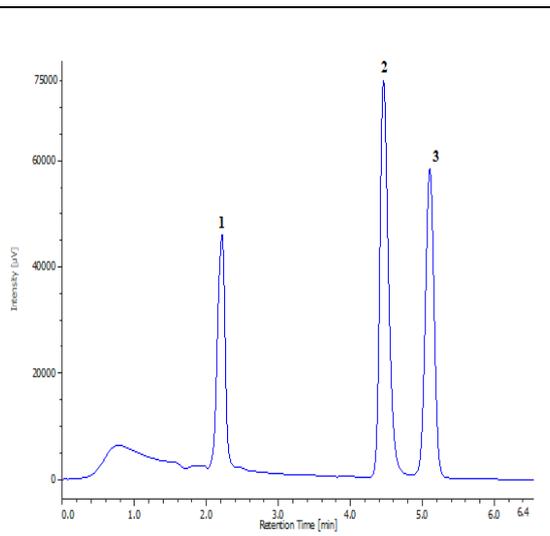


Figure 6.5.4.6

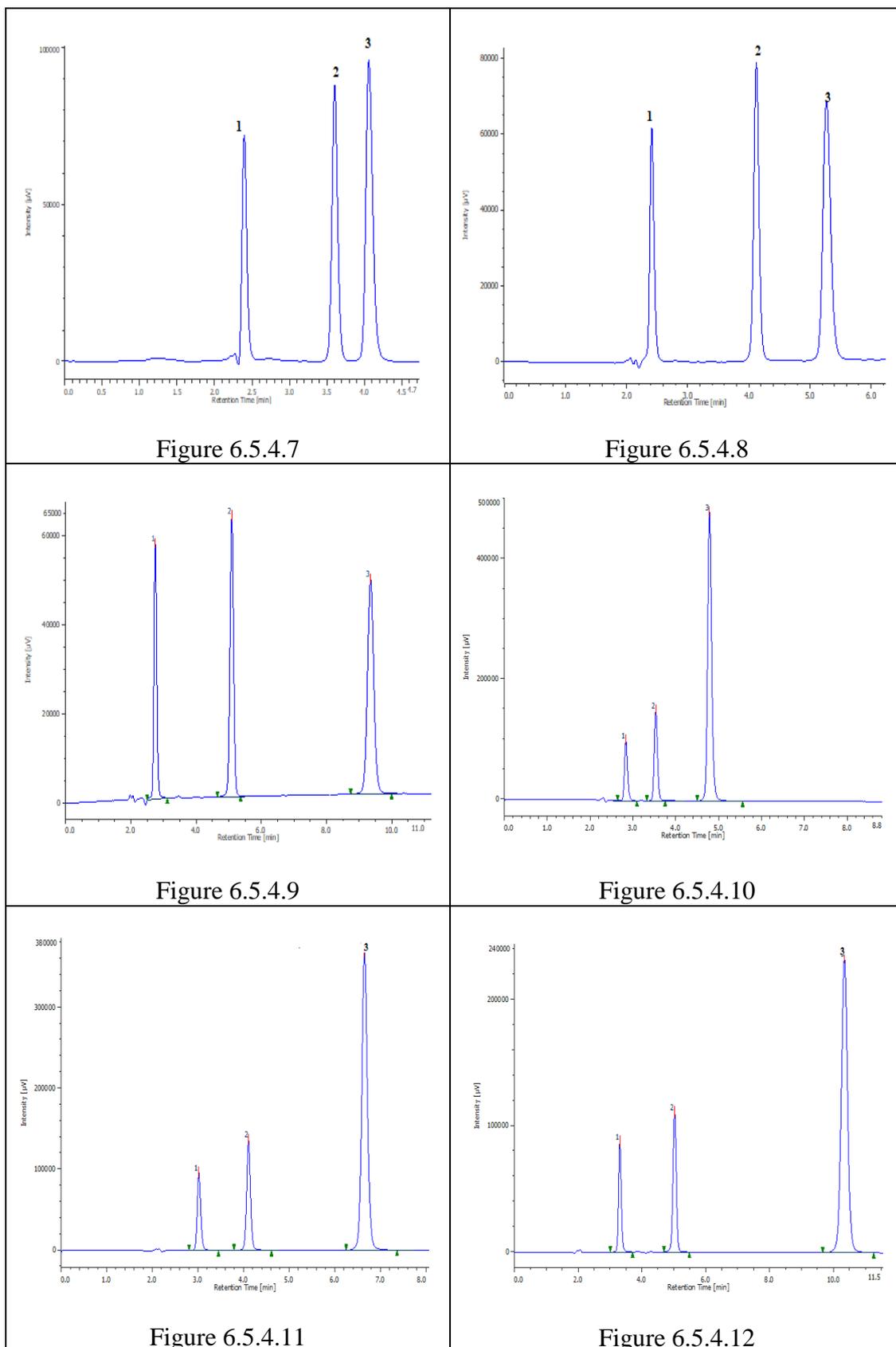


Figure 6.5.4: Representative chromatograms of exploratory trials for optimization of mobile phase composition for TA and impurities (A and B)

It was observed that when the pH of the buffer was maintained at 3.5, 5.5 and 6.5 efficient separation due to peak merging or partial elution seen or early elution (peaks eluted in dead volume) was seen. However at pH 2.5 separations were satisfactory. All the peaks were well resolved from each other and from drug peak as seen in Fig 6.5.4.11.

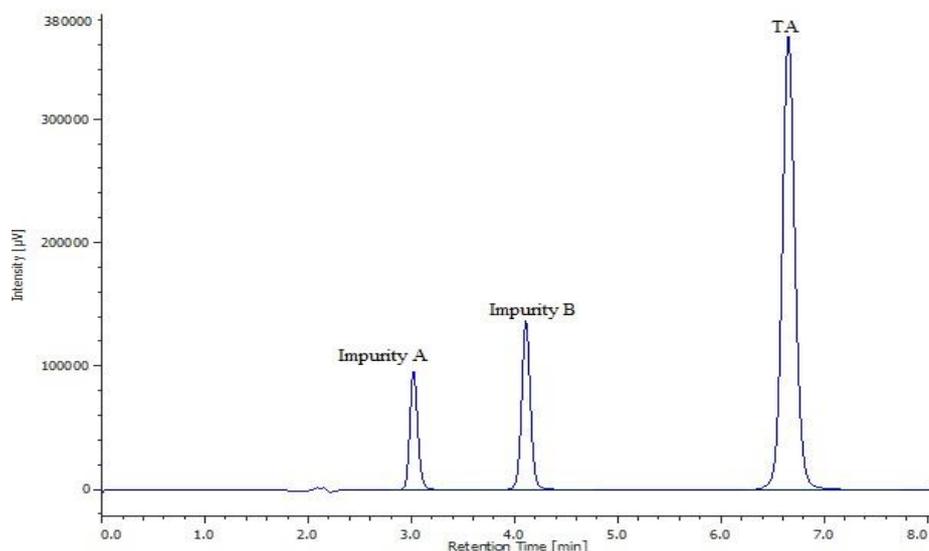


Figure 6.5.4.11: Optimized chromatogram of TA and impurities (A and B)

Chromatographic conditions were optimized from exploratory trial on Waters Sunfire C-18 column. Best fit mobile phase for separation of TA from Imp A and Imp B was selected best of satisfactory resolution of peaks on smooth baseline. Final optimized chromatographic condition is shown in Table 6.5.4.2.

Table 6.5.4.2: Optimized chromatographic conditions for separation of TA and impurities (A and B)

Mobile phase	Mixture of acetonitrile and 10 mM ammonium dihydrogen ortho phosphate buffer (pH adjusted to 2.5 with ortho phosphoric acid) in the ratio of 80:20 % v/v
Column	Waters -Sunfire ODS C18 (250 mm x 4.6, 5 µm)
Flow rate	1 ml/min
Wavelength	205 nm
Injector loop size	10 µL

6.6. FORCED DEGRADATION STUDIES

The forced degradation studies were conducted for the drug TA by exposing to hydrolysis (HCl, NaOH and neutral), oxidation, elevated temperature and photolytic stress. Series of samples were prepared for each condition as per routine protocols (Table 6.6.). Sample subjected to stress were obtained and analyzed by the proposed method.

Table 6.6: Protocol for stress degradation of TA

Samples	Hydrolysis			Oxidative Deg.	Thermal Deg.	Photo Deg.
	Acid	Alkaline	Neutral			
Blank stored under normal condition	√	√	√	√	-----	-----
Blank subjected to stress condition	√	√	√	√	-----	-----
Drug / drug solution stored under normal condition	√	√	√	√	√	√
Drug / drug solution subjected to stress condition*	√	√	√	√	√	√

* To get desired degradation, initially degradation was carried out at room temperature and if necessary samples were subjected to higher temperature or strength of stress reagent was increased.

6.6.1. Hydrolytic degradation

Hydrolytic studies were carried with HCl, NaOH and water to stimulate acidic, alkaline and neutral conditions respectively. The acid and base degradation was carried out using hydrochloric acid and sodium hydroxide, initially with strength of N/10 and followed by 1N if no degradation is seen at lower strength. The studies were performed initially at room temperature followed by heating the solution of drug with acid, base and water at 70 °C on water bath equipped with thermostat for extended time intervals, if no degradation was seen at RT.

The drug solution treated with acid and base was neutralized by using base and acid respectively of the same strength and diluted up to the mark with the mobile phase. These solutions were then subjected to analysis by the proposed method.

6.6.1.1. Acid degradation

Acid degradation was carried out by using hydrochloric acid. Samples were prepared by taking 0.1 ml of stock solution of drug (1000 µg/ml) and 1 ml N/10 HCl in 10 ml V.F and kept at RT and at higher temperature of 70 °C for extended time interval around 6 hr. Further the study was conducted using 1ml of 1N HCl under the RT and 70 °C. The samples were cooled and then neutralized by 1 ml of same strength of NaOH and volume up to the mark was made with mobile phase. The solutions were then analyzed by the proposed method. Observation of hydrolytic study of TA with varied concentration of HCl at different temperature and time is presented in Table 6.6.1.1 and representative chromatogram as Fig 6.6.1.1.

Table 6.6.1.1: Degradation study of TA with HCl

Concentration of HCl	Temperature	Time	% of active drug after degradation
0.1N	RT	6 hr	96.56
1N	RT	6 hr	95.28
0.1N	70 °C	6 hr	95.64
1N	70 °C	6 hr	90.50

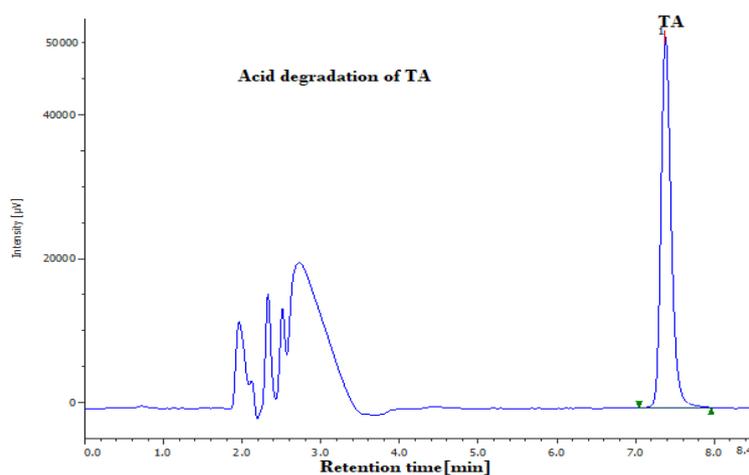


Figure 6.6.1.1: Chromatogram of TA (10 µg/ml) treated with 1N HCl for 6 hr at 70 °C

Peak name	CH	R _t (min)	Area(µV-sec)	NTP	Symmetry
TA	1	7.375	467482	16641	1.29
Control	1	7.408	516554	16437	1.29

6.6.1.2. Base degradation

Base degradation was carried out by using sodium hydroxide. Samples were prepared by taking 0.1 ml of stock solution of drug (1000 $\mu\text{g/ml}$) and 1 ml N/10 NaOH in 10 ml V.F and kept at RT and at higher temperature of 70 $^{\circ}\text{C}$ for extended time interval around 6 hr. Further the study was conducted using 1ml of 1N NaOH at RT and 70 $^{\circ}\text{C}$. The samples were cooled and neutralized by 1 ml of same strength of HCl and volume up to the mark was made with mobile phase. The solutions were then analyzed by the proposed method. Observation of hydrolytic study of TA with varied concentration of NaOH at different temperature and time is presented in Table 6.6.1.2 and representative chromatogram as Fig 6.6.1.2.

Table 6.6.1.2: Degradation study of TA with NaOH

Concentration of NaOH	Temperature	Time	% of active drug after degradation
0.1N	RT	6 hr	96.30
1N	RT	6 hr	95.02
0.1N	70 $^{\circ}\text{C}$	6 hr	92.96
1N	70 $^{\circ}\text{C}$	6 hr	90.05

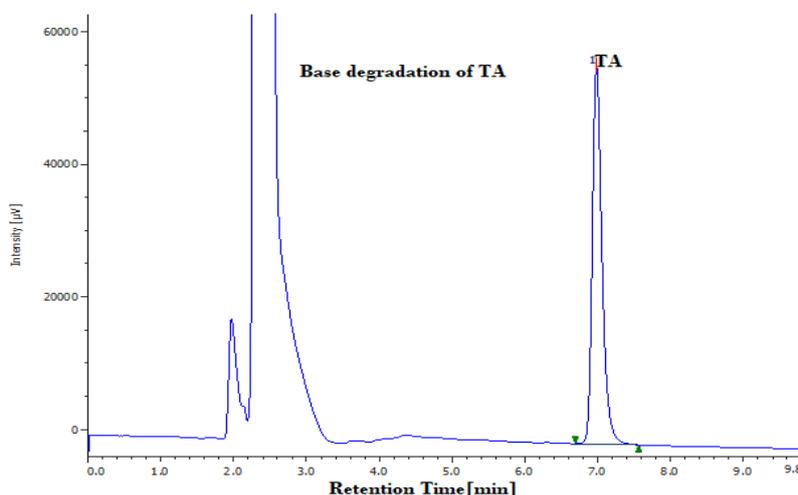


Figure 6.6.1.2: Chromatogram of TA (10 $\mu\text{g/ml}$) treated with 1N NaOH for 6 hr at 70 $^{\circ}\text{C}$

Peak name	CH	R _t (min)	Area($\mu\text{V}\cdot\text{sec}$)	NTP	Symmetry
TA	1	6.983	509415	14289	1.30
Control	1	7.125	562890	16943	1.19

6.6.1.3. Neutral degradation

Neutral degradation was carried out by using distilled water. Samples were prepared by taking 0.1 ml of stock solution of drug (1000 µg/ml) and 1 ml of distilled water in 10 ml V.F and kept at RT and at higher temperature of 70 °C for extended time interval around 6 hr. The samples were cooled and volume up to the mark was made with mobile phase. The solutions were then analyzed by the proposed method. Observation of hydrolytic study of TA with water at different temperature and time is presented in Table 6.6.1.3 and representative chromatogram as Fig 6.6.1.3.

Table 6.6.1.3 Degradation study of TA with water

Temperature	Time	% of active drug after degradation
RT	6 hr	99.01
70 °C	6 hr	98.47

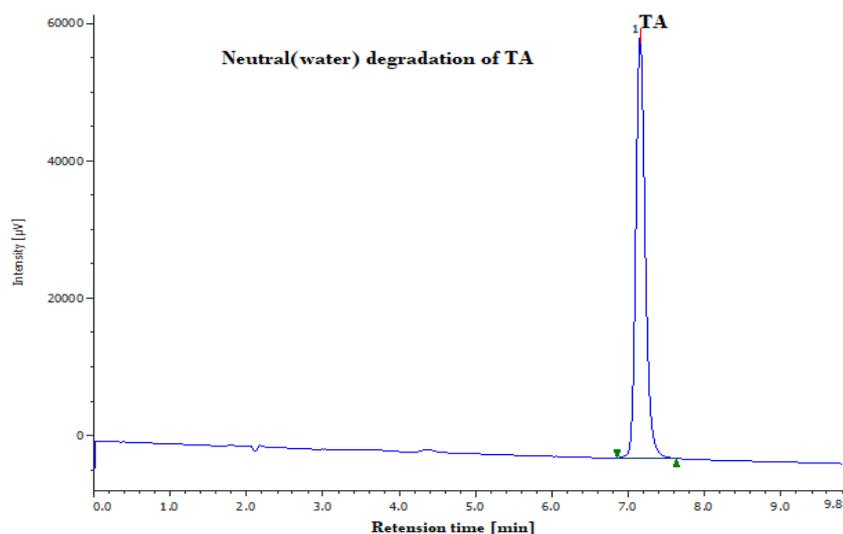


Figure 6.6.1.3: Chromatogram of TA (10 µg/ml) treated with water for 6 hr at 70 °C

Peak name	CH	R _t (min)	Area(µV-sec)	NTP	Symmetry
TA	1	7.167	505020	17321	1.25
Control	1	7.167	512864	17385	1.23

6.6.2. Oxidative Degradation

Oxidative degradation was carried out by using hydrogen peroxide. Samples were prepared by taking 0.1 ml of stock solution of drug (1000 µg/ml) and 1 ml of hydrogen peroxide (10 % v/v) in 10 ml V.F at RT for specified time interval (7 days) to obtain sufficient degradation. After required exposure samples were diluted up to the mark by mobile phase and subjected for analysis by the proposed method. Observation of oxidative study of TA with 10 % v/v H₂O₂ is presented in Table 6.6.2 and representative chromatogram as Fig 6.6.2.

Table 6.6.2: Degradation study of TA with H₂O₂

Temperature	Time	% of active drug after degradation	RRT of additional peak formed
RT	2 days	85.61	1) 0.54 (DP I)

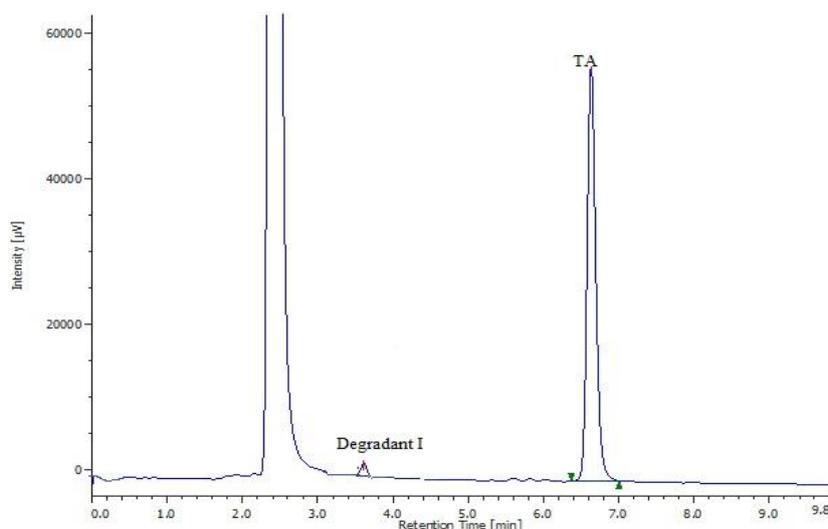


Figure 6.6.2: Chromatogram of TA (10 µg/ml) treated with 10 % H₂O₂ at RT for 2 days

Peak name	CH	R _t (min)	Area(µV-sec)	NTP	Symmetry
TA	1	6.633	459239	16586	1.15
DPI	1	3.608	10174	9875	1.00

6.6.3. Thermal degradation

Drug sample 10 mg each was taken in two 10 ml V.F and closed with stopper. One flask was exposed to dry heat in hot air oven for specified temperature (80 °C) and time interval (4 days) and another was kept as control. After required exposure two separate solutions were prepared by weighing appropriate amount of sample exposed to thermal stress and control to produce concentration of 10 µg/ml. The samples were diluted up to the mark with mobile phase and injected separately for analysis by the proposed method. Observation of thermal study of TA in hot air oven (80 °C) is presented in Table 6.6.3 and representative chromatogram as Fig 6.6.3.

Table 6.6.3: Degradation study of TA in hot air oven

State	Temperature	Time	% of active drug after degradation
Solution	70 °C	6 hr	98.47
Solid	80 °C	4 days	92.93

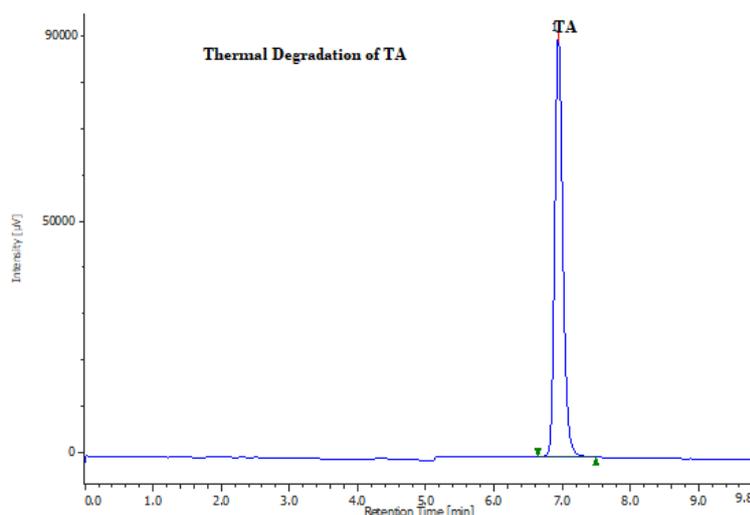


Figure 6.6.3: Chromatogram of TA (10 µg/ml) in oven for 4 days at 80 °C

Peak name	CH	R _t (min)	Area(µV-sec)	NTP	Symmetry
TA	1	6.942	474737	17300	1.23
Control	1	6.917	510820	17292	1.20

6.6.4. Photo degradation

Drug in sufficient amount was taken in 10 ml volumetric flask and was exposed to direct sunlight for a specified time period (7 days). Another flask containing drug was kept as control. After required exposure two separate solutions were prepared by weighing appropriate amount of sample exposed to stress and control to produce concentration of 10 $\mu\text{g/ml}$. The samples were diluted up to the mark with mobile phase and injected separately for analysis by the proposed method. Observation of photo light study of TA after direct exposure to sunlight is presented in Table 6.6.4 and representative chromatogram as Fig 6.6.4.

Table 6.6.4: Degradation study of TA with direct exposure to sunlight

Temperature	Time	% of active drug after degradation
Sunlight	4 days	95.34

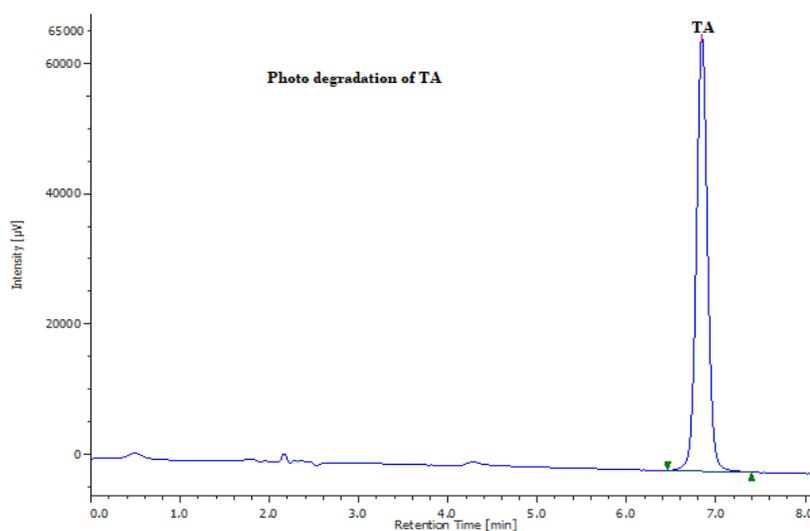


Figure 6.6.4: Chromatogram of TA (10 $\mu\text{g/ml}$) exposed to direct sunlight for 7 days

Peak name	CH	R _t (min)	Area($\mu\text{V-sec}$)	NTP	Symmetry
TA	1	6.842	593127	15418	1.023
Control	1	6.900	622118	12919	0.962

Forced degradation study of TA showed that there was formation of one degradant (DP I) under oxidative stress conditions. DP I showed an RRT value of 0.54. The RRT values of DP I was different from the RRT values of the pharmacopoeial impurities after application of the developed method. Hence the RP HPLC developed for separation of TA and its pharmacopoeial impurities is a stability indicating method.

6.7. VALIDATION OF DEVELOPED STABILITY INDICATING ANALYTICAL METHOD

Validation is an act of confirming that a method does what it is intended to do. According to USP SIAM is grouped under category II (Analytical methods for determination of impurities in APIs or for determination of degradation products in final drug products). Parameters recognized by ICH for method validation (Table 6.7).

Table 6.7: Validation parameters and acceptance criteria

Sr.No.	Validation Parameters	Acceptance Criteria
1	Specificity	Peak (s) of degradation products and analyte should be pure and well separated from one other.
2	Linearity	Correlation coefficient not less than 0.999
3	Accuracy (across the specified range)	Recovery (%) between 98% to 102%
4	Precision	
	4.1) Intra-day precision	RSD (%) of replicate injections not more than 2.0
	4.2) Inter-day precision	
5	Robustness	
6	System Suitability Test	1) Resolution \leq 2.0 2) % RSD of replicate injections \leq 2.0 3) Theoretical plate number \leq 2000 4) Asymmetry of peak should not be more than 2.0

6.7.1. System suitability parameters

Results of the study for system suitability parameters when proposed method was applied for analysis of TA in presence of Imp A, Imp B and degradation product (DP I) is presented in Table 6.7.1. Values obtained meet the acceptance criteria Table 6.7.

Table 6.7.1: System suitability testing parameters of the proposed RP HPLC method

Sr.no	Components	RRT	Peak asymmetry	Theoretical plate
1	Tolfenamic acid	1.00	1.098	14400
2	Imp A	0.453	1.213	7515
3	Imp B	0.617	1.069	11607
4	DP I(oxidative condition)	0.54	1.009	9875

6.7.2. Specificity and Selectivity

Results of specificity and selectivity study undertaken on TA along with Imp A, Imp B and DP I are presented in Table 6.7.2 and are in agreement with acceptance criteria defined in Table 6.7.

Table 6.7.2 Selectivity of the HPLC method

Component	Peak (RRT)
Tolfenamic acid	1.00
Imp A	0.453
Imp B	0.617
DP I(oxidative condition)	0.54

6.7.3. Linearity and Range

Study involving determination of linearity range was undertaken with working/standard solution of TA, Imp A and Imp B diluted to appropriate concentrations as mentioned under section 6.9.6.2. Results of the study are tabulated (Table 6.7.3.1) and linearity graphs presented by various figures as follow.

Table 6.7.3.1: Linearity Range

Compound	Linearity range (µg/ml)	R ²	Reference
TA	13.77-100	0.9987	Fig 6.7.3.1
Imp A	0.1-1	0.9984	Fig 6.7.3.3
Imp B	0.12-1	0.9989	Fig 6.7.3.5

Table 6.7.3.2: Linearity data of TA

Sr.No	Conc. (µg/ml)	Peak Area				RSD (%)
		Injection 1	Injection 2	Injection 3	Average of 3 injection	
1	10	556774	559444	558765	558327	0.24
2	20	1073110	1066281	1067454	1068948	0.34
3	40	2150673	2157929	2181249	2163283	0.73
4	60	2995976	2995999	2996006	2995994	0.09
5	80	4016739	4076136	4050060	4046437	0.73
6	100	4936623	4930285	4930385	4932431	0.07

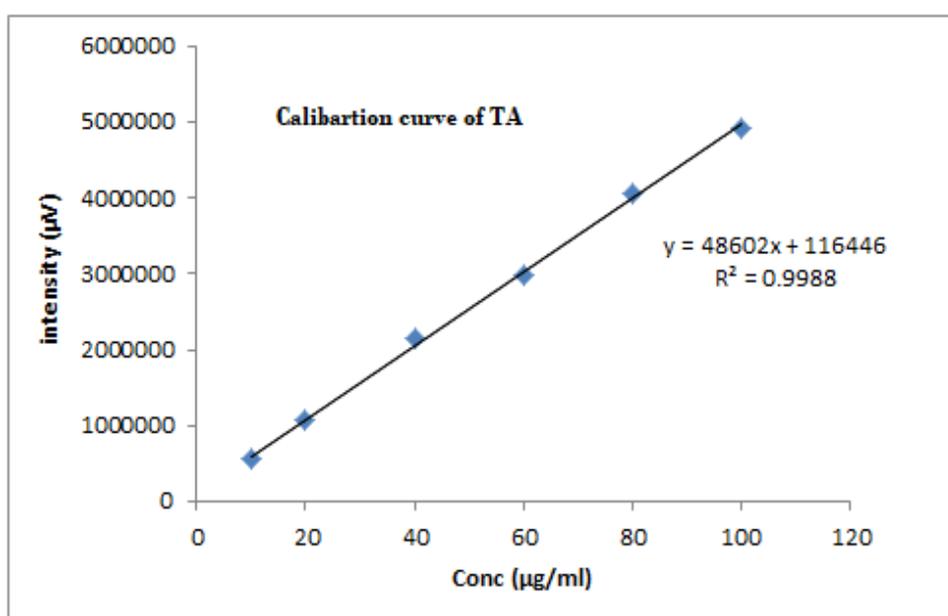


Figure 6.7.3.1: Linearity graph of TA

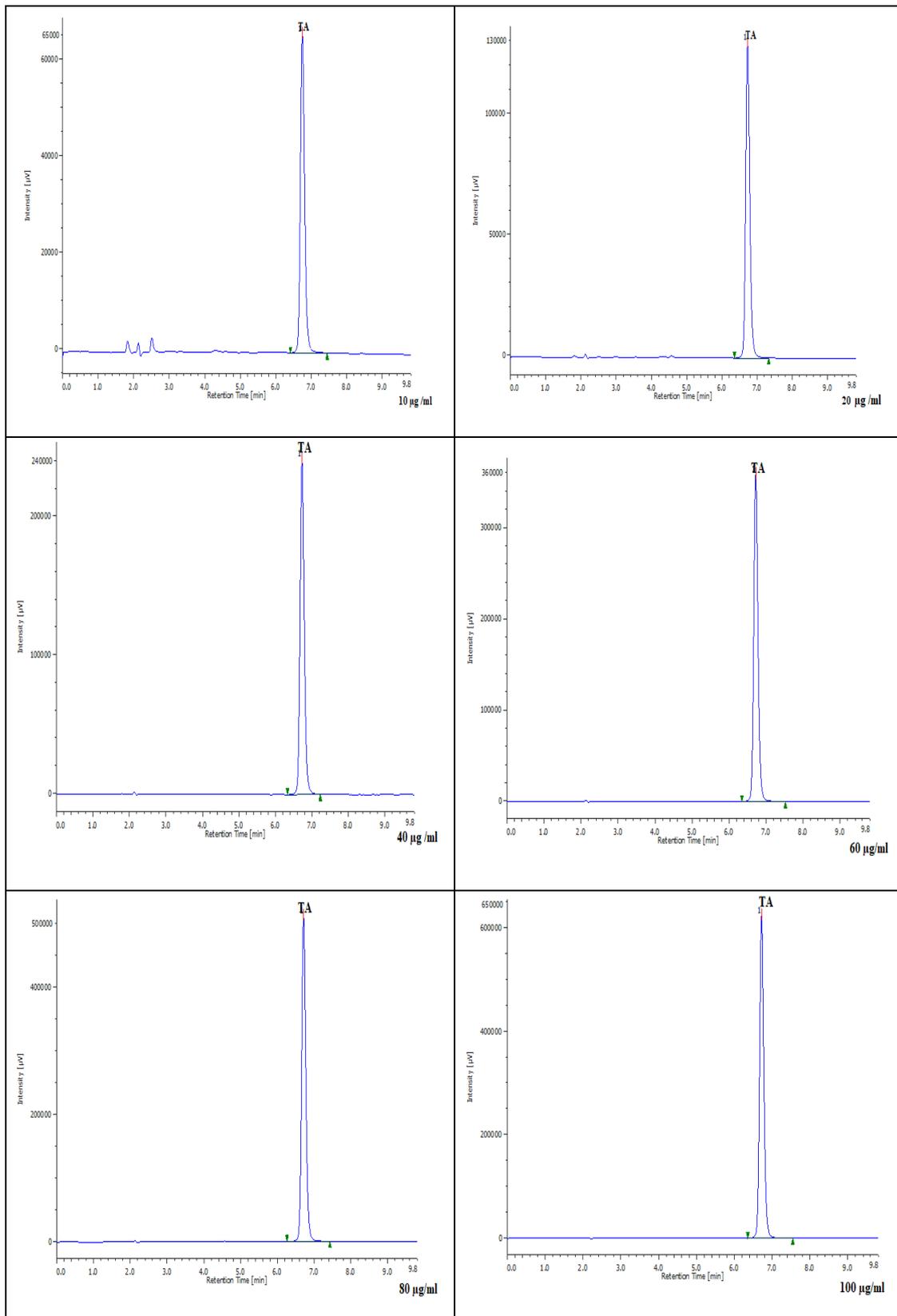


Figure 6.7.3.2: Representative chromatograms of TA (Conc. 10, 20, 40, 60, 80, 100 µg/ml) after first injection

Table 6.7.3.3: Linearity data of Imp A

Sr.No	Conc. (µg/ml)	Peak Area				RSD (%)
		Injection 1	Injection 2	Injection 3	Average of 3 injections	
1	0.1	12079	12087	12056	12074	0.13
2	0.2	19025	19555	19194	19258	1.40
3	0.4	36425	36713	36800	36646	0.56
4	0.6	52937	52407	52722	52688	0.51
5	0.8	71195	71868	70215	71092	1.16
6	1.0	89591	90102	90251	89981	0.38

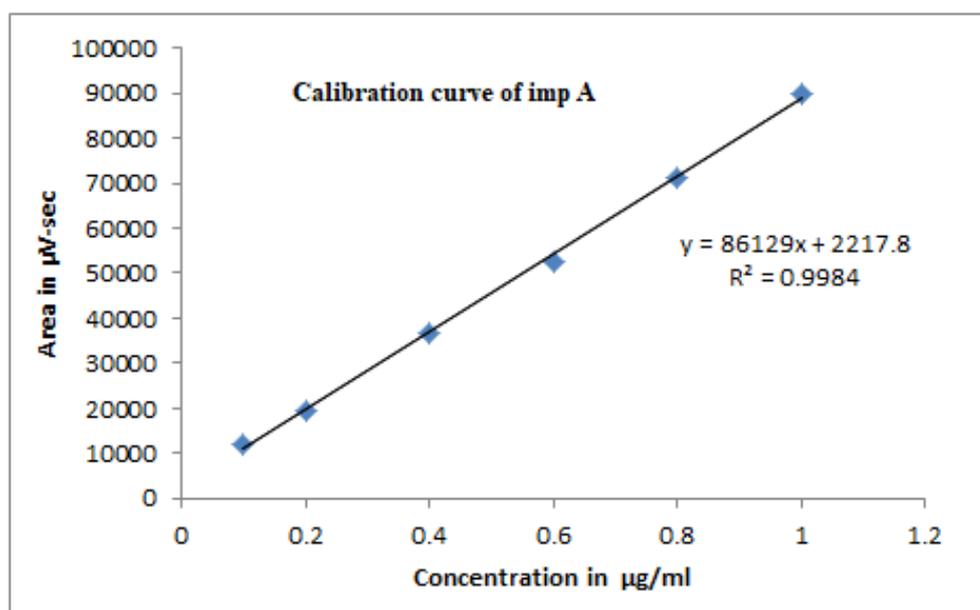


Figure 6.7.3.3: Linearity graph of Imp A

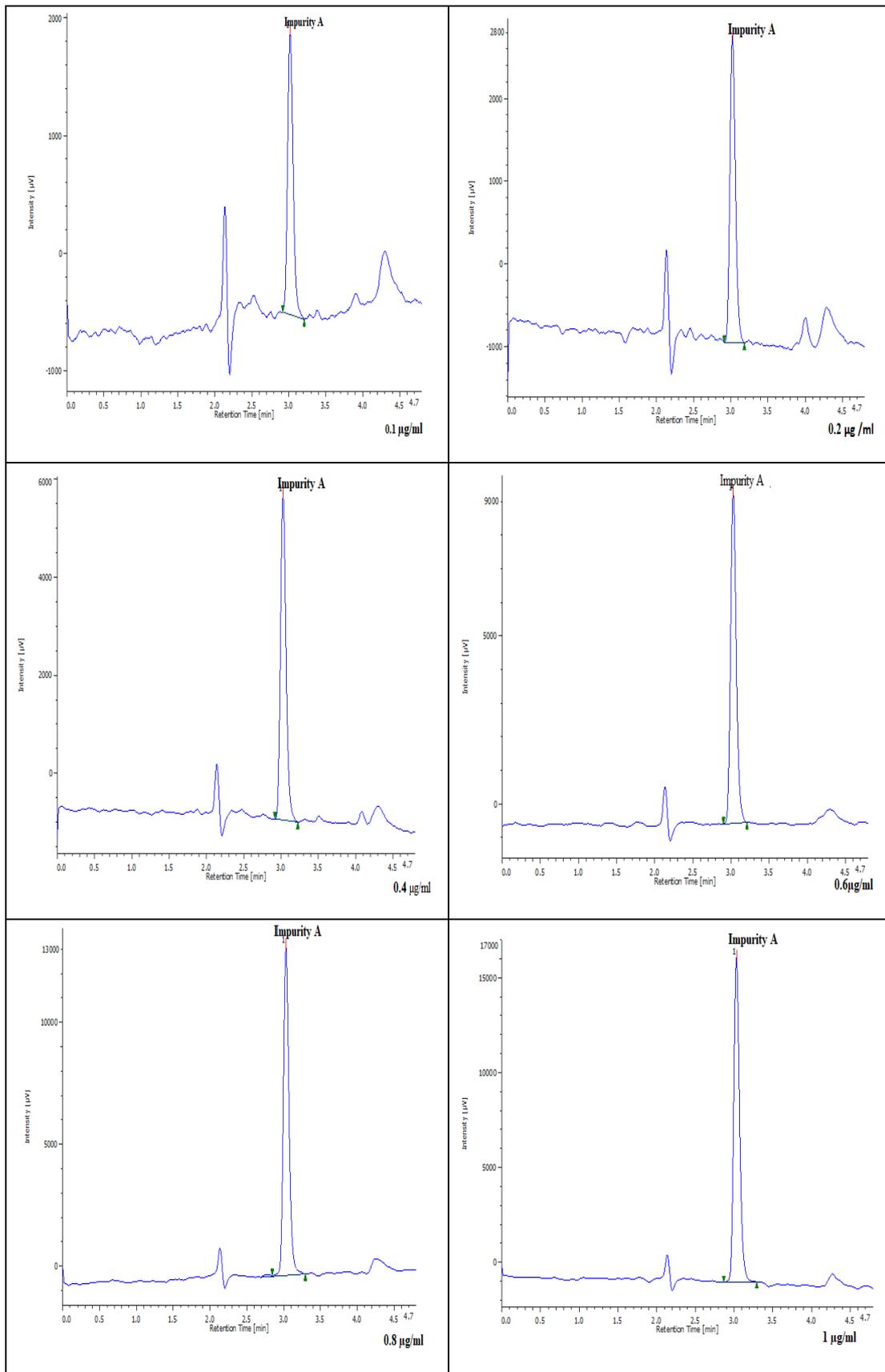


Figure 6.7.3.4: Representative chromatogram of Imp A (Conc. 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 µg/ml) after first injection

Table 6.7.3.4: Linearity data of Imp B

Sr.No	Conc. (µg/ml)	Peak Area				RSD (%)
		Injection 1	Injection 2	Injection 3	Average of 3 injections	
1	0.1	11972	11821	11870	11888	0.64
2	0.2	24412	24822	24633	24622	0.83
3	0.4	46735	45863	46137	46244	0.96
4	0.6	72390	72726	72600	72572	0.23
5	0.8	98370	98470	98628	98488	0.13
6	1.0	118990	119300	118351	118880	0.40

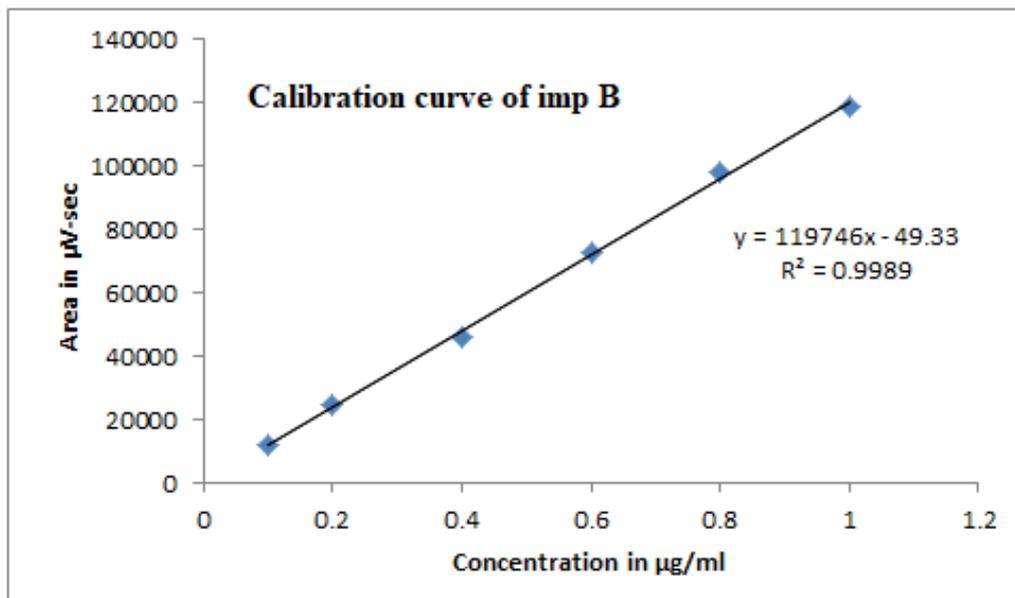


Figure 6.7.3.5: Linearity graph of Imp B

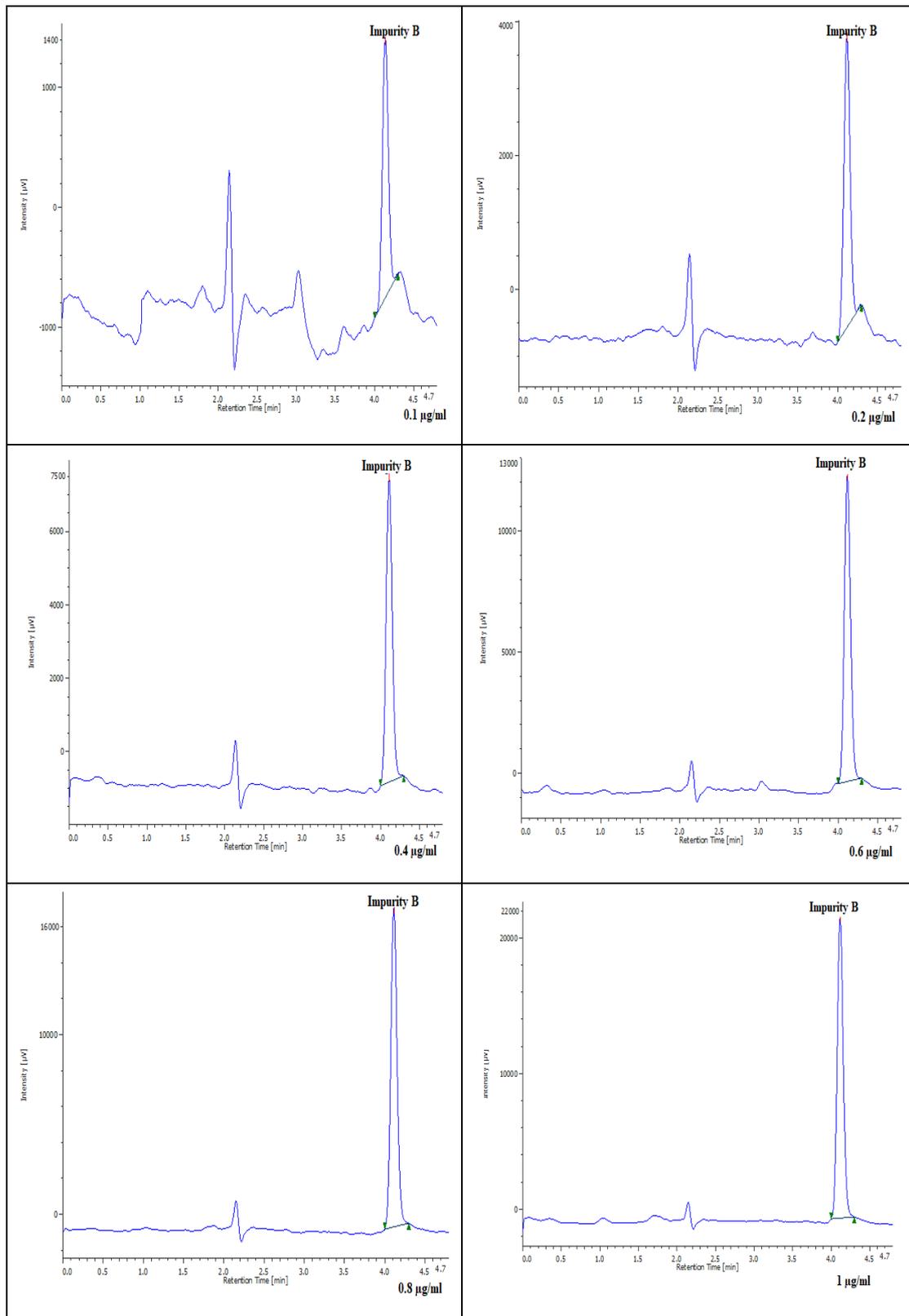


Figure 6.7.3.6: Representative chromatogram of Imp B (Conc, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 $\mu\text{g/ml}$) after first injection

6.7.4. Recovery / Accuracy

Accuracy of the proposed method was determined by recovery studies (**standard addition method**). The percentage recovery studies of TA, Imp A and Imp B was carried out in triplicate at 3 different levels ranging from 80 % - 120 %, by spiking standard samples as described under procedure at section 6.9.6.3. Result of recovery study is presented in Table 6.7.4.

Table 6.7.4: Recovery studies (n=3)

Component	Amount of sample (µg/ml)	Average peak area of sample	Level of spiking (%)	Amount of standard spiked (µg/ml)	Average Peak area* after standard addition	Peak area of recovered standard	Amount Recovered (µg/ml)	% Recovery
TA	40	2418828	80	32	4331840	1913012	31.635	98.86
			100	40	4830656	2411828	39.884	99.71
			120	48	5358774	2939946	48.617	101.28
Imp A	0.4	50693	80	0.32	90824	40131	0.316	98.95
			100	0.4	100938	50245	0.396	99.11
			120	0.48	112405	61712	0.486	101.44
Imp B	0.4	39971	80	0.32	71481	31510	0.315	98.54
			100	0.4	79900	39929	0.399	99.89
			120	0.48	88768	48797	0.488	101.73

*Average of three readings

6.7.5. Precision and Sensitivity

Intraday repeatability of the method was evaluated by analyzing three concentrations of TA (20, 40 and 80 µg/ml), and three concentration of Imp A and Imp B (0.2, 0.4 and 0.8 µg/ml). Interday precision was evaluated by assaying the three chosen same sets of concentration of TA, Imp A and Imp B in triplicates on two successive days using the procedure stated under section 6.9.6.4 and 6.9.6.5. Results of the study are presented in Table 6.7.5.1 and Table 6.7.5.2.

Table 6.7.5.1: Intra and Interday Precision data (n=3)

Component		Intra-day		Inter-day	
		mean peak area ±SD	% R.S.D	mean peak area ±SD	% R.S.D
TA concentration (µg/ml)	20	1128241± 4402	0.39	1068948 ± 3651	0.34
	40	2068060 ±17262	0.83	1963283 ± 15975	0.81
	80	4176141 ± 16259	0.38	4046437± 29772	0.73
Imp A concentration (µg/ml)	0.2	24946 ± 216	0.86	19258 ± 270	1.40
	0.4	49508 ± 112	0.22	34646 ± 196	0.56
	0.8	104303 ± 522	0.50	71092 ± 831	1.16
Imp B concentration (µg/ml)	0.2	28289 ± 419	1.48	23622 ± 205	0.86
	0.4	47911 ± 800	1.66	45911 ± 205	0.44
	0.8	98416 ± 302	0.30	96749± 280	0.28

Table 6.7.5.2: Sensitivity of the method (LOD and LOQ)

Parameters	TA	Imp A	Imp B
LOD(µg/ml)	4.54	0.035	0.042
LOQ(µg/ml)	13.77	0.106	0.128

6.7.6. Robustness

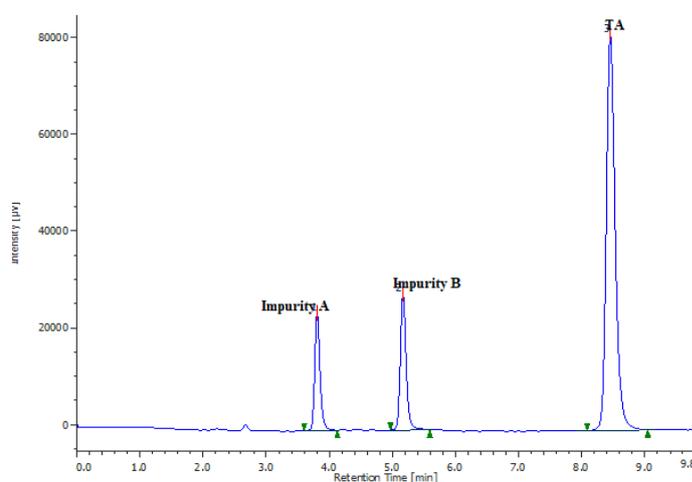
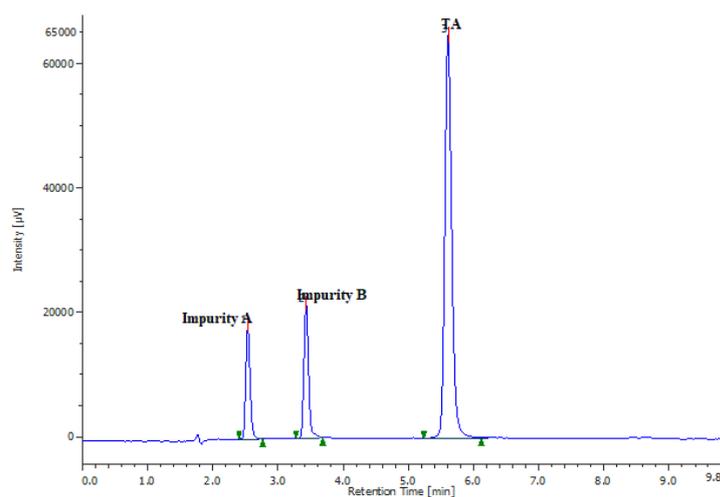
Robustness of an analytical method is the measurement of the methods ability to remain unchanged by minute but intended alterations in the methods variables and provide reliability during normal use. The robustness of the method was studied by making deliberate changes in flow rate, and composition of mobile phase ratio.

6.7.6.1. Variation in the flow rate (± 0.2 ml/ min of the optimized flow rate)

The optimized flow rate was for the method was set at 1ml/min. For robustness study, the flow rate was varied to 0.8 ml/min and 1.2 ml/min as per section 6.9.6.6. The solutions were then injected into the chromatograph equilibrated with the flow rate of 0.8 ml/min and 1.2 ml/min. The results obtained upon the flow rate variation are displayed in Table 6.7.6.1 with representative chromatogram (Fig 6.7.6.1.1 and Fig 6.7.6.1.2).

Table 6.7.6.1: Results of flow rate variation

	TA	Imp A	Imp B
Flow rate	Average area (n=3)	Average area (n=3)	Average area (n=3)
0.8ml	549254	88651	124420
1 ml	558327	89981	121609
1.2 ml	539579	89538	122249
% RSD	1.70	0.75	1.20

Figure 6.7.6.1.1: Chromatogram with flow rate 0.8 ml/min
(Optimized 1.0ml/min)Figure 6.7.6.1.2: Chromatogram with flow rate 1.2 ml/min
(Optimized 1.0ml/min)

6.7.6.2. Variation in the mobile phase composition ($\pm 2\%$ of the optimized ratio)

The optimized isocratic elution program was Acetonitrile: Ammonium di hydrogen Ortho Phosphate buffer (pH adjusted to 2.5 with OPA acid,) in ratio of 80:20 % v/v. For robustness study, organic phase ratio was varied to 78:22 % v/v (- 2 % of the optimized ratio) and 82:18 % v/v (+2 % of the optimized ratio). The results obtained for robustness study wherein organic phase ratio of mobile phase was varied is presented in Table 6.7.6.2 with representative chromatograms (Fig. 6.7.6.2.1 and Fig. 6.7.6.2.2).

Table 6.7.6.2: Results of mobile phase variation

	TA	Imp A	Imp B
Composition	Average area (n=3)	Average area (n=3)	Average area (n=3)
78:22% v/v	556096	88275	124159
80:20% v/v	558327	89981	121609
82:18% v/v	557084	89500	123934
% RSD	0.20	0.98	1.14

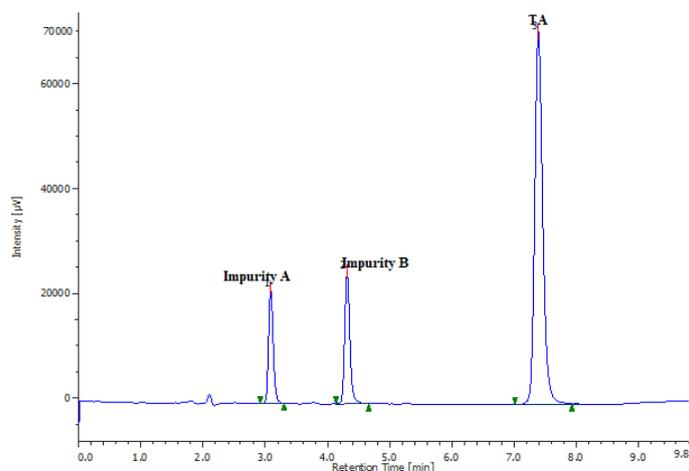


Figure 6.7.6.2.1: Chromatogram with organic phase ratio altered to 78:22 % v/v (Optimized 80:20 % v/v)

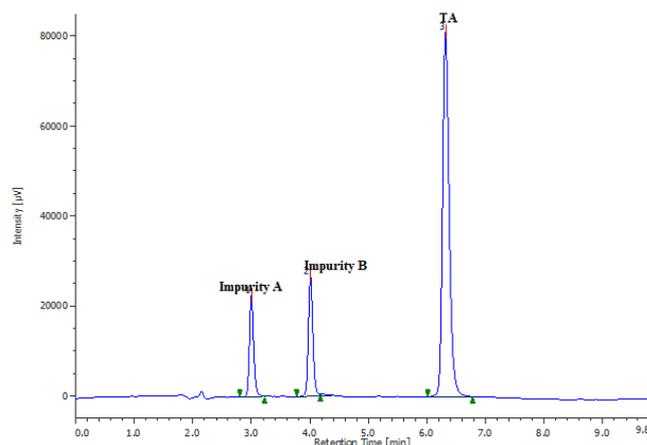


Figure 6.7.6.2.2: Chromatogram with organic phase ratio altered to 82:18 % v/v (Optimized 80:20 % v/v)

6.8. ANALYSIS OF MARKETED PRODUCT

The proposed HPLC method was extended for the determination of TA in marketed dosage (Clotan 200 mg by Pure and Cure Healthcare Pvt Ltd) as per the procedure in section 6.9.7. The assay results showed 99.97 % compliance to label claim. Results are summarized in Table 6.8. No detectable peaks were found at the RRTs of Pharmacopoeial impurities (A and B) and degradation product (DP I), which signifies their absence in the detectable concentration in the marketed product as seen in Fig 6.8.

Table 6.8: Result of TA in marketed product (Clotan 200 mg)

Sr. no	Conc. of sample solution ($\mu\text{g/ml}$)	Peak area	Mean peak area	% assay
1	40	2265376	2265718	99.97
2		2263092		
3		2268687		

**Average of analysis in triplicate*

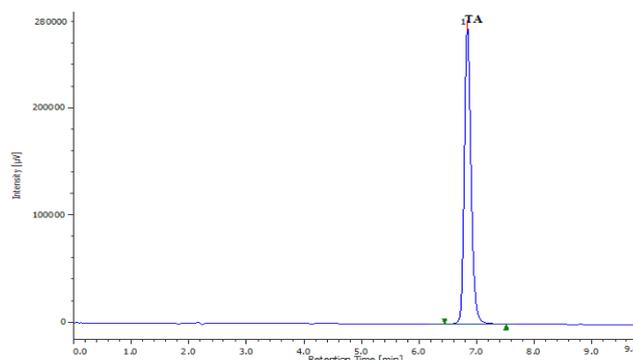


Figure 6.8: Chromatogram of TA sample (Clotan-200 mg)

Thus the new validated RP HPLC stability indicating analytical method could be used for the estimation of TA, two pharmacopoeial impurities (A and B) and degradation product.

6.9. METHODOLOGY FOR DETERMINATION OF TOLFENAMIC ACID AND ITS IMPURITIES

6.9.1. Instrumentation

Instrument	Source
HPLC System	Jasco LC-4000 series quaternary pump system (PU-4180), an on-line degasser, an auto-sampler (AS-4050), a column temperature controller (CO-4061) and a diode array detector (MD-4010). Software: Jasco Chrom NAV software
Column	Waters -Sunfire ODS C18 (4.6 x 250mm,5 μ m) column.
Sonicator	Citizon Ultrasonic Cleaner
pH meter	Digital pH Meter, Labtronics, LT-10
Electronic balance	Wensar Digital Electronic Balance MAB 220
HPLC water purification system	Bio-age Water purification system
Hot air oven	Universal Hot air Owen
Constant temperature water bath	Tempo

6.9.2. Chemicals and Reagents

- 1) Acetonitrile (HPLC grade), Rankem, India.
- 2) Water (HPLC grade), obtained from Bio age water purification system.
- 3) Ammonium Dihydrogen Ortho Phosphate (AR grade), S.D. Fine-Chem Ltd., Mumbai, India.
- 4) Glacial acetic acid (AR grade), Qualigens fine chemicals, Mahape, India.
- 5) Hydrochloric acid (AR grade), Qualigens fine chemicals, Mahape, India.
- 6) Sodium Hydroxide (AR grade), Qualigens fine chemicals, Mumbai, India.
- 7) Hydrogen peroxide, S.D. Fine-Chem Ltd., Mumbai, India.

6.9.3. Working standard

6.9.3.1. Tolfenamic acid (Pure and Cure Healthcare Ltd, Uttarakhand, India),

2 Chlorobenzoic acid (TA- Imp A) and 3-Chloro-2-methylaniline (TA- Imp B) (Sigma Aldrich, USA).

6.9.4. Solution preparation

6.9.4.1. Preparation of stock solution of TA

TA (100 mg) was weighed and transferred into 100 ml V.F., dissolved in 50 ml of acetonitrile and volume made up to the mark to obtain solution concentration 1000 µg/ml. Further dilutions were made from stock as per the requirements.

6.9.4.2. Preparation of stock solution of impurities

An amount of 100 mg of TA- Imp A and TA- Imp B was weighed and transferred into two separate 100 ml V.F. 75 ml of acetonitrile added, mixed well and sonicated for 10 min. Final volume was made up to the mark to obtain concentration of each impurity 1000 µg/ml.

6.9.4.3. Preparation of working standard solution of impurities

1ml of stock solution of each impurity was transferred into three different 10 ml V.F. Acetonitrile was added in the flasks to the mark to get concentration of 100 µg/ml of each impurity. Lower concentration was prepared by appropriate dilution.

6.9.4.4. Preparation of buffer – 10 mM ammonium dihydrogen ortho phosphate (pH = 4.0)

Ammonium dihydrogen ortho phosphate (1.15 gm) was dissolved in 1000 ml of HPLC grade water and pH adjuster to 2.5 with ortho phosphoric acid. The solution was filtered through 0.45 µ membrane filter.

6.9.4.5. Preparation of 1N sodium hydroxide solution

Sodium hydroxide flakes (4 gm) were dissolved in 50 ml of water in 100 ml V.F and volume made up to the mark with water to get 1N sodium hydroxide solution. Lower strength was prepared by appropriate dilutions.

6.9.4.6. Preparation of 1N hydrochloric acid solution

Hydrochloric acid (1N) was prepared by diluting 8.5 ml of Conc. HCl to 100 ml with water in V.F. Lower strength were prepared by appropriate dilutions.

6.9.4.7. Preparation of 10 % hydrogen peroxide solution

Hydrogen peroxide (10 % v/v) was prepared by appropriately diluting 30 % v/v H₂O₂ to 100 ml with water in V.F.

6.9.4.8. Diluent

Mixture of ACN and ammonium dihydrogen orthophosphate buffer (pH adjusted to 2.5 with ortho phosphoric acid) in the ratio 80:20 % v/v was used as diluent for dilution of samples.

6.9.5. Stress studies

The forced degradation studies were carried out with objectives to achieve substantial degradation of the drug. The drug was exposed to hydrolytic, oxidative, thermal and photolytic stress. The results were obtained by comparing four samples which were generated for every stress condition except thermal and photolytic stress viz., the blank stored under normal condition (blank untreated), the blank subjected to stress in same manner as that of drug solution (blank treated), zero time sample containing the drug (standard untreated) and the drug solution subjected to stress treatment. For thermal and photolytic stress conditions, only two samples were generated one sample exposed to stress condition and other is control.

6.9.5.1. Hydrolytic degradation

The hydrolytic degradations were carried out in acidic, alkaline and neutral conditions. Samples were prepared by taking 0.1 ml of stock solution of drug (1000 µg/ml) and 1ml of hydrolytic agent (HCl / NaOH / water) in 10 ml volumetric flask. Initially 0.1N strength of HCl and NaOH was used for study followed by 1N strength, if no degradation was seen in 0.1N strength of hydrolytic agent. The study was carried out at room temperature. If required, the samples were heated on constant temperature water bath at 70 °C for specified time intervals. After required exposure samples were neutralized by using equal strength of acid or alkali which ever was required. Finally volume was made up to the mark by using diluent and subjected for HPLC analysis by proposed method.

6.9.5.2. Oxidative degradation

Oxidative degradation was carried out using H₂O₂. Samples were prepared by using 0.1 ml of stock solution of drug (1000 µg/ml) and 1 ml of hydrogen peroxide (10 % v/v) in 10 ml V.F at RT for varied time intervals (7 days) Further the samples were processed by diluting up to the mark using diluent and subjected for HPLC analysis by proposed method.

6.9.5.3. Thermal degradation

Drug sample 10 mg each was taken in two 10 ml V.F and stoppered. One flask was exposed to dry heat in hot air oven for specified temperature (80 °C) and time interval (4 days) and another was kept as control. After exposure two separate solutions were prepared by weighing appropriate amount of sample exposed to thermal stress and control to produce concentration of 10 µg/ml. The samples were diluted up to the mark with the help of diluent and subjected to analysis by proposed HPLC method.

6.9.5.4. Photo degradation

Drug in sufficient amount was taken in 10 ml V.F and was exposed to direct sunlight for a specified time period (7 days). Another flask containing drug was kept as control. After required exposure two separate solutions were prepared by weighing appropriate amount of sample exposed to stress and control to produce concentration of 10 µg/ml. The samples were diluted up to the mark with the help of diluent and injected and analyzed by proposed HPLC method.

6.9.6. Validation studies

Validation of optimized stability indicating method was carried out with respect to parameters recommended under ICH guideline Q2 (R1).

6.9.6.1. Specificity and Selectivity

Establishment of resolution factor between the drug peak, pharmacopoeial impurity peaks and other obtained degradation peak was explored to determine the specificity of the method. Method Specificity was evaluated by comparing relative retention time (RRT) of TA, Imp A, Imp B and stressed samples of TA on the developed method. Chromatograms were recorded and RRT were calculated.

6.9.6.2. Linearity and Range

To establish linearity and range, stock solution of drug (1000 µg/ml) was further diluted with the help of diluent to get the drug concentration range of 10-100 µg/ml. The samples in triplicate were injected into the HPLC. For impurities, each impurity was diluted from working standard solutions to get a concentration range of 0.1-1 µg/ml. The samples were in triplicate were injected into the HPLC.

6.9.6.3. Accuracy

Accuracy of the proposed method was estimated using standard addition method (recovery studies). The percentage recovery studies of TA, TA-Imp A and TA-Imp B was carried out in triplicate at 3 different levels of 80 %, 100 % and 120 %, by spiking standard samples. Standard drug solutions of concentration 32 µg/ml (80 %), 40 µg/ml(100 %) and 48 µg/ml(120 %) were prepared by transferring 0.32 ml, 0.4 ml and 0.48 ml respectively from stock solution of drug into a series of 10 ml V.F each containing 0.4 ml of sample stock solution. The volume up to the mark was made with mobile phase. The solutions prepared were injected (n=3) into the chromatograph and the concentration were determined from the peak areas obtained from the chromatograms. Similarly study was carried out for individual impurity by preparing solutions of concentration 0.32 µg/ml (80 %), 0.4 µg/ml (100 %) and 0.48 µg/ml (120 %), from respective working standard of each impurity.

6.9.6.4. Precision

Intraday repeatability of the method was evaluated by analyzing three concentrations of TA (20, 40 and 80 µg/ml), and three concentration of TA-Imp A and TA-Imp B (2, 4 and 8 µg/ml). Interday precision was evaluated by assaying the chosen sets of TA, TA-Imp A and TA-Imp B in triplicate on two successive days using the same procedure stated under chromatographic conditions. % RSD of peak area was calculated.

6.9.6.5. Sensitivity

The sensitivity of the developed method was obtained by calculating LOD and LOQ for TA and its two impurities. LOD and LOQ were calculated based on the Standard deviation of the Response and the Slope as mentioned below.

LOD = 3.3 X Standard deviation of the response/ Slope of the calibration curve

LOQ = 10 X Standard deviation of the response/Slope of the calibration curve

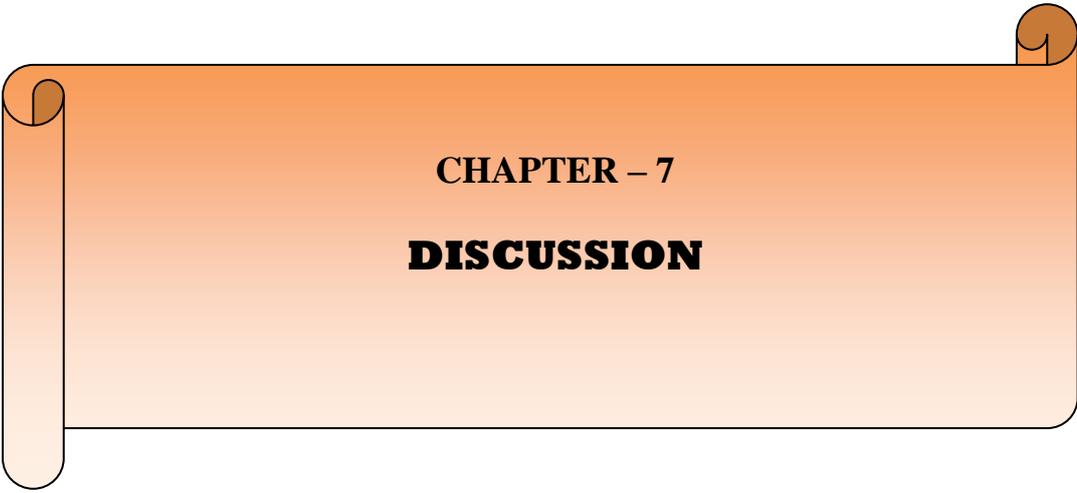
6.9.6.6. Robustness

Robustness of the developed method was determined by making three injections of mixture containing TA and pharmacopoeial impurities and by making deliberate change in method parameters like flow rate, and composition of mobile phase ratio. The values of percent relative standard deviation (% RSD) of peak area was recorded. Required aliquots were taken from stock solution of TA and working standard solution of each impurity in 10 ml V.F. Volume was made up to the mark using diluent to get concentration of solution containing 50 µg/ml of TA and 5 µg/ml of each impurity. To study the effect of variation in flow rate on the method, ± 0.2 ml of the optimized flow rate (1 ml) was studied on the method. The prepared solution was analyzed in triplicate by the proposed method by altering the flow rate of the proposed method to 0.8 ml (- 0.2 ml) and 1.2 ml (+ 0.2 ml).

To analyze the effect of change in composition of mobile phase ratio on the proposed method, the composition of organic phase in the mobile phase was altered by ± 2 % of the optimized composition. Hence the mobile phase used for study was ACN: buffer at 78: 22 % v/v (- 2 %) and 82:18 % v/v (+ 2 %). The prepared solution was analyzed in triplicate by the proposed method, carrying out the necessary changes in the mobile phase composition.

6.9.7. Analysis of Marketed product

For analysis of tablets, 10 tablets were weighed individually and their average weight determined. Tablets were then crushed to fine powder and powder equivalent to 40 mg was transferred to 100 ml V.F and dissolved in 75 ml acetonitrile with vigorous shaking for 15 minutes. The solution was sonicated for 10 min. The volume up to the mark was made with acetonitrile. The solution was then filtered through Whatman filter paper (#1). From this sample solution, 1 ml was transferred into 10 ml V.F and diluent was added up to the mark to obtain a concentration of 40 µg/ ml. The solution prepared were injected (n=3) into the chromatograph to determine the peak area. From the peak area of the standard solution and the tablet sample solution, the percentage content of TA in the marketed tablets was calculated.



CHAPTER – 7
DISCUSSION

7. DISCUSSION

The major goal of HPLC separation is to obtain complete separation of analytes of interest. Resolution between two analyte peaks is governed by the separation efficiency (N), analyte retention (k) and separation selectivity (α). Largest impact on resolution is contributed by α and hence optimizing the separation selectivity is a necessity during analytical method development. Parameters affecting selectivity are the experimental variables viz., stationary phase present in columns, organic phase ratio in mobile phase influencing solvation properties, physiological properties like temperature, pH, viscosity and so on. Selection of appropriate phases for separation is determined by system suitability parameters.

The search for such mobile phases is considered challenging in situations that present mixture of compounds for separations with diverse physiochemical properties. With options of varying composition of stationary phase being limited in HPLC, a number of options are possible to alter composition of mobile phase through measured changes in volumes or strength of the components to arrive at most appropriate system to achieve best possible separation. Mobile phase pH is said to have a profound effect on analyte retention and selectivity. Hence series of trials should be undertaken by varying pH of the mobile phase over a wide range and the separation profiles studied elaborately. The pH of mobile phase supports the ionization state in case of ionisable analytes. Varying mobile phase pH is a typical tool for controlling critical chromatographic parameters like retention time, shape of peak and also selectivity. Hypothetically, for establishing method robustness, it is generally recommended to apply a mobile phase pH of ± 2 pH units with respect to analyte pKa. However such hypothesis is difficult in complex situations that arise due to presence of multiple numbers of analytes with overlapping pKa values.

It becomes necessary to strengthen the testing methodology by providing robust mobile phase that could be resistant to being influenced by slight or wider range of pH. Examining the effect of changes in pH on the separation profiles is recommended to assess the method robustness. Analyte retention in RP-HPLC is influenced by hydrophobicity of the analyte. When ionisable analytes are present in a sample, the selectivity between analytes is said to be influenced by mobile phase pH.

For ionisable analytes, with an increase in the degree of ionization, the retention is likely to decrease. For basic analytes, at mobile phase pH below their pKa, the analyte is positively charged. At higher pH (i.e., above their pKa), they will be in their neutral form and is likely to be better retained by reversed-phase. On the contrary, strongest retention is showed by acidic species with a mobile phase below their pKa and thus more weakly retained at high pH, in their deprotonated form.

It should be noted that at low pH, the acidic analytes are present in their non-ionized, neutral form and therefore show strongest retention. As the mobile phase pH is increased to the analytes pKa and beyond, the degree of ionization increases and a gradual decrease in retention is observed. Conversely, at low pH the basic analytes are positively charged and hence show shorter retention. As the pH increases, the ionization is suppressed and analyte retention increases. Protonated basic analytes exhibit low retention and/or poor peak shape when analyzed at low pH. Performing the analysis at higher pH is said to improve both peak shape, retention, differences in selectivity is observed, peaks elution order is also affected & also provide improved sensitivity.

Mobile phase pH can thus provide useful selectivity especially in samples containing both acidic and basic components thereby addressing resolution issues. Optimization of pH is utilized during method development to alter selectivity and help optimize the separation. For maximizing column lifetime, silica based RP columns are useful within a limited pH range of approx. 2-8 and are therefore not preferred to work at higher pH. The separation parameters such as pH, ratio of organic to aqueous phase, buffer concentration and temperature was proposed to be altered during the study and changes in analyte retention behavior monitored for improved understanding.

Generally mobile phase pH is controlled with buffers of high buffer capacity. Buffer capacity expresses strength of the buffer. The capacity specifies the quantum of hydronium ions or hydroxyl ions that are essential to change the pH of the buffer by some value. Higher the buffer capacity more is the concentration of acid or base that can be withstood by system with fluctuations in pH. With the use of phosphate buffer no buffering capacity is noticed at around pH 4.5 as pKa values of phosphate are at 2 and 7. Even addition of small amount of H⁺ and OH⁻ in the range of pH 4 to 5 to mobile phase causes wide change in pH.

This causes an impact on the retention profile of analyte and reflects on the resolution. Judicious use of acid and base in such a sensitive range could be one of the ways to cause differential ionization and hence migration to cause separation with good resolution.

Mefenamic acid was chosen for the study as it provides impurities, few of them including drug having pKa's between 4.0 to 4.7 and one at 2.9. Similarly Tolfenamic acid was selected as its impurities were limited in number having overlapping pKa's and diverse in nature.

7.1. Optimizing separation of compounds with closer pKa values. Case study- Impurity profiling of Mefenamic acid

7.1.1. Identification of process related impurities of mefenamic acid

From synthetic schemes of mefenamic acid, it was found that 2, 3-Dimethylaniline, 2-Chlorobenzoic acid and Benzoic acid appears as reactants/intermediates. The presence of these compounds in MA in trace amounts are listed as specified impurities A (2, 3-Dimethylaniline), C (2-Chlorobenzoic acid) and D (Benzoic acid) in the BP monograph as their presence beyond permissible limits is shown to express undesirable effects in physiological system. Also in the monograph of MA, two other impurities are listed and mentioned as 'other detectable impurities'. It is inferred that Imp A, Imp C and Imp D are 'Process related impurities' and are likely to be present with MA after synthesis unless purification methods have been undertaken. These compounds as Imp A (pKa = 4.70), Imp C (pKa = 2.89), and Imp D (pKa = 4.19), were considered for separation along with the active compound MA (pKa = 4.20) in the process of analytical development. The samples of Imp A, Imp C and Imp D were procured in purest form and used as standards during method development.

7.1.2. Development of new HPLC method for estimation of Mefenamic acid in presence of its pharmacopoeial impurities

The Pharmacopoeial method for assay of MA represented an isocratic elution mode of analysis with ternary solvent as mobile phase comprising of 50 mM Phosphate buffer (pH adjusted to 5 with dilute ammonia): Acetonitrile: THF (40:46:14 % v/v) on C18 column with a flow rate of 1 ml/min, at detection wavelength of 254 nm. The method when applied in our laboratory showed several noise peaks in the vicinity of impurity peaks when applied to solution containing mixture of MA with impurities of research interest.

The solvent did not appear to provide any specific advantage nor produce satisfactory smooth baseline (Fig 7.1.2.1) necessitating improvisation. Despite its strong solubilization power and eluotropic strength, THF generally avoided/minimized in reversed-phase LC. Problems related to toxicity and safety issues of THF due to peroxide formation prevent its widespread use except in gel permeation chromatography.

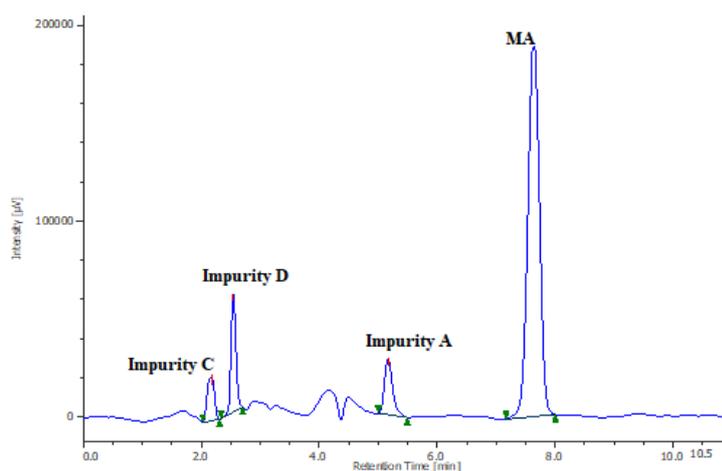


Figure 7.1.2.1: Chromatogram of MA and impurities (A, C and D) as per BP method.

A new RP-HPLC method was developed for quantification of MA and its pharmacopoeial impurities (Imp A, Imp C and Imp D) on Waters Sunfire C18 column with acetonitrile: phosphate buffer pH 4 (55:45 % v/v) as mobile phase at detection wavelength of 225 nm, in isocratic mode. Since the pKa values of the drug and its impurities were in the range of 2.89 to 4.70, various exploratory trials at different pH of the buffer solution (3 to 6) were performed to optimize the pH of mobile phase and the ratio of buffer with organic phase (acetonitrile).

With the use of phosphate buffer the sensitive region between pH 4 to 5 provided good platform for undertaking systematic study on separation principles as MA and three of its impurities had pKa ranging from 4 to 5. Study concerned with mining of peak parameters for the four components of the study including the drug MA that were coeluted with mobile phase at varied pH and at different composition of mobile phase at fixed pH is graphically presented as Fig 7.1.2.2 and Fig. 7.1.2.3.

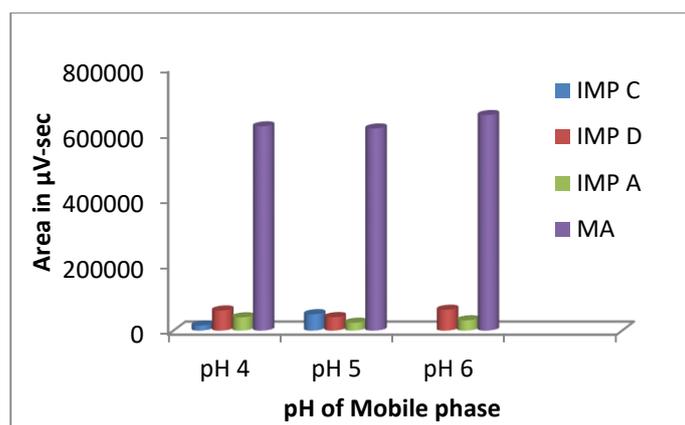


Figure 7.1.2.2: Peak areas of MA, Imp A, Imp C and Imp D varying with pH of buffer (mobile phase)

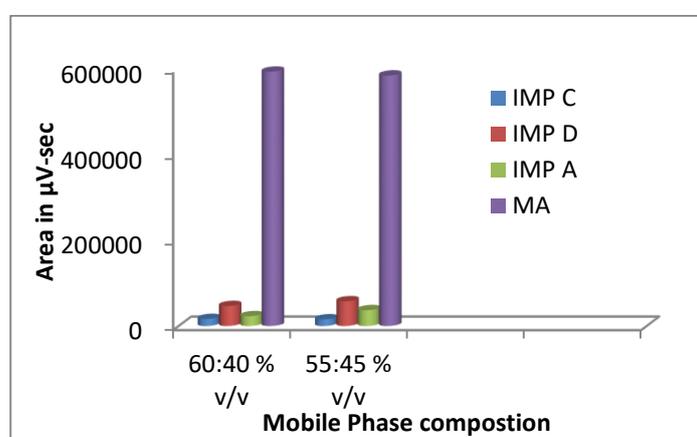


Figure 7.1.2.3: Peak areas of MA, Imp A, Imp C and Imp D varying with organic phase ratio (mobile phase)

Retention time of the drug and its pharmacopoeial impurities (A, C and D) in mixture were seen to be affected by the pH of the mobile phase as seen from Fig. 7.1.2.4.

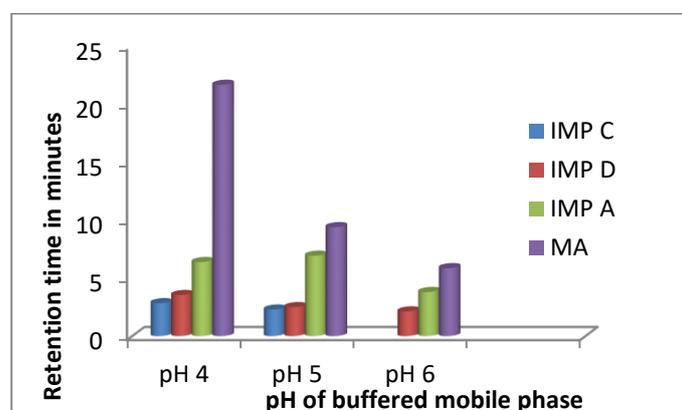


Figure 7.1.2.4: Retention time of MA, Imp A, Imp C and Imp D varying with pH of buffered mobile phase

It has been noticed that at pH 4 the acidic compounds (MA, Imp C and Imp D) below their pKa values show greater retention as compared to pH higher than their pKa values. Interestingly weakly basic compound (Imp A) showed less retention at pH lower than its pKa value and more retention at pH higher (pH 5) than its pKa value. Rt of MA dropped sharply from 20.65 min at pH 4 to 5.86 min at pH 6 indicating ionization of acidic analyte. At pH 4 retention is high at 20.65 min and with increase of pH there was gradual decrease in retention observed indicating increase in degree of ionization of MA. Similar effects were observed in cases of other acidic analytes Imp C and Imp D.

Conversely Imp A, a basic compound by nature showed low retention at acidic pH and showed poor ionizing ability thereby showing higher retention at higher pH. The new HPLC method developed and optimized minimized the noise peaks interfering with measurement of peak areas of MA and its impurities on a smooth base line (Fig 7.1.2.5). Also, THF as a component of mobile phase could be avoided. The developed new RP HPLC method was validated as per ICH Q2R1 guidelines.

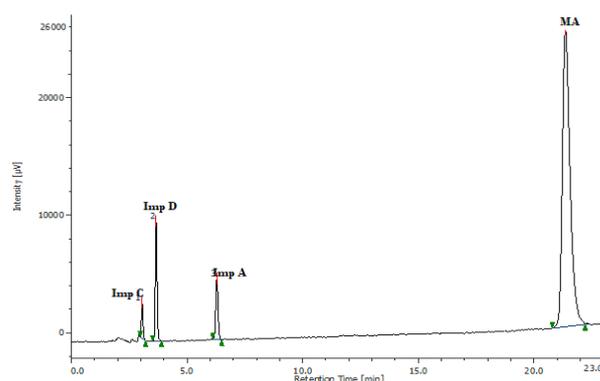


Figure 7.1.2.5: Optimized chromatogram of Mefenamic acid and impurities (A, C and D)

7.1.3. Forced degradation study of Mefenamic acid

Degradation was seen in MA samples when subjected to oxidative stress condition. Two prominent additional peaks were seen in the chromatogram along with MA peak. The two additional peaks had RRTs of 0.46 and 0.74 for DP I and DP II respectively which were not identical from the RRTs of the three specified impurities, thus indicating possible oxidative degradation leading to formation of two degradation products (DP I and DP II) different from the specified impurities (for possible degradation pathway see Appendix).

Decrease in drug peak area was low and insignificant when subjected to hydrolysis (acid, base and neutral), thermal and photo stress conditions, with no additional peak seen in the chromatogram for a run time period of 25 min as shown in Figure 7.1.3.

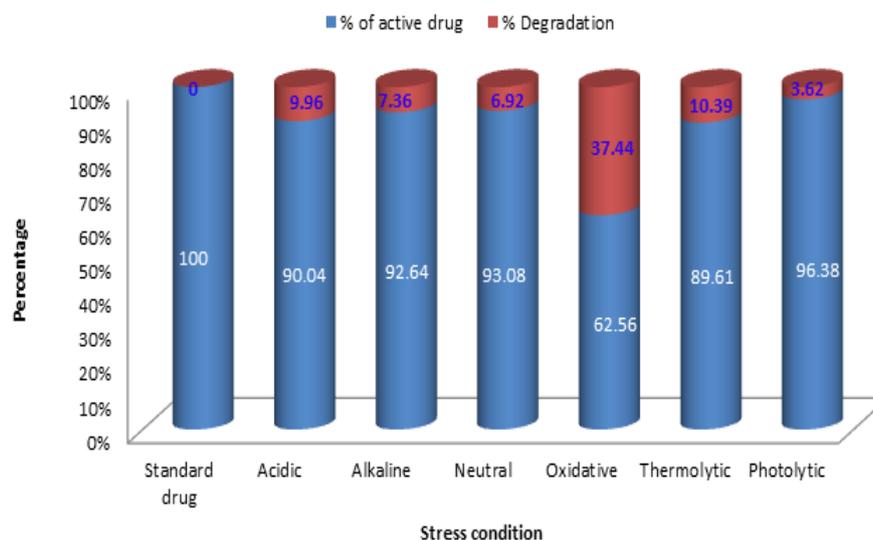


Figure 7.1.3: Degradation study profile of Mefenamic acid

7.2. Analysis of drug in presence of its metabolite. Case study- Impurity profiling of Carbimazole

Drug analysis is an indispensable critical component of scientific research. Majority of research findings incorporate a section on analysis for interpreting results. In terms of structure and chemical properties, drugs are very diverse compounds. The beneficiary action of these compounds is highly dose-dependent. Each drug has a well-defined therapeutic range, and their existence at concentrations lower than the minimum level elicits very weak beneficial therapeutic effects in patients, while concentrations above the threshold or maximum limit invites side effects, which may be undesirable in sick patients. Therapeutic drug monitoring is frequently applied in clinical practice. The establishment of powerful testing methodologies for drug monitoring in biological matrices is essential for determining patient safety.

Thus, highly efficient techniques involved in the drug or metabolite extraction and enrichment, separation, and sensitive cum selective detection are essential for their reliable determination in complex biological samples. An essential and important part of the drug development process is assessing the metabolic fate of drugs.

Research on drug metabolism pathways, chemistry of drug-drug interactions, polymorphism effects have been shown to influence phase I and/or II metabolism of a drug. During drug evaluation, the research on drug metabolism is of extreme importance especially when metabolites are pharmacologically active or toxic, or when a drug highly sensitive to metabolism. During the developmental phase drug discovery focuses on identification of drug metabolism and pharmacokinetic issues at the earliest possible stage to reduce the attrition rate of drug candidates. Metabolic fate of drugs can be accountable for issues correlated with their bioavailability. Drug-metabolite interactions compromise on beneficial pharmacological activity or induce undesirable toxicity. For a suitable metabolic assessment of drugs the known identity of metabolites is the prerequisite. **Suitable bioanalytical methods have to be developed and validated** for the successful monitoring of drug metabolism.

To determine the concentrations of drugs and their metabolites, several methods have been proposed in which selection depends on the complexity of the sample and the chemical nature of the analytes and the matrices. Biological matrices which often exist at low concentrations in samples are complex and often contain proteins, lipids, salts, acids, bases, and various other organic and inorganic compounds properties similar to the analytes. Generally methods based on combining a very efficient separation technique like HPLC/UPLC or HPTLC with a sensitive detection method is used for analyzing drugs belonging to various therapeutic classes in different biological samples.

LC methods are efficient and robust, specific, with merits of convenience, operation, strong separation ability, and diverse sample application. HPLC with UV-VIS detector; fluorescence, diode array (DAD), and mass spectrometric detection methods have been used for the analysis of different drugs in various biological samples. For the analysis of drugs in biological samples preparation of samples before chromatographic analysis is an important stage. It enables accompanying undesirable components to be removed and the drug to be enriched or concentrated. Before chromatographic analysis the choice of a sample preparation method is closely related to the properties of the investigated drugs, metabolites, and matrices.

In the current study, efforts were made to identify a drug that can readily breakdown to its metabolite and interfere with analytes detection and quantification. Carbimazole is one such drug that easily breaks down to Methimazole, a metabolite with similar therapeutic

activity but of a different magnitude. Incidentally methimazole by itself is used in the synthesis of Carbimazole.

7.2.1. Identification of process related impurities of Carbimazole

Methimazole is generally used as starting material for the synthesis of Carbimazole as seen from synthetic schemes of CZ. Trace amount of MZ is listed as specified impurity A in the BP monograph of CZ. Thus MZ was identified as a process related impurity of CZ. Imp A was considered for developing a new RP-HPLC method for separation of CZ from MZ and their estimation. Pure sample of Imp A were procured and used for the study.

7.2.2. Development of new HPLC method for estimation of Carbimazole in presence of its pharmacopoeial impurity

The Pharmacopoeial analytical method for CZ represented an isocratic elution program mode of analysis with binary solvent as mobile phase comprising of acetonitrile: water (10:90 % v/v) on C18 column with a flow rate of 1 ml/min at detection wavelength of 254 nm. An additional peak in the vicinity of Imp A peak was seen (Fig 7.2.2.1). Absence of buffer of specific pH in the mobile phase expected to promote variation in pH during the course of study when exposed to different environmental atmospheric conditions.

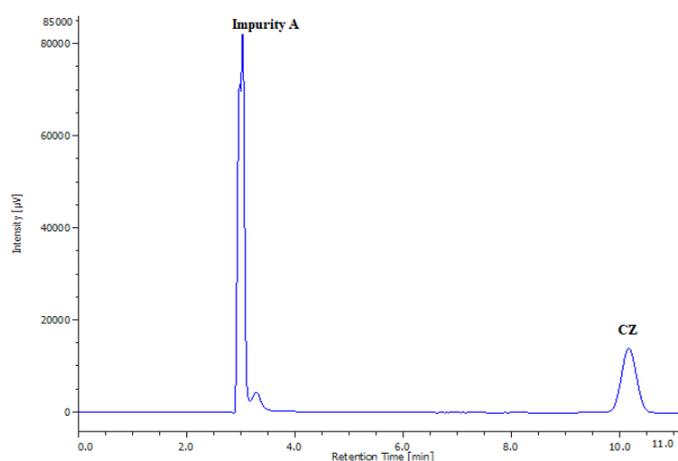


Figure 7.2.2.1: Chromatogram of CZ and Imp A as per BP method.

For quantification of CZ and its pharmacopoeial impurity A, a new RP-HPLC method was developed on Waters Sunfire C18 column with acetonitrile: phosphate buffer pH 2.5 (50:50 % v/v) as mobile phase at detection wavelength of 260 nm, in isocratic mode.

Various exploratory trials at different pH of the buffer solution (2.5 to 6.5) were performed to optimize the mobile phase and ratio of buffer with organic phase (acetonitrile). The new HPLC method could overcome the formation of additional peak in the vicinity of impurity peak (Fig 7.2.2.2). Also total run time was shorter compared to official method. Buffered mobile phase produced reproducible chromatograms inspite of varying conditions deliberately. The new method was validated as per ICH Q2R1 guidelines.

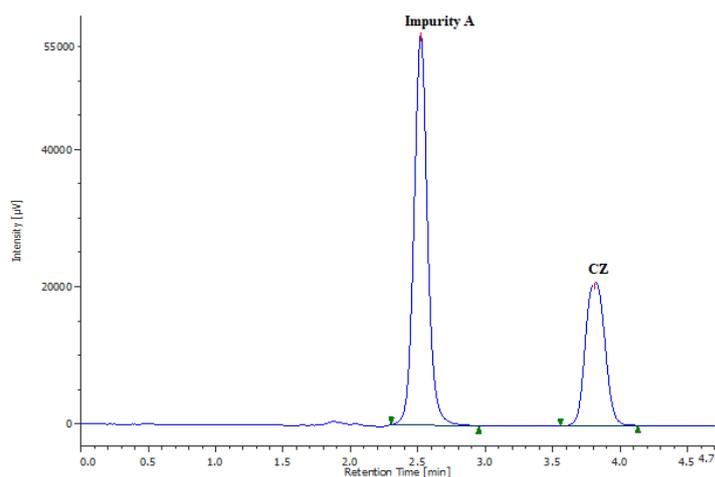


Figure 7.2.2.2: Optimized chromatogram of CZ and Imp A

7.2.3. Comparison of phosphate buffer method with acetate buffer method applied for analysis of Carbimazole

The system suitability parameters of the two methods developed for analysis of Carbimazole is presented below.

Table 7.2.3: Comparison of experimental variables for analysis of Carbimazole with phosphate buffer method and acetate buffer method

Parameters	Phosphate buffer method	Acetate buffer method
Column	Sunfire C-18	Sunfire C-18
Mobile Phase	ACN: 10 mM Ammonium dihydrogen orthophosphate buffer (50:50 % v/v)	ACN: 10 mM Ammonium dihydrogen orthophosphate buffer (50:50 % v/v)
pH of the buffered mobile phase	2.5	2.5
Detection wavelength	260	260
Retention time	3.81	3.78
Theoretical plate number	7791	4342
Peak symmetry	1.05	1.03

The new developed method was carried in acetate buffer with pH 2.5 in order to characterize the impurity by LCMS. Fig 7.2.3 highlights the chromatogram of CZ and its impurity in acetate buffer.

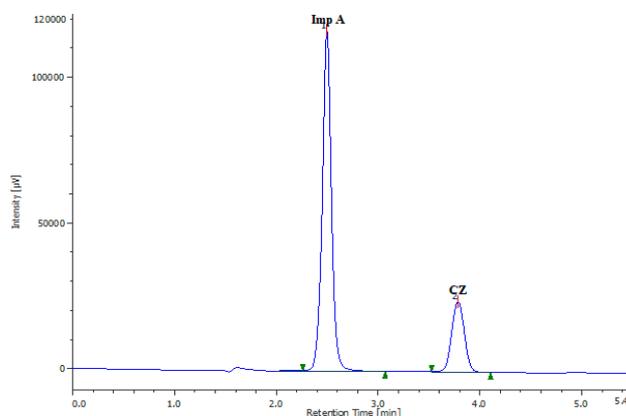


Figure 7.2.3: Optimized chromatogram of CZ and Imp A in acetate buffer

7.2.4. Forced degradation study of Carbimazole

Carbimazole was highly susceptible to degradation in hydrolytic, oxidative and thermal stress conditions with formation of one additional peak. The RRT of the additional peak (DP I) 0.66 min matched with the RRT of pharmacopoeial impurity A 0.66 min, thus confirming the formation of Imp A when CZ is exposed to hydrolytic, oxidative and thermal conditions. Decrease in drug peak area was seen to an extent of less than 10 % when it was subjected to photo stress conditions although no additional peak could be seen (Fig 7.2.4.1). Thus confirming the stability of CZ in thermal stress conditions.

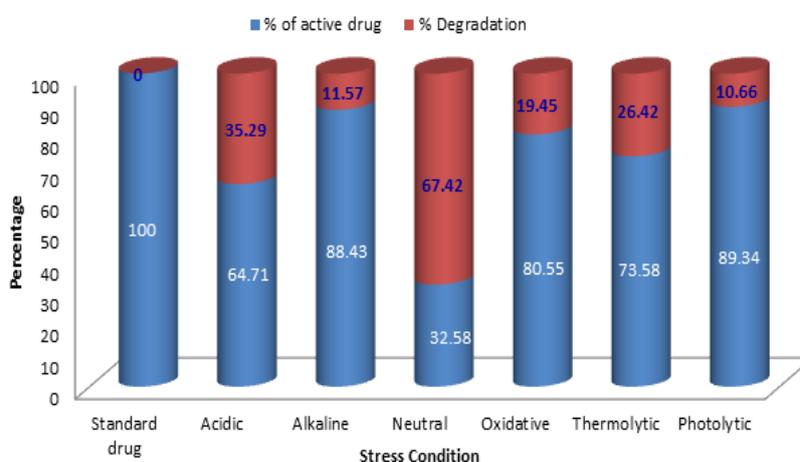


Figure 7.2.4: Degradation study profile of Carbimazole

7.2.5. Identification of degradation products of Carbimazole by LCMS

The common degradation product (DP I) of CZ obtained in various stress conditions was confirmed by LC-MS study. The positive ions, $[M+H]^+$ at m/z value of 115, was the molecular ion of the DP I. The molecular weight of the DP I was identified as 114 with respect to its molecular ion. The molecular weight of specified Imp A is 114.17 which approximately match with the DP I molecular weight. Thus confirming that the common degradant product (DP I) formed under various stress conditions is specified impurity A.

7.3. Use of Special techniques-Derivatization to explore compounds having less detection ability and application of LC-MS for Characterization. Case study - Impurity profiling of Cyclizine Hydrochloride

7.3.1. Identification of process related impurities of Cyclizine Hydrochloride

BP Monograph of Cyclizine HCl lists Imp A (1-methyl piperazine) and Imp B (benzhydrol) as specified impurities. Synthetic schemes of CY shows the use of 1-methyl piperazine and benzhydrol as starting material for its synthesis. Thus Imp A and Imp B was identified as a process related impurity of CY. RP-HPLC method was proposed to be developed for separation and estimation of CY and its impurities (Imp A and Imp B). Pure samples of Imp A and B were procured and used for the study.

7.3.2. Development of new HPLC method for estimation of Cyclizine HCl in presence of its pharmacopoeial impurities

BP monograph of CY lists Gas chromatography (GC) as an analytical method for estimation of related substances in CY. GC method requires the compound to be volatile and thermally stable during the process of analysis. Also any other impurity including degradant if formed can get detected in GC only if such compounds are volatile and remain thermally stable. Versatility of GC methods as such is lower and impurity profiling type of study requires a large number of compounds both volatile and non-volatile to be simultaneously analyzed when present in the mixture. That makes LC methods to be most popular in profiling.

The safety of a drug product depends not only on the intrinsic ability of the molecule to demonstrate desirable therapeutic efficacy but also on its toxicological properties. The risk reward ratio eventually determines the acceptability of a molecule as a miracle pill.

In spite of such issues, drugs are often screened for purity with respect to product and process related impurities and degradation products. These can often be controlled provided they are detected and their presence demonstrated through rugged testing methods. Hence testing methods should be robust enough to detect, determine and propose strategies to control the presence of these undesirable compounds (impurities) in drug substances and the products. Building up of the quality into the product is expected to commence from earliest possible steps, perhaps from the stage of synthesis of the API itself. The quality and quantity of the drug product and the substances used during its manufacturing are typically determined via different analytical methods, such as volumetric, chromatographic, spectroscopic, and electrochemical methods. The role played by the hyphenated systems in detecting components present even at the sub micro levels have given opportunities to the scientific community to predict and witness all possible entities co-existing with the drug like never before.

Among the chromatographic techniques, liquid chromatography (LC) today dominates the area of pharmaceutical analysis and has been a big boon to pharmaceutical industry and the mankind to confirm the quality of drugs produced and marketed. LC-based techniques due to their high resolving power help in the structure determination and quantitation of degradation products and impurities in APIs and pharmaceutical formulations. In this respect, the choice of the detector is critical to guarantee the versatility of not missing out on the detectability of components present even in most complex mixtures. The most commonly used detector in LC is the ultraviolet (UV) detector. It is capable of monitoring several wavelengths simultaneously by applying a multiple wavelength scanning program. However, it can hardly impart a distinct identification. The addition of further detection capabilities to LC separations have brought in significant opportunities to support pharmaceutical development efficiently and ensure the quality and safety of pharmaceuticals. The hyphenation of LC and mass spectrometry (MS) has taken a key role in drug discovery and development due to the high throughput, selectivity and sensitivity.

Impurities in pharmaceutical products are undesirable and their presence is responsible for change in quality especially with respect to safety and efficacy. Sources of impurities could be diverse and includes starting materials/ reactants, active reagents, catalysts that

promote synthetic reactions, intermediate compounds, solvents used at different stages of manufacture in multiple steps and degradation products formed during manufacture and long term storage of the drug. Impurities are classified into several classes that depend upon their origin, composition and biological safety. LC–MS is considered to be an indispensable tool for structure characterization. LC-MS combines the separation power of LC (production of pure fractions from chemical mixture) with the mass analysis capabilities of MS (for characterization of compounds with high molecular specificity and detection sensitivity for compounds in multiple stages of drug development (discovery, preclinical and clinical phases), metabolism studies (both in vitro and in vivo), and for the identification of impurities and degradation products.

It is universally accepted that HPLC is a method of choice for the qualitative and quantitative determination of multi-components at microgram levels. The search for sensitive techniques for detection and determination of product related impurities and degradants co-existing with active drugs have brought in situations wherein the complete matrix of the sample can be visualized. However inability to establish mass balance has been a matter of great concern especially molecules lacking adequate absorptivity due to poor chromophoric effects in them. Many substances of interest go undetected and cannot be directly analyzed by HPLC. One possible solution for such issues brings in derivatization of such molecules as a possibility to extend the analytical applications.

Derivatization introduces characteristic groups into sample molecules to increase their sensitivity for UV absorption and thereby their detection. By using organic or electrochemical reactions, such as oxidation and reduction, or by displacement or addition reactions derivatization can be obtained. The derivatization reaction are generally performed before injection into LC system although automation can also help in reducing sample preparation time and increase the process standardization (in terms of precision of the reagent dispensation and homogeneity of the reaction time) for the best reproducibility. Various chemical changes involving enrichment of an analyte with chromophores is performed with appropriate chemical reaction/s in order to make it suitable for a specific mode of detection. Commonly derivatization process preferred is an instantaneous chemical reaction online or post column based on reaction of the reagent with a functional entity of the analyte.

This ensures no impact on the separation profile/ property of the concerned molecule. The factors which determine the choice of chemical change include the nature of the analyte, the sample matrix, the required changes in the analyte properties, and the analytical method to be used.

In chemistry derivatization is a technique where in a chemical compound is transformed into a product of similar chemical structure, called a derivative. Most commonly a particular functional group of the analyte participates in the derivatization reaction and transforms itself to a derivate having different reactivity, solubility, boiling point, melting point, aggregate state, or chemical composition. In HPLC derivatization brings in characteristic groups into analyte/s for the purpose of increasing the sensitivity to UV absorption and eventually the detection. Such reactions could be organic or electrochemical, such as oxidation - reduction, or displacement or addition reactions. The resulting products can be used favorably for quantification after separation in the column in case of precolumn derivation. Sometimes a sample may produce a chemical reaction only with the analytes upon addition of a reagent without affecting the matrix. But some matrix components are derivatized unintentionally. Thus it is preferable to have only the analytes derivatized to obtain a better separation from the matrix. A wide range of derivatization reagents and procedures are listed where the reagents carrying specific moieties provide a desired property to the analytes, also specific reactive groups which permit the reaction with the analyte. Multiple step derivatization as well as derivatization followed by a second one is known.

Derivatization for HPLC is often directed toward amino acids, amines, saccharides, thiols, carboxylic acids, steroids, alcohols, fatty acids, and several inorganic species, that are difficult to detect at low levels by absorbance, luminescence, or electrochemical means. Upon pre derivatization small hydrophilic molecules are often converted into larger more hydrophobic compounds, making reversed-phase HPLC easier or feasible. Pre column derivatization has been applied to the analysis of many compounds, including amino acids using various reagents like Fluorescein, o-Phthalaldehyde Naphthalenedialdehyde, 3-(4-Carboxybenzoyl)-2-quinolinecarboxaldehyde(CBQCA), Fluorescein isothiocyanate (FITC) 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), 4-Chloro-7-nitrobenzofurazan (NBD chloride), Dansyl chloride, Dansyl hydrazine Bromomethylcoumarin Phenylglyoxal Thiazole orange etc.

The reaction of an analyte or group of analytes, with the derivatization reagent must be complete or close to complete. Also the reaction must take place in a length of time that is not prohibitive, with a very little loss of the analyte by formation of artifacts or decomposition products. Only when such conditions are met a specific chosen derivatization is applied successfully. The stability of the derivative is also important. The percent derivatization should be as near to 100 % as possible. It is also important that the reaction yield only one derivative per analyte, so that co elutions of extra peaks does not occur, and so solute identification and quantitation are accurate. Since highly sensitive procedures that lead to formation of derivatives of reasonable stability using low-cost reagents are very desirable when analyzing small samples or samples with low concentration of the amine by HPLC, NBD-Cl and NBD-F as derivatizing agents has become quite popular.

The reaction of the fluorogenic reagents 7-Chloro-4-nitrobenzoxadiazole (NBD-Cl) and 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) with amines and amino acids produces a highly fluorescent NBD-product, that are relatively stable (Fig 7.3.2.1). Thus becoming a standard method for analysis of very low levels of amines. A significant amount of studies have been carried out using these dyes for developing methods that can be used for quantification of amines and amino acids in pharmaceutical and biological samples by labeling the amine functional groups.

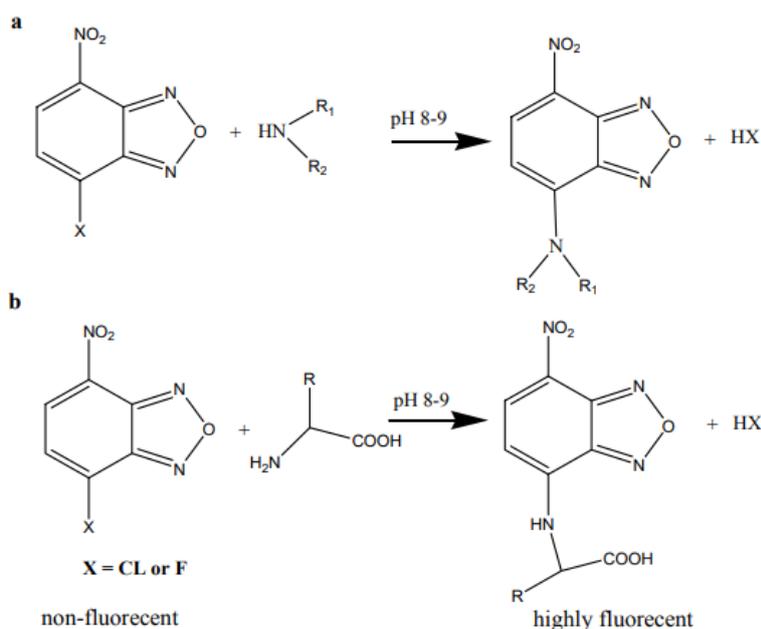


Figure 7.3.2.1: Schematic diagram for the reaction of amines and amino acids with NBD-Cl and NBD-F

The strong fluorescence of the NBD-amines is the best observed in solvents of low polarity. NBD-Cl derivatives show an optimum excitation wavelength of 464 nm. As compare to dansyl chloride (DNS), NBD chloride is considered to be more stable to moisture and also more soluble in aqueous solutions. Because of distinct advantages NBD chloride is used for detecting and determining small quantities of amines and amino acids. NBD-Cl is widely used for the analysis of compounds with primary or secondary amino groups owing to its low cost in spite of its lower reactivity towards amino acids and a longer reaction time compared to NBD-F. Few of the compounds which are estimated by HPLC using NBD-Cl as derivatization agent are Amlodipine, Tyramine, Lisinopril, Amlodipine Fluoxetine (FL), Norfluoxetine (NFL), Tianeptine, Gabapentin, Insulin, Topiramate, Reboxetine, Fluvoxamine, Trimetazidine, Paroxetine, Sertraline, Hydroxyproline, Amino acids, Domoic acid etc.

An alternative RP-HPLC method was developed for quantification of CY and its Pharmacopoeial impurities (Imp A, and Imp B) on Waters Sunfire C18 column with Acetonitrile: Phosphate buffer pH 6.5 (80:20 % v/v) as mobile phase at detection wavelength of 225 nm, in isocratic mode. Imp A lacked a chromophore in its chemical structure resulting in the need for its derivatization. Derivatized Imp A showed absorption in the UV region. Buffered mobile phase of pH 6.5 (phosphate buffer) provided satisfactory elution of CY with pKa 8.51. Several trials were performed to optimize the mobile phase and the ratio comprising of buffer and acetonitrile. The new HPLC method could provide peak areas of CY and its impurities on a smooth base line and over a short runtime period as seen in Fig 7.3.2.2, enabling quick and accurate analysis. The method was validated as per ICH Q2R1 guidelines.

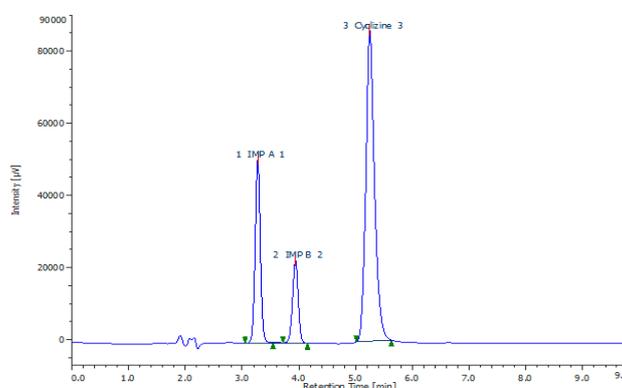


Figure 7.3.2.2: Optimized chromatogram of CY and impurities (A and B)

7.3.3. Comparison of phosphate buffer method with acetate buffer method applied for analysis of Cyclizine Hydrochloride

The system suitability parameters of the two methods developed for analysis of Cyclizine Hydrochloride is presented below.

Table 7.3.3: Comparison of experimental variables for analysis of Cyclizine Hydrochloride with phosphate buffer method and acetate buffer method

Parameters	Phosphate buffer method	Acetate buffer method
Column	Sunfire C-18	Sunfire C-18
Mobile Phase	ACN: 10 mM ammonium dihydrogen orthophosphate buffer (80:20 % v/v)	ACN: 10 mM ammonium dihydrogen orthophosphate buffer (80:20 % v/v)
pH of the buffered mobile phase	6.5	6.5
Detection wavelength	225	225
Retention time	5.24	6.63
Theoretical plate number	6405	5976
Peak symmetry	1.23	1.52

For the characterization of all organic, inorganic and biological compounds, LC-MS is considered to be one of the most important tools. Because of its very high sensitivity and selectivity LC-MS finds many applications. Success of the LC/MS experiment is ensured by a careful choice of an appropriate acid or buffer. Presence of non-volatile aqueous components, like salts, acids, bases, or buffers, will greatly affect and even decrease and prevent the detection of analyte ions. Ion sources and vacuum regions of mass spectrometers are also affected by these non-volatile buffers. Hence the use of non-volatile phosphate or citrate buffers is strongly discouraged during ionization and for practical reasons.

Due to deposition of non-volatile components, frequent cleaning of the instrument is often required. When non-volatile buffers are used, ion suppression and decreased sensitivity is observed. In most cases, volatile acids or buffers are used. Ammonium acetate or formate buffers with concentrations ranging from 2 to 50 mM can be used, with a maximum concentration of 10 – 20 mM is endorsed to avoid ion suppression.

Hence the developed method was carried in acetate buffer with pH 6.5 in order to characterize the impurities by LCMS. Fig 7.3.3 highlights the chromatogram of CY and its impurities in acetate buffer.

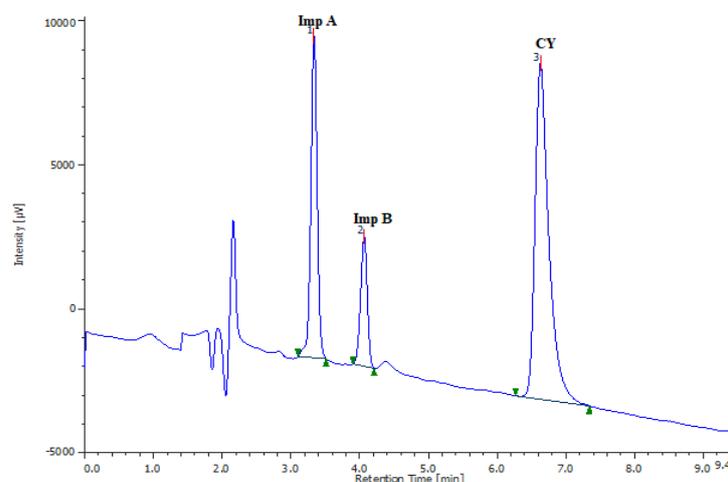


Figure 7.3.3 : Optimized chromatogram of CY and impurities (A and B) in acetate buffer

7.3.4. Forced degradation study of Cyclizine HCl

Cyclizine HCl showed degradation in acid and oxidation stress conditions with formation of one additional peak apart from the drug peak in acidic stress condition and two additional peaks apart from drug peak in oxidative stress conditions. The RRT of the additional peak (DP I) formed in the acidic medium matched with the RRT of pharmacopoeial impurity B, thus confirming the formation of Imp B when CY is exposed to acidic conditions. The RRT of DP I formed under oxidation condition were different from the RRTs of specified Imp A, Imp B and DP I formed in acidic condition. The RRT of the other peak (DP II) matched with the RRT of pharmacopoeial impurity B, thus confirming the formation of Imp B.

Thus confirming formation of Imp B in acid and oxidation stress conditions. Decrease in drug peak area was seen to an extent of 8 % when it was subjected to basic neutral, thermal and photo stress conditions with no additional peak thus confirming the stability of CY (Fig 7.3.4).

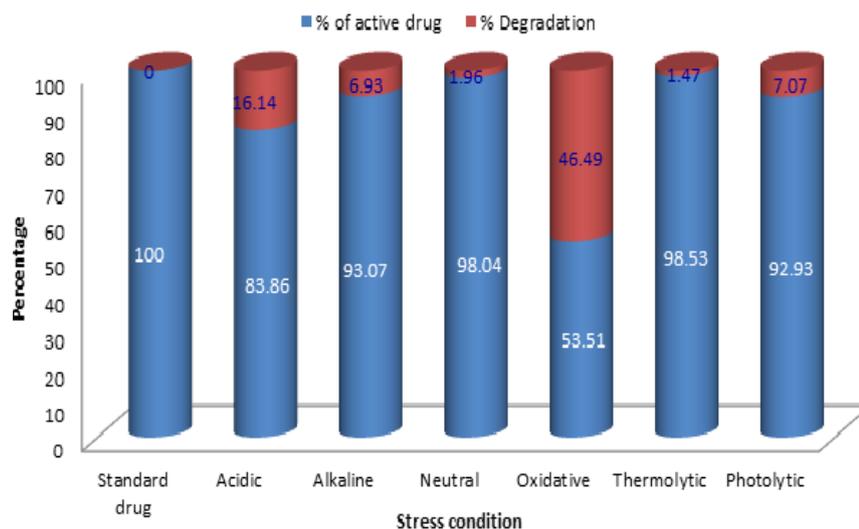


Figure 7.3.4: Degradation study profile of Cyclizine HCl

7.3.5. Identification of degradation products of Cyclizine HCl by LCMS

The degradation products of CY formed in acidic and oxidation stress condition were confirmed by LC-MS study. The fragment ion peak of common degradant (RRT = 0.72) obtained in acidic and oxidative stress medium appeared at m/z value of 167.1. The peak represented the molecular mass of degradant (RRT = 0.72) which was found to be 184.23, due to loss of alcohol group, which approximately matches with the specified Imp B. The m/z of second degradant (RRT = 0.54) formed under oxidative stress condition appeared with m/z value of 283.2. The m/z of degradant (RRT = 0.54) was found to be 282.38 confirming formation of N-oxide of Cyclizine. Thus it was identified that the common degradant formed under acid hydrolysis and oxidative stress condition is Imp B and the other degradant formed in oxidation medium is the N-oxide of Cyclizine.

7.4. HPLC method development for multicomponent mixture with variable acid dissociation constants. Case study - Tolfenamic Acid determination in presence of Imp A and Imp B

7.4.1 Identification of process related impurities of Tolfenamic acid

Schemes for synthesis of TA were surveyed from literature. It was found that 2-Chlorobenzoic acid and 2-Chloro 3-methylaniline are used as starting material for the synthesis of TA. The presence of these substances are listed as Imp A (2-Chlorobenzoic acid) and B (2-chloro 3-methylaniline) in the BP monograph of TA. Also Imp C (3-Chloro-4-methyl-9-oxo-9,10-dihydroacridine) is mentioned in the monograph of TA.

Imp A and B are 'Process related impurities' since they are likely to remain with drug TA after synthesis involving use of said compounds as raw materials and purification methods being inadequate. These compounds as Imp A ($pK_a = 2.89$) and B ($pK_a = 3.47$) along with drug TA ($pK_a = 5.11$) was used for developing a new RP HPLC method for separation of drug from its impurities and their estimation. Pure samples of Imp A and B were procured and used for the study.

Mobile phases in HPLC are optimized on basis of the pK_a (acid dissociation constant) of the drug. The pH of the buffered mobile phase is generally taken as ± 2 of the pK_a value of the concerned analytes of research interest. However when mixtures of analytes with variable acid dissociation constants are present, difficulty arises in predicting and controlling the migration of the ionized analytes when buffers are used as component of mobile phase. In the case of Tolfenamic acid, the pK_a of 5.11 is more than a unit above Imp A and Imp B with values of 2.89 and 3.47, respectively. In such a situation it is desirable to optimize mobile phase composition by using buffer of appropriate pH that provide a balanced ionization property to the component that establish complete separation with satisfactory resolution.

7.4.2 Development of new HPLC method for estimation of Tolfenamic acid in presence of its pharmacopoeial impurities

The Pharmacopoeial analytical method for TA represented an isocratic elution program mode of analysis with tertiary solvent as mobile phase comprising of glacial acetic acid : water : ethanol (2:350:650 % v/v/v) on C18 column with a flow rate of 0.8 ml/min and injection volume of 20 μ l at detection wavelength of 232 nm.

Method development for TA was based on rational basis. Drug and its two impurities (Imp A and Imp B) were considered for separation on Waters Sunfire C18 column. Due to differences in pK_a values of drug and the two impurities (Imp A and Imp B), buffer solutions of diverse pH (2.5 to 6.5) were selected to optimize the composition of mobile phase that provide best separation as acidic and basic analytes are sensitive to changes in pH of mobile phase component.

Peak parameters for the three components of the study including the drug TA that was coeluted with mobile phase at varied pH and at different composition of Mobile phase at fixed pH is presented in Fig 7.4.2.1 and Fig 7.4.2.2.

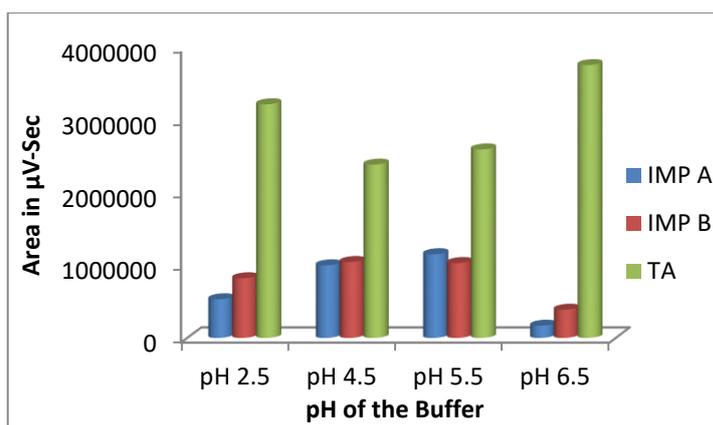


Figure 7.4.2.1: Peak areas of TA, Imp A and Imp B varying with pH of buffer (mobile phase)

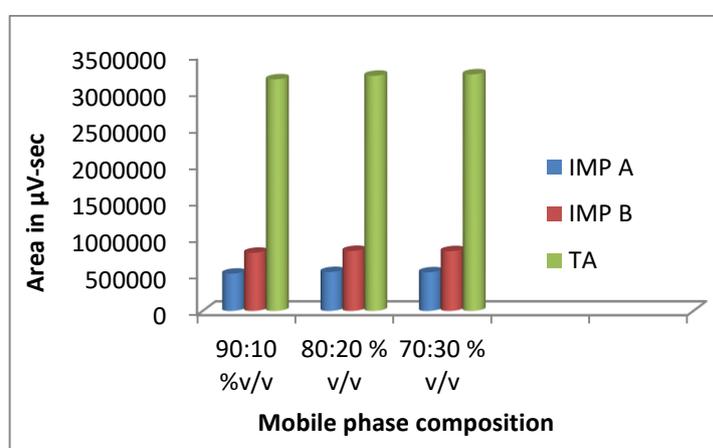


Figure 7.4.2.2: Peak areas of TA, Imp A and Imp B varying with organic phase ratio (mobile phase)

Retention time of the drug and its impurities (A and B) in mixture were seen to be affected by the pH of the mobile phase as seen from Fig. 7.4.2.3.

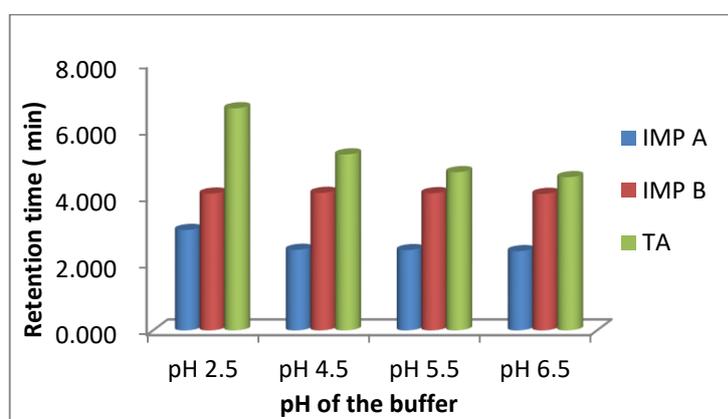


Figure 7.4.2.3: Retention time of TA, Imp A and Imp B varying with pH of buffered mobile phase

It may be observed that at pH 2.5 the acidic compounds (TA and Imp A) below their pKa values show greater retention as compared to pH higher than their pKa values. Whereas basic compound (Imp B) showed less retention at pH lower than its pKa value which more or less increases at pH higher than its pKa value. Rt of TA dropped sharply from 6.65 min at pH 2.5 to 4.59 min at pH 6.5 indicating ionization of acidic analyte. At pH 2.5 retention is high at 6.65 min and with increase of pH there was gradual decrease in retention observed indicating increase in degree of ionization of TA. Similar effects were observed in cases of other acidic analytes Imp A. Conversely Imp B, a basic compound by nature showed low retention at acidic pH and lose its ionizing ability showing higher retention at higher pH.

The developed new HPLC method use a simple mobile phase with two components i.e. mixture of acetonitrile and 10 mM ammonium dihydrogen ortho phosphate buffer (pH adjusted to 2.5 with ortho phosphoric acid) in the ratio of 80:20 % v/v and helped in reducing the retention time of TA to half with a short run time as compared to official method (Fig 7.4.2.4). The developed new RP HPLC method was validated as per ICH Q2R1 guidelines.

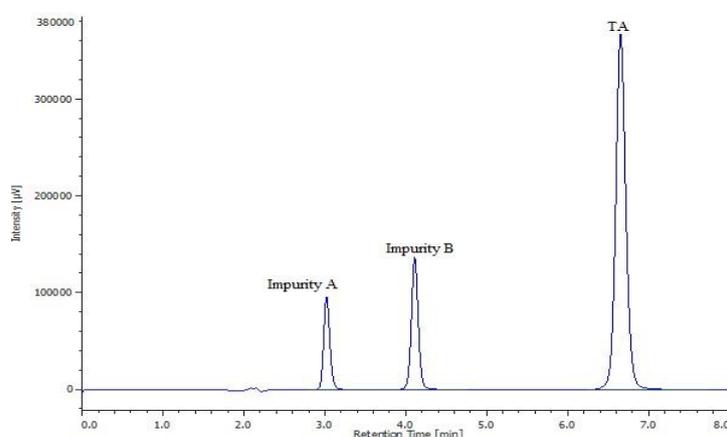


Figure 7.4.2.4: Optimized chromatogram of Tolfenamic acid and impurities (A and B)

7.4.3. Forced degradation study of Tolfenamic acid

Degradation to an extent of 15 % was seen in TA samples when subjected to oxidative stress condition with one additional peak (DP I) seen in the chromatogram along with TA peak.

The RRT value of the DP I was different from the RRTs of the two pharmacopoeial impurities thus confirming that oxidative degradation leads to formation of one degradation products (DP I) different from the specified impurities (for possible degradation pathway see Appendix). Decrease in drug peak area around 10 % was seen when subjected to hydrolysis (acid, base and neutral), thermal and photo stress conditions with no additional peak seen in the chromatogram for a run time period of 10 min as seen in Fig 7.4.3. Thus TA showing its stability towards hydrolysis, thermal and photo stress conditions.

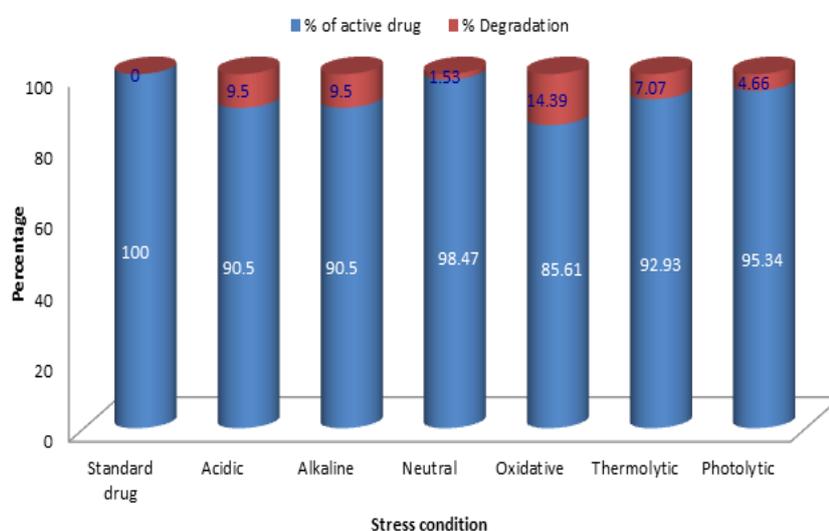


Figure 7.4.3: Degradation study profile of Tolfenamic acid

7.5. Profile of impurities and degradants

7.5.1. Detection and determination profile of impurities and degradation products of Mefenamic acid

Impurity	Detection (RRT)	Determination by HPLC method
2, 3-Dimethylaniline) (Imp A)	0.29	A binary mobile phase consisting of acetonitrile and 10 mM phosphate buffer pH 4 in the ratio of 55:45 % v/v with flow rate of 1 ml/min at detection wavelength of 225 nm, in isocratic mode on Waters Sunfire C18 column (250 x 4.6 mm ,5 µm)
2-Chlorobenzoic acid (Imp C)	0.14	
Benzoic acid (Imp D)	0.17	
DP I	0.46	
DP II	0.74	

7.5.2. Detection and determination profile of impurities and degradation products of Carbimazole

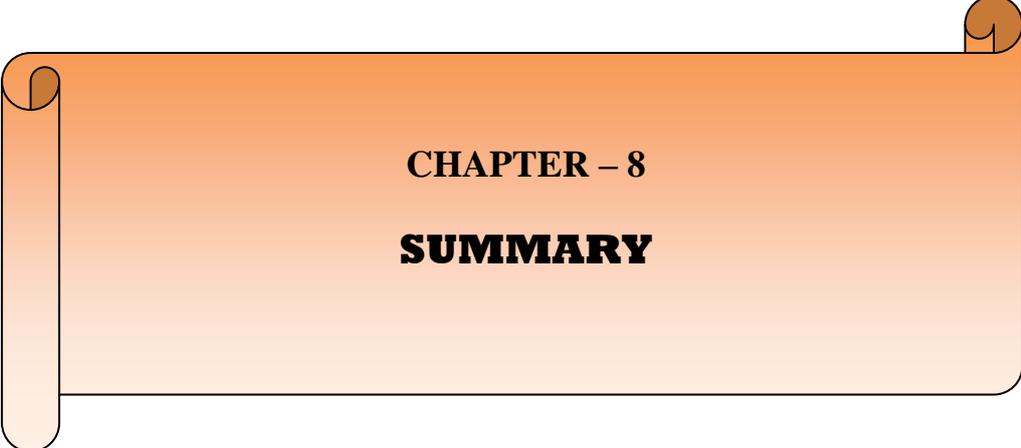
Impurity	Detection (RRT)	Determination by HPLC method
1-methyl-1H-imidazole-2-thiol (Methimazole) (Imp A)	0.66	A binary mobile phase consisting of acetonitrile and 10 mM phosphate buffer pH 2.5 in the ratio of 50:50 % v/v with flow rate of 1 ml/min at detection wavelength of 260 nm, in isocratic mode on Waters Sunfire C18 column (250 x 4.6 mm ,5 µm)
DP I / Imp A	0.66	

7.5.3. Detection and determination profile of impurities and degradation products of Cyclizine HCl

Impurity	Detection (RRT)	Determination by HPLC method
1-Methylpiperazine (Imp A)	0.54	A binary mobile phase consisting of acetonitrile and 10 mM phosphate buffer pH 6.5 in the ratio of 80:20 % v/v with flow rate of 1 ml/min at detection wavelength of 225 nm, in isocratic mode on Waters Sunfire C18 column (250 x 4.6 mm ,5 µm)
Diphenylmethanol (Imp B)	0.68	
DP I	0.54	
DP II / Imp B	0.68	

7.5.4. Detection and determination profile of impurities and degradation products of Tolfenamic acid

Impurity	Detection (RRT)	Determination by HPLC method
2-Chlorobenzoic acid (Imp A)	0.45	A binary mobile phase consisting of acetonitrile and 10 mM phosphate buffer pH 2.5 in the ratio of 80:20 % v/v with flow rate of 1 ml/min at detection wavelength of 205 nm, in isocratic mode on Waters Sunfire C18 column (250 x 4.6 mm ,5 µm)
3-Chloro-2-methylaniline (Imp B)	0.62	
DP I	0.54	



CHAPTER – 8

SUMMARY

8. SUMMARY

The search for appropriate testing methods for analysis of drug substances and products has been a fascinating experience in the laboratory. Strengthening of the testing methodologies has always been of prime importance, a challenging situation and indispensable responsibility for an intelligent analyst. *With development of science and access to modern technology, wider choices have been offered to choose an appropriate process that has opened up newer horizons and helped in visualizing compounds amidst its matrices that could never have been seen nor perceived earlier.* The modern analytical laboratory today supports detection and determination of compounds at sub microgram levels too, thereby providing options to look beyond purity of a substance and search for minute, elusive and intricate unwanted entity, the elimination of which could lead to significant improvement in the quality of drug substances as well as products in general.

One of the remarkable discoveries in separation science – High Performance Liquid Chromatograph has been widely used for determination of compounds, whether it be for ascertaining content uniformity or for determination of related substances or for estimating the purity of drug substances or products. With pharmacopoeia and the regulatory bodies acknowledging the opportunities provided by advancement of science and technology, the monographs of the drug substances list additional parameters that include ‘undesirable entities’ to confirm beyond doubt in the mind of the stakeholder i.e. doctor and the patient eagerly willing to invest in quality medicines for better health.

The quality conscious industry has accepted the challenges of the society and has invested heavily in the technology and is looking for development of strong testing methods to enable thorough assessment of drug substances and products for purity. Appropriate HPLC methods have been felt desirable for robust testing of compounds. This requires dedicated efforts of analysts to develop appropriate mobile phases that are resistant to being influenced by experimental and environmental factors - that a drug is likely to experience during its shelf life and in the biological systems. Such issues could extend from exposure of drug to solutions of diverse pH, humidity, temperature, light, oxidation, etc. The influence of some of such variables needs to be provided from the initial point of method development itself. Such issues have been explored in the proposed study during method development to enable rugged testing protocols.

Examining the effect of changes in pH on the separation profiles is recommended to assess the method robustness. Analyte retention in RP-HPLC is also influenced by hydrophobicity of the analyte. When ionisable analytes are present in a sample, the selectivity between analytes is said to be influenced by mobile phase pH.

The search for appropriate mobile phases is considered challenging in situations that present complex mixture of compounds for separations with diverse physiochemical properties. With options of varying composition of stationary phase being limited in HPLC, a number of possibilities could be enabled to alter composition of mobile phase through measured changes in volumes or strength of the components to arrive at most appropriate system to achieve best possible separation.

Mefenamic acid was chosen for the study as it provides impurities, few of them including drug having pKa's ranging between 4.0 to 4.7 and one at 2.9. For a similar objective of study, Tolfenamic acid was selected as its reported impurities are limited and have pKa's that are wide apart.

As mobile phase pH is said to have a profound effect on analyte retention and selectivity, series of trials were undertaken by varying pH of the mobile phase over a wide range and the separation profiles studied elaborately.

From the synthetic schemes of mefenamic acid, it was found that 2, 3-Dimethylaniline, 2-Chlorobenzoic acid and Benzoic acid are used as reactants/intermediates. The presence of these compounds in MA in trace amounts are listed as specified impurities A (2, 3-Dimethylaniline), C (2-Chlorobenzoic acid) and D (Benzoic acid) in the BP monograph as their presence beyond permissible limits is shown to express undesirable effects in physiological system. Also in the monograph of MA, two other impurities are listed and mentioned as 'other detectable impurities'. It is inferred that Imp A, C and D are 'Process related impurities' and are likely to be present with MA after synthesis unless appropriate purification methods have been undertaken. These compounds viz., Imp A (pKa = 4.70), C (pKa = 2.89), and D (pKa = 4.19), were considered for separation along with the active compound MA (pKa = 4.20) in the process of analytical development. The samples of Imp A, C and D were procured in the purest form and used as standards during method development.

It should be noted that the pharmacopoeial method for assay of MA represented an isocratic elution mode of analysis with ternary solvent as mobile phase comprising of 50 mM Phosphate buffer (pH adjusted to 5 with dilute ammonia): acetonitrile: THF (40:46:14 % v/v) on C18 column with a flow rate of 1 ml/min, detection wavelength of 254 nm. The method when applied during the study showed several noise peaks in the vicinity of impurity peaks. The mobile phase did not appear to provide any specific advantage nor produce satisfactory smooth baseline necessitating improvisation. Despite its strong solubilization power and eluotropic strength, THF is generally avoided/minimized in reversed-phase LC. It is understood that issues related to toxicity and safety of THF - due to peroxide formation prevent its widespread use except in gel permeation chromatography.

Hence a new RP-HPLC method was developed for quantification of MA and its Pharmacopoeial impurities (Imp A, Imp C and Imp D) on Waters Sunfire C18 column in isocratic mode with Acetonitrile: Phosphate buffer pH 4 (55:45 % v/v) as mobile phase, detection wavelength 225 nm. Since the pKa values of the drug and its impurities were in the range of 2.89 to 4.70, various exploratory trials at different pH of the buffer solution (3 to 6) were performed to optimize the pH of mobile phase and the ratio of buffer with organic phase (acetonitrile).

With the use of phosphate buffer the sensitive region between pH 4 to 5 provided good platform for undertaking systematic study on separation principles as MA and three of its impurities had pKa ranging from 4 to 5. Study concerned with mining of peak parameters for the four components of the study including the drug MA that were coeluted with mobile phase at varied pH and at different composition of mobile phase at fixed pH. As per ICH guidelines the developed method was validated. (Table 8.1).

Table 8.1: Summarized result of validation parameters in analytical development of MA and impurities (A, C and D)

Sr. No	Validation Parameters	MA	Imp A	Imp C	Imp D
1	System suitability				
1A	RRT	1.00	0.29	0.14	0.17
1B	Resolution	NA	39.37	4.8	16.29
1C	Peak asymmetry	1.45	1.05	1.30	1.19
1D	Theoretical plate	21212	19264	8696	10387
2	Specificity	No peaks	No peaks	No peaks	No peaks

3	Linearity				
3A	Range ($\mu\text{g/ml}$)	12.42-100	0.67-10	1.66-10	1.04-10
3B	Regression coefficient	0.999	0.999	0.998	1.00
3C	Regression equation	$y=52423x-45446$	$y=31772x-761.1$	$y=14321x+946.2$	$y=51500x+1287.6$
4	Limit of detection ($\mu\text{g/ml}$)	4.09	0.22	0.54	0.34
5	Limit of quantification ($\mu\text{g/ml}$)	12.42	0.67	1.66	1.04
6	Precision				
6A	Intra-day precision	0.41 %	0.90 %	0.92 %	0.30 %
6B	Inter-day precision	0.37 %	0.78 %	0.99 %	0.45 %
7	Accuracy (% Recovery)				
7A	Level I- 80 %	99.08 %	98.76 %	98.95 %	98.99 %
7B	Level II- 100 %	99.95 %	99.04 %	100.66 %	100.45 %
7C	Level III- 120 %	100.27 %	100.95 %	101.99 %	101.22 %
8	Robustness				
8A	Flow rate variation	1.31 %	1.33 %	1.39 %	0.98 %
8B	Mobile phase ratio variation	0.87 %	1.04 %	0.21 %	0.79 %

Degradation was seen in MA samples when subjected to oxidative stress condition. Two prominent additional peaks were seen in the chromatogram along with MA peak. The two additional peaks had RRTs of 0.46 and 0.74 for DP I and DP II respectively which were not identical from the RRTs of the three specified impurities, thus indicating possible oxidative degradation leading to formation of two degradation products (DP I and DP II) different from the specified impurities (Fig 8.2).

Table 8.2: Summarized result of stress study of MA

Sr. No	Stress condition	% of active drug after degradation	No. of peaks of specified impurity /degradants	RRT of specified impurity/ degradants
1	1N HCl at 70 °C for 6 hr	90.04	NIL	---
2	1N NaOH at 70 °C for 6 hr	92.64	NIL	----
3	Water at 70 °C for 6 hr	93.08	NIL	----
4	H ₂ O ₂ (10 % v/v) at RT for 3 days	62.56	02	1) 0.46 (DP I) 2) 0.74 (DP II)
5	Thermal, at 80°C for 4 days	89.61	NIL	---
6	Direct sunlight for 7 days	96.38	NIL	---

The new method developed for analysis of MA was thus stability indicating and also produced chromatogram with complete separation of drug from the selected impurities and degradants formed under stress degradation studies.

On similar lines, Schemes for synthesis of TA were surveyed from literature. It was found that 2-Chloro benzoic acid and 2-Chloro 3-methylaniline are used as starting material for the synthesis of TA. The presence of these substances are listed as Imp A (2-Chlorobenzoic acid) and Imp B (2-chloro 3-methylaniline) in the BP monograph of TA. Also Imp C (3-Chloro-4-methyl-9-oxo-9, 10-dihydroacridine) is mentioned in the monograph of TA.

Imp A and Imp B are 'Process related impurities' since they are likely to remain with drug TA after synthesis involving use of said compounds as raw materials and purification methods being inadequate. These compounds as Imp A (pKa = 2.89) and B (pKa= 3.47) along with drug TA (pKa= 5.11) was used for developing a new RP HPLC method for separation of drug from its impurities and their estimation. Pure samples of Imp A and Imp B were procured and used for the study.

Mobile phases in HPLC are optimized on basis of the pKa (acid dissociation constant) of the drug. The pH of the buffered mobile phase is generally taken as ± 2 of the pKa value of the concerned analytes of research interest. However when mixtures of analytes with variable acid dissociation constants are present, difficulty arises in predicting and controlling the migration of the ionized analytes when buffers are used as component of mobile phase. In the case of Tolfenamic acid, the pKa of 5.11 is more than a unit above Imp A and Imp B with values of 2.89 and 3.47, respectively. In such a situation it is desirable to optimize mobile phase composition by using buffer of appropriate pH that provide a balanced ionization property to the component that establish complete separation with satisfactory resolution.

Method development for TA was based on rational basis. Drug and its two impurities (Imp A and Imp B) were considered for separation on Waters Sunfire C18 column. Due to differences in pKa values of drug and the two impurities (Imp A and Imp B), buffer solutions of diverse pH (2.5 to 6.5) were selected to optimize the composition of mobile phase that provide best separation as acidic and basic analytes are sensitive to changes in pH of mobile phase component.

The developed new HPLC method uses a simple mobile phase with two components i.e. mixture of acetonitrile and 10 mM ammonium dihydrogen orthophosphate buffer (pH adjusted to 2.5 with ortho phosphoric acid) in the ratio of 80:20 % v/v and brought down the retention time of TA by half, with a short run time as compared to official method. The developed method was validated for various parameters as per ICH guidelines (Table 8.3).

Table 8.3: Summarized result of validation parameters in analytical development of TA and impurities (A and B)

Sr.No	Validation Parameters	TA	Imp A	Imp B
1	System suitability			
1A	RRT	1.00	0.45	0.61
1B	Resolution	NA	7.54	13.62
1C	Peak asymmetry	1.09	1.21	1.06
1D	Theoretical plate	14400	7515	11607
2	Specificity	No peaks	No peaks	No peaks
3	Linearity			
3A	Range ($\mu\text{g/ml}$)	13.77-100	0.1-10	0.12-1
3B	Regression coefficient	0.9997	0.9988	0.9994
3C	Regression equation	$y=48602x-16446$	$y=86129x+2217.4$	$y=119746x+49.33$
4	Limit of detection ($\mu\text{g/ml}$)	4.54	0.035	0.042
5	Limit of quantification ($\mu\text{g/ml}$)	13.77	0.106	0.12
6	Precision			
6A	Intra-day precision	0.81	0.56	0.44
6B	Inter-day precision	0.83	0.22	1.66
7	Accuracy (% Recovery)			
7A	Level I- 80 %	98.86 %	98.95 %	98.54 %
7B	Level II- 100 %	99.71 %	99.11 %	99.89 %
7C	Level III- 120 %	101.28 %	101.44 %	101.73 %
8	Robustness			
8A	Flow rate variation	1.70 %	0.75 %	1.20 %
8B	Mobile phase ratio	0.20 %	0.98 %	1.14 %

Degradation to an extent of 15 % was seen in TA samples when subjected to oxidative stress condition with one additional peak (DP I) seen in the chromatogram along with TA peak. The RRT value of the DP I was different from the RRTs of the two Pharmacopoeial impurities thus confirming that oxidative degradation leads to formation of one degradation products (DP I) different from the specified impurities.

Decrease in drug peak area around 10 % was seen when subjected to hydrolysis (acid, base and neutral), thermal and photo stress conditions with no additional peak seen in the chromatogram for a run time period of 10 min. Thus TA showing its stability towards hydrolysis, thermal and photo stress conditions (Table 8.4).

Table 8.4: Summarized result of stress study of TA

Sr.No	Stress condition	% of active drug after degradation	No. of peaks of impurity/ degradants	RRT of impurity/ degradants
1	1N HCl at 70 °C for 6 hr	90.50	NIL	---
2	1N NaOH at 70 °C for 6 hr	90.50	NIL	----
3	Water at 70 °C for 6 hr	98.47	NIL	----
4	H ₂ O ₂ (10 %) at RT for 2 days	85.61	01	1) 0.54 (DP I)
5	Thermal, at 80 °C for 4 days	92.93	NIL	---
6	Direct sunlight for 4 days	95.34	NIL	---

The new method developed for analysis of TA was thus stability indicating and also produced chromatogram with complete separation of drug from the selected impurities and degradants formed under stress degradation studies.

Highly efficient techniques involved in the drug or metabolite extraction and enrichment, separation, and sensitive cum selective detection are essential for their reliable determination in complex biological samples. Assessing the metabolic fate of drugs is an essential and important part of the drug development process. LC methods are efficient and robust, specific, with merits of convenience, operation, strong separation ability, and diverse sample application. HPLC with UV-VIS detector; fluorescence, diode array (DAD), and mass spectrometric detection methods have been used for the analysis of different drugs in various biological samples.

In the current study, efforts were made to identify a drug that can readily breakdown to its metabolite and interfere with analytes detection and quantification. Carbimazole is one such drug that easily breaks down to Methimazole, a metabolite with similar therapeutic activity but of a different magnitude. Incidentally methimazole by itself is used in the synthesis of Carbimazole.

The pharmacopoeial analytical method for CZ represented an isocratic elution program mode of analysis with binary solvent as mobile phase comprising of acetonitrile: water (10:90 % v/v) on C18 column with a flow rate of 1 ml/min at detection wavelength of 254 nm. An additional peak in the vicinity of Imp A peak was seen. Absence of buffer of specific pH in the mobile phase expected to promote variation in pH during the course of study when exposed to different environmental atmospheric conditions.

For quantification of CZ and its pharmacopoeial impurity A, a new RP HPLC method was developed on Waters Sunfire C18 column with acetonitrile: phosphate buffer pH 2.5 (50:50 % v/v) as mobile phase at detection wavelength of 260 nm, in isocratic mode.

Various exploratory trials at different pH of the buffer solution (2.5 to 6.5) were performed to optimize the mobile phase and ratio of buffer with organic phase (acetonitrile). The new HPLC method could overcome the formation of additional peak in the vicinity of impurity peak. Also total run time was shorter compared to official method. Buffered mobile phase produced reproducible chromatograms inspite of varying conditions deliberately. The new method was validated as per ICH Q2R1 guidelines (Table 8.5). The new developed method was carried in acetate buffer with pH 2.5 in order to characterize the impurity by LCMS.

Table 8.5: Summarized result of validation parameters in analytical development of CZ in presence of Imp A

Sr.No.	Validation Parameters	CZ	Imp A
1	System suitability		
1A	RRT	1.00	0.66
1B	Resolution	NA	5.77
1C	Peak asymmetry	1.05	1.07
1D	Theoretical plate	7791	4693
2	Specificity	No additional peaks	No additional peaks
3	Linearity		
3A	Range ($\mu\text{g/ml}$)	10.66-100	1.71-10
3B	Regression coefficient	0.9990	0.9997
3C	Regression equation	$y=3047.7x-3409.5$	$y=48836x+18636$
4	Limit of detection($\mu\text{g/ml}$)	3.52	0.51
5	Limit of quantification ($\mu\text{g/ml}$)	10.66	1.71
6	Precision		
6A	Inter-day precision	0.44 %	1.29 %
6B	Intra-day precision	1.23 %	0.52 %
7	Accuracy (% Recovery)		

7A	Level I- 80 %	98.85 %	98.08 %
7B	Level II- 100 %	99.16 %	99.01 %
7C	Level III- 120 %	101.4 1%	100.64 %
8	Robustness		
8A	Flow rate variation	0.95 %	1.89 %
8B	Mobile phase ratio	1.47 %	1.09 %

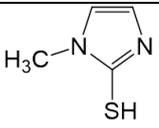
Carbimazole was highly susceptible to degradation in hydrolytic, oxidative and thermal stress conditions with formation of one additional peak. The RRT of the additional peak (DP I) 0.66 min matched with the RRT of pharmacopoeial impurity A 0.66 min, thus confirming the formation of Imp A when CZ is exposed to hydrolytic, oxidative and thermal conditions. Decrease in drug peak area was seen to an extent of less than 10 % when it was subjected to photo stress conditions although no additional peak could be seen (Table 8.6).

Table 8.6: Summarized result of stress study of CZ

Sr.No	Stress condition	% of active drug after degradation	No. of peaks of specified impurity/ degradants	RRT of specified impurity/ degradants
1	0.1N HCl at RT for 2h	64.71	01	0.65 (Imp A)
2	0.001N NaOH at RT for 5 min	88.43	01	0.66 (Imp A)
3	Water at 70 °C for 10 min	32.58	01	0.65 (Imp A)
4	H ₂ O ₂ (0.1 %) at RT for 1day	80.55	01	0.66 (Imp A)
5	Thermal, at 80 °C for 6 hr	73.58	01	0.65 (Imp A)
6	Direct sunlight for 7 days	89.34	NIL	--

The common degradation product (DP I) of CZ obtained in various stress conditions was confirmed by LCMS study. The positive ions, [M+H]⁺ at m/z value of 115, was the molecular ion of the DP I. The molecular weight of the DP I was identified as 114 with respect to its molecular ion. The molecular weight of specified Imp A is 114.17 which approximately match with the DP I molecular weight. Thus confirming that the common degradant product (DP I) formed under various stress conditions is specified impurity A (Table 8.7).

Table 8.7: Summary of LC-MS results for Carbimazole and its degradation product

Sr.no	Stress condition (RRT)	ESI (+ ve) m/z value of CZ	Characteristic degradation product Ion (m/z)	Identification
1	Hydrolysis (Acid, Base Neutral) (0.66)	187	115	 1-methyl-1H-imidazole-2-thiol (Imp A) Molecular Formula: C ₄ H ₆ N ₂ S Molecular weight: 114.17
2	Oxidation (0.66)			
3	Thermal (0.65)			

The new method developed for analysis of CZ was thus stability indicating and also produced chromatogram with complete separation of drug from its impurities and degradants formed under stress degradation studies.

HPLC methods require analyst to apply certain special techniques like derivatization and application of moderate heat for extending the applications. Robust separation process coupled with hyphenated system like MS helps in characterization of the compound. Applications of such models are desirable to identify the undesirable compounds formed during the degradation of the drug substances.

An alternative RP-HPLC method was developed for quantification of CY and its Pharmacopoeial impurities (Imp A and Imp B) on Waters Sunfire C18 column with Acetonitrile: Phosphate buffer pH 6.5 (80:20 % v/v) as mobile phase at detection wavelength of 225 nm, in isocratic mode. Imp A lacked a chromophore in its chemical structure resulting in the need for its derivatization. Derivatized Imp A showed absorption in the UV region. Buffered mobile phase of pH 6.5 (phosphate buffer) provided satisfactory elution of CY with pKa 8.51. Several trials were performed to optimize the mobile phase and the ratio comprising of buffer and acetonitrile.

The new HPLC method could provide peak areas of CY and its impurities on a smooth base line and over a short runtime period, enabling quick and accurate analysis. The method was validated as per ICH Q2R1 guidelines (Table 8.8).

Table 8.8: Summarized result of validation parameters in analytical development of CY, Imp A and Imp B

Sr.No.	Validation Parameters	CY	Imp A	Imp B
1	System suitability			
1A	RRT	1.00	0.62	0.75
1B	Resolution	NA	3.80	5.91
1C	Peak asymmetry	1.23	0.99	0.99
1D	Theoretical plate	6405	6303	7479
2	Specificity	No peaks	No peaks	No peaks
3	Linearity			
3A	Range ($\mu\text{g/ml}$)	16.65-100	1.87-10	2.46 -10
3B	Regression coefficient	0.9997	0.9988	0.9994
3C	Regression equation	$y=15968x-6467.4$	$y=63253x-45955$	$y=29519x+11647$
4	Limit of detection ($\mu\text{g/ml}$)	5.49	0.56	0.74
5	Limit of quantification ($\mu\text{g/ml}$)	16.65	1.87	2.46
6	Precision			
6A	Intra-day precision	0.88 %	0.41 %	0.18 %
6B	Inter-day precision	0.15 %	1.19 %	0.63%
7	Accuracy (% Recovery)			
7A	Level I- 80 %	98.94 %	98.70 %	98.99 %
7B	Level II- 100 %	100.43 %	99.13 %	100.24 %
7C	Level III- 120 %	101.24 %	101.85 %	101.28 %
8	Robustness			
8A	Flow rate variation	1.71 %	1.29 %	1.97 %
8B	Mobile phase ratio	1.13 %	1.12 %	1.57 %

Cyclizine HCl showed degradation in acid and oxidation stress conditions with formation of one additional peak apart from the drug peak in acidic stress condition and two additional peaks apart from drug peak in oxidative stress conditions. The RRT of the additional peak (DP I) formed in the acidic medium matched with the RRT of pharmacopoeial impurity B, thus confirming the formation of Imp B when CY is exposed to acidic conditions. The RRT of DP III formed under oxidation condition were different from the RRTs of Imp A, Imp B and DP I formed in acidic condition. The RRT of the other peak (DP II) matched with the RRT of pharmacopoeial impurity B, thus confirming the formation of Imp B in acid and oxidation stress conditions (Table 8.9).

Decrease in drug peak area was seen to an extent of 8 % when it was subjected to basic neutral, thermal and photo stress conditions with no additional peak thus confirming the stability of CY.

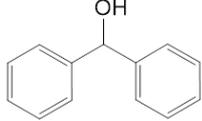
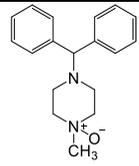
Table 8.9: Summarized result of stress study of CY

Sr.No	Stress condition	% of active drug after degradation	No. of peaks of specified impurity/ degradants	RRT of specified impurity/ degradants
1	1N HCl at RT for 4 hr	83.86	01	1) 0.72 (DP I)
2	1N NaOH at 70 °C for 6 hr	93.07	NIL	---
3	Water at 70 °C for 6 hr	98.04	NIL	----
4	H ₂ O ₂ (10 %) at RT for 2 day	53.51	02	1) 0.54 (DP III) 2) 0.68 (DP II)
5	Thermal, at 80 °C for 4 days	98.53	NIL	----
6	Direct sunlight for 7 days	92.93	NIL	--

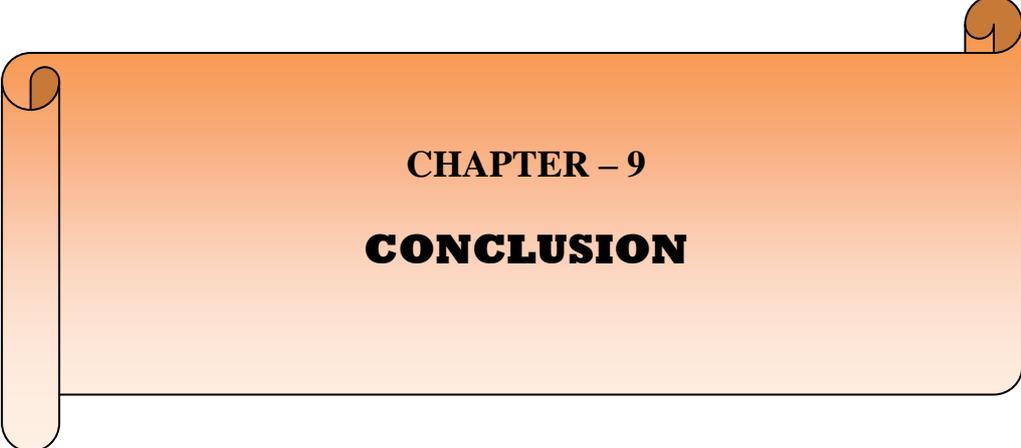
The degradation products of CY formed in acidic and oxidation stress condition were confirmed by LC-MS study. The fragment ion peak of common degradant (RRT = 0.72) obtained in acidic and oxidative stress medium appeared at m/z value of 167.1. The peak represented the molecular mass of degradant (RRT = 0.72) which was found to be 184.23, due to loss of alcohol group, which approximately matches with the specified Imp B. The m/z of second degradant (RRT = 0.54) formed under oxidative stress condition appeared with m/z value of 283.2. The m/z of degradant (RRT = 0.54) was found to be 282.38 confirming formation of N-oxide of Cyclizine.

Thus it was identified that the common degradant formed under acid hydrolysis and oxidative stress condition is Imp B and the other degradant formed in oxidation medium is the N-oxide of Cyclizine (Table 8.10).

Table 8.10: Summary of LCMS results for Cyclizine HCl and its degradation products

Sr.no	Stress condition (RRT)	ESI (+ ve) m/z value of CY	Characteristic degradation product Ion (m/z)	Identification
1	Acid Hydrolysis (0.72)	302.8	167.1	 <p>Diphenylmethanol (benzhydrol) (Imp B) Molecular Formula: C₁₃H₁₂O Molecular weight: 184.23</p>
2	Oxidation (0.68)			 <p>4 benzhydryl-1-methylpiperazine 1-oxide Molecular Formula: C₁₈H₂₂N₂O Molecular weight: 282.83</p>
3	Oxidation (0.54)		283.2	

The new method developed for analysis of CY was thus stability indicating and also produced chromatogram with complete separation of drug from the selected impurities and degradants formed under stress degradation studies.



CHAPTER – 9
CONCLUSION

9. CONCLUSION

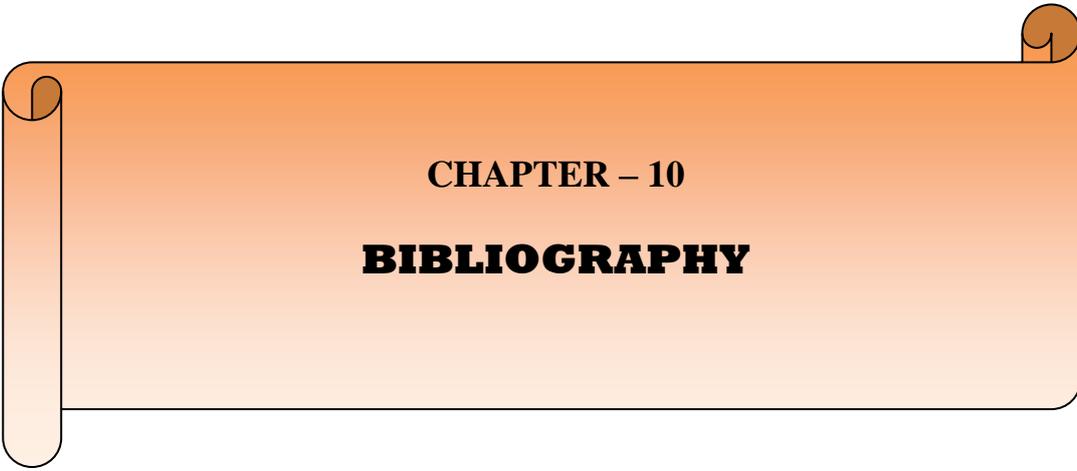
Method development for analysis of drug substances in presence of their specified impurities and degradants are challenging. In the evolving global scenario the testing methods are required to be sturdy and rugged with objective to predict impurities likely to form during its shelf life as formulation and through stress induced studies. Such compounds are in addition to specified impurities that molecule are likely to co-exist during its life time.

The search for study methods has invoked systematic method development with behavior of drugs and their impurities assessed for variation of migration parameters with change in their physicochemical/chemical properties. Properties that lead to a change in migration parameters could be exploited for identifying a mobile phase that provides complete separation of compounds as seen in their chromatograms with good base line separation for all the peaks.

Certain special techniques like derivatization were applied for identifying compounds with poor chromophoric properties and MS detector exploited for characterization of impurities that arised from stress induced studies.

As case studies four drugs were considered Viz., Mefenamic acid, Carbimazole, Cyclizine Hydrochloride and Tolfenamic acid for stability indicating HPLC method development. Limitations of existing methods were identified and improvisations with objectives of these attain ruggedness for simultaneous estimation of drug in presence of impurities and degradants established. Major limitations of existing methods collectively were with regard to resolution between peaks, unstable drifting baselines, peaks with reduced areas due to weakly absorbing chromophore and new impurities could be overcome in the proposed methods.

The future study could focus upon use of sensitive, versatile detection to enable mass balance and also suggest preferably an environment friendly analytical process with minimum use of such organic solvents that are not ecologically viable.



CHAPTER – 10

BIBLIOGRAPHY

10. BIBLIOGRAPHY

1. Nagpal S, Upadhyay AT. Bhardwaj RT and Thakkar A. A Review on Need and Importance of Impurity Profiling. *Curr. Pharm. Anal.* 2011; 7(1): 62-70. DOI: 10.2174/157341211794708749.
2. Ayre A, Varpe D, Nayak R and Vasa N. Impurity Profiling of Pharmaceuticals. *Int. J. Adv. Res. Pharm.* 2011; 1(2): 76-90.
3. Roy J. Pharmaceutical Impurities—a mini review. *AAPS PharmSciTech.*2002; 3(2): 1-8. DOI: 10.1208/pt030206.
4. Ahuja S and Alsante KM. Handbook of Isolation and Characterization of Impurities in Pharmaceuticals, Vol. 5. Separation Science and Technology, Academic press. 2003.
5. Ahuja S. Impurities Evaluation of Pharmaceuticals, Marcel Dekker, Inc. New York, 2006.
6. Ahuja S and Scypinski S. Handbook of Modern Pharmaceutical Analysis, Vol. 3. Separation Science and Technology, Academic press. 2003.
7. Ahuja SS. Assuring Quality of Drugs by Monitoring Impurities. *Advanced Drug Delivery Review.* 2007; 59(1): 3-11. DOI: 10.1016/j.addr.2006.10.003.
8. Venkatesan P and Valliappan K. Impurity Profiling: Theory and Practice. *Journal of Pharmaceutical Sciences and Research.* 2014; 6(7): 254- 259.
9. International Conference on Harmonization, Q3A (R2), Impurities in New Drug Substances, October 2006
10. International Conference on Harmonization, Q3B (R2), Impurities in New Drug Products, July 2006.
11. The United States Pharmacopoeia (32) – National Formulary (27), The United States Pharmacopoeial Convention, 12601 Twinbrook, Parkway, Rockville, Vol. 1 May 2009.
12. Richard JS and Micheal LW. Analysis of drug impurities. Blackwell publishing. 2007; 1-19.
13. International Conference on Harmonization, Q7, Good Manufacturing Practice for Active Pharmaceutical Ingredients, November 2000.
14. International Conference on Harmonization, Q1A (R2), Stability Testing Guidelines: Stability testing of new Drug Substances and Products, August 2003.

15. International Conference on Harmonization, Q6A, Specifications: Test procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances, May 2000.
16. Guidance for Industry, NDAs : Impurities in Drug Substances, U.S. department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, 2000.
17. Guidance for Industry, ANDAs : Impurities in Drug Products, U.S. department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, 2010.
18. Radhika R. Review of regulatory guidance on impurities. *Separation Science and Technology*. 2004; 5: 27-37. DOI: 10.1016/S0149-6395(03)80004-1.
19. International Conference on Harmonization, Q3C, Impurities: Guidelines for Residual Solvents, July 1997.
20. Gorog S (Ed.), *Identification and Determination of Impurities in Drugs*, Elsevier Science B.V, Amsterdam 2000.
21. Hovorka SW and Schoneich C. Oxidative degradation of pharmaceuticals: theory, mechanisms and inhibition. *J. Pharm. Sci.* 2001; 90:253–269. DOI: 10.1002/1520-6017(200103)90:3<253::AID-JPS1>3.0.CO;2-W.
22. Rao RN and Nagaraju V. An overview of the recent trends in development of HPLC methods for determination of impurities in drugs. *J Pharm Biomed Anal.* 2003; 33(3):335-377. DOI: 10.1016/s0731-7085(03)00293-0.
23. Sándor G. The role of impurity profiling in drug research, development and production. *Progress in Pharmaceutical and Biomedical Analysis*. 2000; 4: 38-47. DOI: 10.1016/S1464-3456(00)80006-X.
24. Liu KT and Chen CH. Determination of Impurities in Pharmaceuticals: Why and How. *Quality Management and Quality Control - New Trends and Developments*. 2010. DOI: 10.5772/intechopen.83849.
25. Bari S, Kadam B, Jaiswal Y, and Shirkhedkar A. Impurity profile: Significance in Active Pharmaceutical Ingredient. *Eurasian J Anal Chem.* 2007; 2(1): 32-53. DOI:10.12973/ejac/78054.
26. Reynolds DW. Forced degradation of pharmaceuticals. *Am Pharm Rev.* 2004; 7: 56–61.
27. Patel RM, Patel PM and Patel NM. Stability indicating HPLC method development - a review. *Int. Res. J. Pharm.* 2011; 2 (5):79-87.

28. Guidance for Industry : INDs for Phase 2 and Phase 3 Studies Chemistry, Manufacturing , and Control Information, U.S. department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, May 2003.
29. Kim Huynh – Ba, Handbook of Stability Testing in Pharmaceutical Development, Springer publication, July 2008, 9 – 21.
30. Karen MA, Akemi A, Roland B, Janice E, Todd DH, Wei K and et al. The Role of Degradant profiling In Active Pharmaceutical Ingredients and Drug Products. *Adv. Drug Deliv. Rev.* 2007; 59: 29 – 37. DOI: 10.1016/j.addr.2006.10.006.
31. Bakshi M and Singh S. Development of Validated Stability Indicating Assay Method – critical review. *J Pharm Biomed Anal.* 2002; 28: 1011–1040. DOI: 10.1016/s0731-7085(02)00047-x.
32. Ruan J, Tattersall P, Lozano R and Shah P. The role of forced degradation studies instability indicating HPLC method development. *Am Pharm Rev.* 2004; 9:46–53.
33. Puthil SP and Vavia PR. Stability Indicating HPTLC Determination of Piroxicam. *J Pharm Biomed Anal.* 2000; 22: 673–677. DOI: 10.1016/s0731-7085(99)00300-3.
34. Salo JP, and Salomices H. High Performance Thin Layer Chromatographic Analysis of Hydrolyzed Thiazole Solutions. *J Pharm Biomed Anal.* 1996; 14: 1261– 1266. DOI: 10.1016/s0731-7085(96)01769-4.
35. Kulkarni SP and Amin P.D. Stability indicating HPTLC Determination of Timolol Maleate as Bulk Drug and in Pharmaceutical Preparations. *J Pharm Biomed Anal.* 2000; 23:983 – 987. DOI: 10.1016/s0731-7085(00)00389-7.
36. Patravale VB, D’Souza S and Narkar Y. HPTLC determination of Nimesulide from Pharmaceutical Dosage Forms. *J Pharm Biomed Anal.* 2001; 25: 685 – 688.
37. Sutar S, Yeligar VC and Patil SS. A Review: Stability Indicating Forced Degradation Studies. *Res J Pharm Technol.* 2019; 12(2):885-890. DOI: 10.5958/0974-360X.2019.00152.5
38. Singh S and Bakshi M. Guidance on Conduct of Stress Tests to Determine Inherent Stability of Drug. *Pharm. Technol,* 2000; 24:1-14.
39. FDA, Guidance for Industry: Stability Testing of Drug Substances and Drug Products (Draft guidance), Food and Drug Administration, Rockville, MD, 1998.
40. International Conference on Harmonization, Q2 (R1), Validation of Analytical procedures: Text and methodology, June 1995.

41. FDA, Guidance for Industry: Analytical Procedures and Methods Validation, U.S. department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, August 2000.
42. Shabir GA. Validation of HPLC methods for Pharmaceutical Analysis Understanding Differences and Similarities Between Validation Requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization, *J Chromatogr A*. 2003; 987(1-2),:57–66.
43. Cazes J. Encyclopedia of Chromatography, Third edition, Vol. I, II and III, CRC press, Taylor and Francis Group, 1118 – 1132.
44. Indian Pharmacopoeia, The Indian Pharmacopoeia Commission Ghaziabad, Government of India Ministry of Health and Family Welfare, Vol. II, 2017.
45. British Pharmacopoeia, Medicines and Healthcare Products Regulatory Agency (MHRA), London, 2013.
46. The United States Pharmacopoeia – National Formulary, United States Pharmacopoeial Convention, Rockville, MD 20852, Vol. 1, 2013.
47. European Pharmacopoeia, Directorate of The Quality of Medicines and Health Care of the Council of Europe, Fifth edition, 2005.
48. Goodman and Gilman's the pharmacological basis of therapeutics. New York: McGraw Hill Publication, 2001.
49. www.drugbank.ca/drugs/DB00999784.
50. Sean C.S., Martindale the complete drug reference, 38th edition, pharmaceutical press, London.
51. Alarfaj NA, Altamimi SA and Almarshady LZ. Spectrophotometric determination of mefenamic acid in pharmaceutical preparations. *Asian J Chem*. 2009; 21:217-226.
52. Singh H, Kumar R and Singh P. Development of UV spectrophotometric method for estimation of mefenamic acid in bulk and pharmaceutical dosage forms. *Int. J. Pharm. Sci*. 2011; 3(2): 237–238.
53. Subramanian N, Devipriyadharshini T, Venkateshwaran K and Chandrasekar P. Spectrophotometric determination of tranexamic acid and mefenamic acid in tablet dosage form using derivatization technique. *Int. J Pharm Biomed Res*. 2011; 1(2): 26-29.

54. Kormosh A, Matviichuk O and Bazel R. Extraction-spectrophotometric determination of mefenamic acid in pharmaceutical preparations. *J. Anal. Chem.* 2014; 69(10): 960–964. DOI: 10.1134/S1061934814100074.
55. Naveed S and Qamar F. Simple UV spectrophotometric assay of mefenamic acid. *Int. j. pharma sci. res.* 2014; 5(7): 364–366.
56. Abachi MQ and Hadi H. Simple, rapid and sensitive method for the determination of mefenamic acid in pharmaceutical preparations. *J. Anal. Chem.* 2014; 69(8): 769–776. DOI: 10.1134/S106193481408005X
57. Maron N and Wright C. Application of Photodiode Array UV Detection in the Development of Stability-Indicating LC Methods: Determination of Mefenamic acid. *J. Pharm Biomed Anal.* 1990; 8:101–105. DOI: 10.1016/0731-7085(90)80014-g.
58. Poirier JM, Lebot M and Cheymol G. Rapid and sensitive liquid chromatographic assay of mefenamic acid in plasma. *The Drug Monit.* 1992; 14:322–326. DOI: 10.1097/00007691-199208000-00011.
59. Murali KR, Buela M and Sahithi A. Estimation of mefenamic acid in pharmaceutical dosage forms by RP-HPLC. *Int. J. Chem Sci.* 2011; 9:1587-1594.
60. Rouini MR, Asadipour A, Ardakani YH and Aghdasi F. Liquid Chromatography Method for Determination of Mefenamic acid in Human Serum. *J Chromatogr B Analyt. Technol Biomed Life Sci.* 2004; 800:189–192. DOI: 10.1016/j.jchromb.2003.09.063.
61. Sato J, Owada E, Ito K, Nidya Y, Wakamatsu A and Umetsu M. Simple, rapid and Sensitive reversed phase High Performance Chromatographic method for the determination of mefenamic acid in plasma. *J Chromatography.* 1989; 493:239–243. DOI: 10.1016/s0378-4347(00)82730-9.
62. Padmalatha H and Vidyasagar G. Validated RP-HPLC method for the estimation of mefenamic acid in formulation. *Int. J. Res Pharm Biomed Sci.* 2011; 2:1261-1265.
63. Wahab SU, Mohamed FP, Mohamed HS, Chandran M and Kadher MS. Development of RP-HPLC method for the simultaneous determination of mefenamic acid and drotaverine HCl combined tablet dosage form. *Int. J. Pharm Sci.* 2011; 3:115-118.
64. AlQaim FF, Abdullah MP, Othman MR and Khalik WA. Development and validation of HPLC analytical assay method for mefenamic acid tablet (Ponstan). *Int. J. Chem. Sci.* 2014; 12(1): 62–72.

65. Oswal T, Bhosale S and Naik S. Development of validated analytical method of mefenamic acid in an Emulgel (topical formulation). *Int. j. pharma sci. res.* 2014; 5(6): 232–237.
66. Prajapati D and Raj H. Simultaneous estimation of mefenamic acid and dicyclomine hydrochloride by RP-HPLC method. *Int. J. Pharm Biosci.* 2012; 3:611-625.
67. Satish YG, Naresh RK, Sachin EP and Kakasaheb RM. HPTLC double development and validation of mefenamic acid and tranexamic acid in combined tablet dosage form. *Der Pharmacia Sinica.* 2013; 4: 16-21.
68. Dusci LJ and Hackett LP. Gas liquid chromatographic determination of mefenamic acid in human serum. *J Chromatogr.* 1978; 161: 340-342. DOI: 10.1016/S0021-9673(01)85249-3.
69. Morcoss MM, Abdelwahab NS, Ali NW and Elsaady MT. Different Chromatographic Methods for Simultaneous Determination of Mefenamic Acid and Two of Its Toxic Impurities. *J. Chromatogr. Sci.* 2017; 55(7):1-7. DOI: 10.1093/chromsci/bmx034.
70. Saleh SF, Dereaya SM and Omar MA. Stability-indicating HPTLC determination of mefenamic acid in bulk drug and pharmaceutical formulations. *Int. J. Anal. Chem.* 2014; 5(1):55-60.
71. Shah S, Mirza A and Shamshad H. Physical and chemical characterization of mefenamic acid in different pharmaceutical dosage forms and their stability studies using novel RP-HPLC method. *Med. Chem. Res.* 2012;21: 3591–3597.
72. Patil PA, Umkar AR, Bari MM and Barhate SD. Stability Indicating RP-HPLC Method for Determination of Drotaverine HCL and Mefenamic Acid in Pure and Pharmaceutical Formulation. *Am. J. Adv. Drug Deliv.* 2015;3(2):110-122.
73. Dhumal BR, Bhusari KP, Tajne MR, Ghante MH and Jain NS. Stability indicating method for the determination of mefenamic acid in pharmaceutical formulations by HPLC. *J. Appl. Pharm. Sci.* 2014; 4: 060–064.
74. https://commons.wikimedia.org/wiki/File:Mefenamic_acid_synthesis.svg.
75. https://commons.wikimedia.org/wiki/File:Mefenamic_acid_synthesis_01.svg.
76. <https://www.chemdrug.com/article/9/3286/16426060.html>.
77. Sean CS, Martindale the complete drug reference, 38th edition, pharmaceutical press, London.

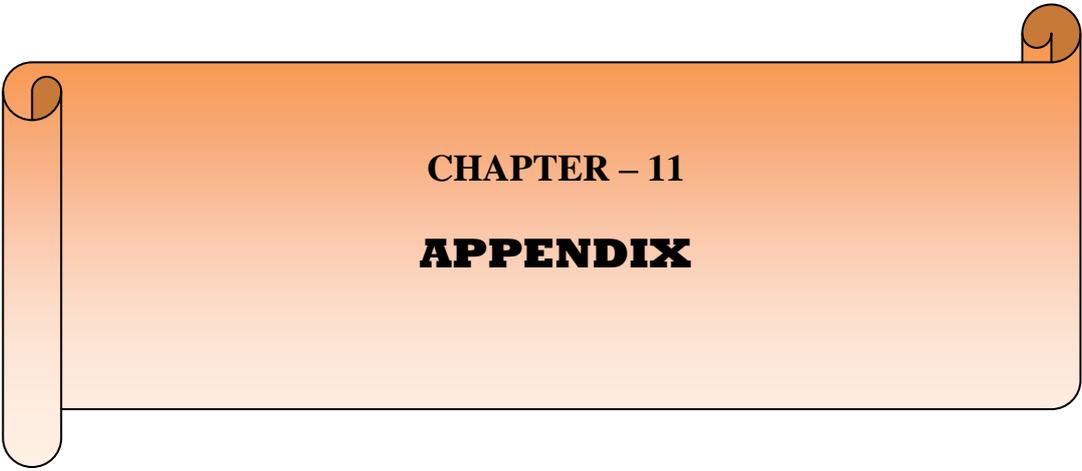
78. <https://www.drugbank.ca/drugs/DB00389>.
79. Amith R and Madhukara HM. Carbimazole-induced Cholestatic Hepatitis in Toxic Multinodular Goiter. *Int. J. Sci. Study*. 2016; 4(5): 252- 254.
80. James JW and Frank QN. Methimazole Toxicity from High Doses. *Ann. Intern. Med.* 1972; 77(3): 411-16.
81. Accetta GS, Fitzmorris AO and Wettingfeld RF. Toxicity of Methimazole (Tapazole). *J Am Med Assoc.* 1954; 155(3):253-254.
82. Pinzauti S, Dal PV and La PE. Potentiometric determination of various thyrostatic drugs in tablets. *Farmaco Prat.* 1973; 28(7): 396-402.
83. Zuman P and Fijalek Z. Polarographic and Spectrophotometric Study of the Base Catalyzed Hydrolysis of Carbimazole. *Anal. Lett.* 1990; 23: 1201-1212.
84. Rama DB and Rao VS. Polarographic determination of carbimazole in pharmaceutical dosage forms. *Indian J. Pharm. Sci.* 1994; 3: 77-80.
85. Ciesielski, W and Krenc A. Potentiometric and Coulometric Determination of Carbimazole. *Anal. Lett.* 2000; 33: 1545-1554.
86. Sultan SM. A Kinetic Method for the Determination of Carbimazole in Pharmaceutical Preparations by Oxidation with Dichromate in Sulfuric Acid. *Anal Sci.* 1992; 8(4): 503-506.
87. Barzegar M, Rahmani A, Jabbari A and Mous MF. A New Kinetic-Photometric Method for Determination of Carbimazole. *J Chin Chem Soc.* 2004; 51: 363-366.
88. El-Bardicy MG, El-Saharty, YS and Tawakkol MS. Bromometric determination of Carbimazole. *Talanta.* 1993; 40:577-583.
89. Korany MA, Bedair MM, Elsayed MA and Fahmy OT. Colorimetric Determination of Pharmaceutical Thio compounds and Allopurinol Using .Mercurochrome. *Anal. Lett.* 1989; 22: 1909-1925.
90. Bedair MM, Korany MA, Elsayed MA and Fahmy OT. Spectrofluorimetric Determination of Three Pharmaceutical Thio compounds and Allopurinol Using Mercurochrome. *Spectrosc. Lett.* 1990; 23:161-173.
91. El-Saharty YS, Abdel KM and El-Bardicy MG. Spectrophotometric and spectrodensitometric determination of Carbimazole. *Spectrosc. Lett*, 2001; 34: 325-334.
92. Ciesielski W and Skowron M. Coulometric Titration of Thiols with Electrogenerated Chlorine. *Chem. Anal. (Warsaw)*. 2004; 49: 619-626.

93. Melander A, Hallengren B, Rosendal HS, Sjöberg AK and Wählén BE. Comparative in Vitro Effects and In Vivo Kinetics of Antithyroid Drugs. *Eur. J. Clin. Pharmacol.* 1980; 17: 295-299. DOI: 10.1007/BF00625803.
94. El-Bardicy MG, El-Saharty YS and Tawakkol MS. Determination of Carbimazole and Methimazole by First and Third Derivative Spectrophotometry. *Spectrosc. Lett.* 1991; 24: 1079-1095. DOI: 10.1080/00387019108018174.
95. Sánchez PC, Albero MI, García MS and Ródenas V. Flow-injection spectrophotometric determination of carbimazole and Methimazole. *Anal. Chim. Acta.* 1995; 308: 457- 461. DOI: 10.1016/0003-2670(94)00606-M.
96. García MS, Albero MI, Sánchez PC and Tobal L. Kinetic determination of carbimazole, methimazole and propylthiouracil in pharmaceuticals, animal feed and animal livers. *Analyst.* 1995; 120: 129-133. DOI: 10.1039/AN9952000129.
97. Fijalek Z and Zuman P. Determination of Methimazole and Carbimazole Using Polarography and Voltammetry. *Anal. Lett.* 1990; 23:1213-1233. DOI: 10.1080/00032719008054349.
98. Pritam SJ, Champalal TP and Sanjay JS. HPTLC and RP-HPTLC Method Development and Validation for the Estimation of Carbimazole in Bulk and Marketed Formulation. *Acta sci. pharm. sci.* 2019; 3: 102-106.
99. Abdelrahman MM. Chromatographic methods development, validation and degradation characterization of the antithyroid drug Carbimazole. *Biomed Chromatogr.* 2019; 33(4). DOI: 10.1002/bmc.4472.
100. Avinash VD, Shruti DD and Sanjay GW. Estimation of Carbimazole in Presence of its Degradants Using RP-HPLC. *Der Pharmacia Sinica.* 2012; 3(3):388-393.
101. Pinet E, Roessle C, Vasseur V, Benakouche S and Bourdon O. Formulation of an oral liquid solution of Carbimazole and validation of a stability indicator assay method. <http://www.gerpac.eu/formulation-of-an-oral-liquid-solution-of-carbimazole-and-validation-of-a-stability-indicator-assay-method>.
102. Madhavee DB, Arunadevi SB, Amol AJ, Amitkumar KJ and Priya DG. Determination of carbimazole in bulk and tablet formulation by using HPLC method. *World J Pharm Sci.* 2020; 9(11): 1321-1339.
103. <https://pharmaceutical-substances.thieme.com/ps/search-results?docUri=KD-03-0034>.
104. <https://www.mugesh.org/research-3>.
105. <https://www.drugbank.ca/drugs/DB01176>.

106. Sean C.S., Martindale the complete drug reference, 38th edition, pharmaceutical press, London.
107. <https://www.glowm.com/resources/glowm/cd/pages/drugs/c090.html>.
108. Backer RC, McFeeley P and Wohlenberg N. Fatality resulting from Cyclizine overdose. *J. Anal. Toxicol.* 1989; 13 (5): 308-309.
109. <https://pubchem.ncbi.nlm.nih.gov/compound/1-Methylpiperazine>.
110. https://www.chemicalbook.com/ChemicalProductProperty_EN_CB4478526.htm.
111. METHYLPIPERAZINE+98%25+500GR&vendorId=VN00032119&countryCode=US&language=en.
112. <https://pubchem.ncbi.nlm.nih.gov/compound/Diphenylmethanol>.
113. <https://www.scbt.com/p/diphenylmethanol-91-01-0>.
114. <https://en.wikipedia.org/wiki/Diphenylmethanol>.
115. Jalal IM, Sa'sa' SI, and Yasin TA. Determination of Ergotamine Tartarate and Cyclizine Hydrochloride in Pharmaceutical Tablets by Reverse Phase HPLC. *Anal. Lett*, 1988; 21(9); 1561-1577. DOI: 10.1080/00032718808066512.
116. Zhi-fang LI and Xiu-wen LU. HPLC Determination of Cyclizine Hydrochloride, Caffeine and Vitamin B6 in Compound Cyclizine Hydrochloride Tablets. *Chinese Journal of Pharmaceutical Analysis*, 2005; 25(2): 241-243.
117. Mohammadi A, Kanfer I and Walker RB. A capillary zone electrophoresis (CZE) method for the determination of Cyclizine Hydrochloride in tablets and suppositories. *J Pharm Biomed Anal.* 2004; 35(1):233-239. DOI: 10.1016/j.jpba.2004.01.011.
118. Walker B and Kanfer I. Sensitive high-performance liquid chromatographic determination of Cyclizine and its demethylated metabolite, norcyclizine, in biological fluids using coulometric detection. *J. Chromatogr. B Biomed. Appl.* 1995; 672 (1): 172-177.
119. <https://patents.google.com/patent/WO2018002696A1/en>.
120. Navaneeswari R and Raveendra P. Analytical method for piperazine in an active pharmaceutical ingredient using chemical derivatization and HPLC-UV. *J. Chem. Pharm. Res.* 2012; 4(6): 2854-2859.
121. <https://www.drugbank.ca/drugs/DB09216>.
122. Sean CS, Martindale the complete drug reference, 38th edition, pharmaceutical press, London.

123. <https://pubchem.ncbi.nlm.nih.gov/compound/3-Chloro-2-methylaniline>.
124. https://www.chemicalbook.com/ChemicalProductProperty_EN_CB4854513.htm.
125. <https://www.sigmaaldrich.com/catalog/product/aldrich/101621?lang=en®ion=IN>.
126. Sofia A, Muhammad AS, Ceyla Y and Ihtesham R. Quantitative determination of Tolfenamic acid and its pharmaceutical formulation using FTIR and UV spectrometry. *Cent. Eur. J. Chem.* 2013; 11(9):1533-1541. DOI: 10.2478/s11532-013-0284-6.
127. Ioannou PC, Rusakova NV, Andrikopoulou DA, Glynou KM and Tzompanaki GM. Spectrofluorimetric determination of anthranilic acid derivatives based on terbium sensitized fluorescence. *Analyst.* 1998; 123: 2839–2843. DOI: 10.1039/A806093B.
128. Gallo P, Fabbrocino S, Dowling G, Salini M, Fiori M, Perretta G, et al. Confirmatory analysis of non-steroidal anti-inflammatory drugs in bovine milk by high performance liquid chromatography with fluorescence detection. *J.Chromatogr. A.* 2010; 1217(17): 2832–2839. DOI:10.1016/j.chroma.2010.02.047.
129. Ibrahim H, Boyer A, Bouajila J, Couderc F and Nepveu F. Determination of non-steroidal anti-inflammatory drugs in pharmaceuticals and human serum by dual-mode gradient HPLC and fluorescence detection. *J. Chromatogr. B.* 2007; 857(1): 59–66. DOI: 10.1016/j.jchromb.2007.07.008.
130. Mikami E, Goto T, Ohno T, Matsumoto H, Inagaki K, Ishihara H, et al. Simultaneous analysis of anthranilic acid derivatives in pharmaceuticals and human urine by high-performance liquid chromatography with isocratic elution. *J. Chromatogr B Biomed Sci Appl.* 2000; 744(1):81–89.
131. Rozou S and Antoniadou VE. An improved HPLC method overcoming Beer's law deviations arising from supramolecular interactions in Tolfenamic acid and cyclodextrins complexes. *J. Pharm. Biomed. Anal.* 1998; 18(4-5): 899–905.
132. Niopas I and Georgarakis M. Determination of Tolfenamic acid in human plasma by HPLC. *J. Liq. Chromatogr.* 1995; 18(13):2675–2682.
133. Papadoyannis I, Georgarakis M, Samanidou V and Zotou A. Rapid assay for the determination of Tolfenamic acid in pharmaceutical preparations and biological fluids by High-performance liquid chromatography. *J. Liq. Chromatogr.* 1991; 14(15): 2951–2967.

134. Papadoyannis IN, Zotou AC and Samanidou VF. Simultaneous reversed-phase gradient-HPLC analysis of anthranilic acid derivatives in anti-inflammatory drugs and samples of biological interest. *J. Liq. Chromatogr.* 1992; 15:1923–1945.
135. Shinozuka T, Takei S, Kuroda N, Kurihara K and Yanagida J. Micro determination of anthranilic acid derivatives of anti-inflammatory drugs by High performance liquid chromatography and its application to forensic chemistry. *Eisei Kagaku* 1991; 37: 461–466.
136. Abdelwahab NS, Ali NW, Zaki MM and Abdelkawy M. Validated chromatographic methods for simultaneous determination of Tolfenamic acid and its major impurities. *J. Chromatogr. Sci.* 2015; 53: 481-491. DOI:10.1093/chromsci/bmu07.
137. <https://patents.google.com/patent/CN102786429A/en>.
138. http://environmentclearance.nic.in/writereaddata/Online/TOR/28_Jun_2017_110706163HW0QXEY2AdditionalAttachment.pdf.



CHAPTER – 11

APPENDIX

11. APPENDIX

11.1. List of Publications

1. Fernandes A, Pai PN Sanjay. An Improved Method for Separation of Pharmacopoeial Specified Impurities of Mefenamic acid by RP-HPLC Method. Latin American Journal of Pharmacy (formerly Acta Farmacéutica Bonaerense). 2019; 38(1): 07-12.
2. Fernandes A, Pai PN Sanjay. Development and Validation of Stability Indicating RP-HPLC Assay Method for Mefenamic Acid. Asian Journal of Chemistry. 2019; 31(3): 656-660.
3. Fernandes A, Pai PN Sanjay. A Validated Stability Indicating RP-HPLC Method for Estimation of Tolfenamic Acid In presence of its Pharmacopoeial Impurities. International Journal of Applied Pharmaceutics. 2019; 11(5):264-270.

11.2. List of Presentations

1. Oral Presentation titled “An Improved Method for Separation of Pharmacopoeial Specified Impurities of Mefenamic acid by RP-HPLC method” at 69th Indian Pharmaceutical Congress, Chitkara University, Chandigarh 2017. The Oral presentation was awarded 8th place and a cash amount of 15,000/-.
2. Oral Presentation titled “Development and Validation of Stability Indicating RP-HPLC Assay Method for Mefenamic acid” at *INNOPHARM3*, Campal, Goa 2018. The oral presentation was awarded 3rd place in Pharmaceutical Chemistry/Pharmaceutical Analysis Category.

11.3. Degradation Pathway of Mefenamic Acid (MA) & Tolfenamic Acid (TA) under conditions of stress induced oxidation.

An Improved Method for Separation of Pharmacopoeial Specified Impurities of Mefenamic acid by RP-HPLC Method

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SUMMARY. A RP-HPLC method for determination of all specified related substances of mefenamic acid (MA) was developed. The separation was carried out on a Sunfire ODS C18 column (4.6 × 250 mm, 5 μm), using acetonitrile and 10 mM ammonium dihydrogen phosphate buffer pH 4 in the ratio of 55:45, v/v at a flow rate of 1 mL/min. The column temperature was kept at 40 °C and analyses were carried out at λ max of 225 nm. The analytical method was validated as per ICH guidelines Q2 (R1). The resolution between three specified impurities and the drug was found to be greater than 2. The method is specific for mefenamic acid, since no interfering peaks were observed with an overall run time of 20 min. The peak symmetry of the drug and all the specified impurities is less than 2. Linearity was obtained in the ranges of 10-100, 0.5-10, 1-10, and 1-10 μg/mL for mefenamic acid, 2,3-dimethylaniline, 2-cholorbenzoic acid, and benzoic acid, respectively. All calibration curves showed good linear correlation coefficient ($r^2 > 0.995$) with the tested ranges. Accuracy reported as % recovery was found to be within limit for mefenamic acid and its specified impurities. The relative standard deviation for inter-day precision (reproducibility), and intra-day precision (repeatability) for MA and its specified impurities was found to be less than 2%. The method was found to be robust for variation in mobile phase flow rate (± 0.1 mL/min) and mobile phase composition ($\pm 2\%$). The proposed method can be used for analysis of specified related substances of mefenamic acid.

RESUMEN. Se desarrolló un método RP-HPLC para la determinación de todas las sustancias relacionadas con el ácido mefenámico (MA). La separación se llevó a cabo en una columna Sunfire ODS C18 (4,6 × 250 mm, 5 μm), usando acetonitrilo y tampón dihidrógeno fosfato de amonio 10 mM, pH 4 en relación de 55:45 v/v con un caudal de 1 mL/min. La temperatura de la columna se mantuvo a 40 °C y los análisis se llevaron a cabo a λ max de 225 nm. El método analítico se validó según las pautas ICH Q2 (R1). Se encontró que la resolución entre tres impurezas especificadas y el fármaco era mayor que 2. El método es específico para el ácido mefenámico, ya que no se observaron picos de interferencia con un tiempo total de ejecución de 20 min. La simetría máxima del fármaco y todas las impurezas especificadas son menores que 2. Se obtuvo linealidad en los rangos de 10-100, 0,5-10, 1-10 y 1-10 μg/ mL para el ácido mefenámico, 2,3-dimetilnilina, ácido 2-cholorbenzoico y ácido benzoico, respectivamente. Todas las curvas de calibración mostraron un buen coeficiente de correlación lineal ($r^2 > 0.995$) con los rangos probados. La precisión reportada como % de recuperación se encontró dentro del límite para el ácido mefenámico y sus impurezas especificadas. La desviación estándar relativa para la precisión (reproducibilidad) entre días y la precisión (repetibilidad) intradía para MA y sus impurezas especificadas fue inferior al 2%. Se encontró que el método era robusto para la variación en la tasa de flujo de la fase móvil (± 0.2 mL/min) y en la composición de la fase móvil ($\pm 2\%$). El método propuesto puede utilizarse para el análisis de sustancias relacionadas específicas del ácido mefenámico.

INTRODUCTION

Mefenamic acid (MA) is 2-[(2, 3-dimethylphenyl) amino] benzoic acid, is a member of the anthranilic acid derivatives class of NSAID drugs ¹. Mefenamic acid is a COX inhibitor and prevents formation of prostaglandins ². Mefenamic acid exhibits anti-inflammatory and analgesic activities ². It is used to relieve pain

arising from rheumatic condition, soft tissue injuries, other painful musculoskeletal conditions and dysmenorrhea ². British pharmacopeia ³ mentions three specified impurities (Fig. 1) and two non-specified impurities (Fig. 2) in the monograph of mefenamic acid. British pharmacopeia monograph carries non-aqueous titrimetric method for analysis of mefenamic acid.

KEY WORDS: ICH guidelines, mefenamic acid, RP-HPLC, specified impurities, validation.

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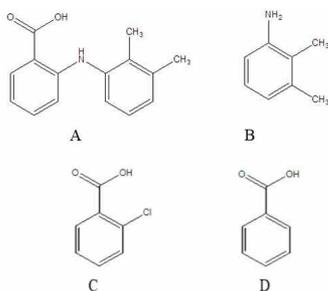


Figure 1. A) Chemical structure of mefenamic acid, B) chemical structure of 2,3- dimethylaniline (impurity A), C) chemical structure of 2 chlorobenzoic acid (impurity C), and D) chemical structure of benzoic acid (impurity D).

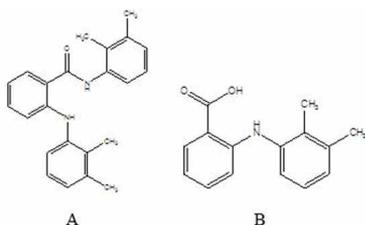


Figure 2. A) Chemical structure of N-(2, 3-dimethylphenyl)-2-[(2,3-dimethyl phenyl)amino] benzamide (impurity B); B) chemical structure of 2,3-dimethyl-N-phenylaniline (impurity E).

Literature survey cited numerous analytical methods for the estimation of mefenamic acid in pharmaceutical formulations or biological fluids either individually or with other drugs, including viz., spectrophotometry ⁴⁻⁹, HPLC ¹⁰⁻¹⁹, HPTLC ^{20,21} and Gas Chromatography ²². Method involving simultaneous determination of MA and two of its specified impurities by RP-HPLC has been reported ²³. No method has been reported in the literature for simultaneous determination of mefenamic acid and all its 3 specified impurities. We report a new simple validated RP-HPLC method for determination of three specified impurities of mefenamic acid.

MATERIALS AND METHOD

Material and reagents

Mefenamic acid was obtained from Blue Cross Laboratories Pvt. Ltd. (Goa, India). 2,3-dimethylaniline (impurity A), 2-chlorobenzoic acid (impurity C) and benzoic acid (impurity D) were purchased from Sigma-Aldrich Co., St. Louis, MO, USA. Tetrahydrofuran (THF) and acetonitrile of HPLC grade were purchased from Rankem, Mumbai, India. Water for HPLC was purified using Bio-age Water purification system. All other chemicals and solvents used were of analytical reagent grade. The solvents and solutions of samples prepared were filtered through 0.45 μ m membrane filter before injecting into the chromatograph.

HPLC instruments and analytical conditions

In the current study Jasco LC-4000 series HPLC system consisting of a quaternary solvent delivery system (PU-4180), an on-line degasser, an auto-sampler (AS-4050), a column temperature controller (CO-4061) and a diode array detector (MD-4010) were used. System control and data analysis were processed with Jasco Chrom NAV software. Other instruments used for this study include Bath sonicator (Citizon Ultrasonic cleaner); Digital Balance (Wensar Digital Electronic Balance) and pH meter (Labtronics, LT-10).

The separation and quantification by RP HPLC were achieved on Sunfire ODS C18 column (4.6 \times 250 mm, 5 μ m) from Waters. The mobile phase used for study involved acetonitrile and 10 mM ammonium dihydrogen phosphate buffer (pH adjusted to 4 with dilute acetic acid solution) in the ratio of 55:45 v/v, with flow rate of 1 mL/min. Studies were performed at 40 $^{\circ}$ C temperature, with the injected volume of 10 μ L and detection wavelength of 225 nm.

Standard solutions

Buffer solution

Ammonium dihydrogen phosphate buffer 10 mM was prepared by dissolving 1.15 g of ammonium dihydrogen phosphate in HPLC water and the volume was made up in a standard one liter volumetric flask. The pH was adjusted to 4 using dilute acetic acid solution. The prepared buffer solution was filtered through 0.45 μ m HPLC membrane filter.

Stock standard solutions (1,000 μ g/mL)

Stock standard solutions of mefenamic acid, impurity A, impurity C and impurity D was prepared by weighing separately and accurately 10 mg of pure powder of each into four separate 10 mL standard volumetric flasks; 7.5 mL of acetonitrile was added in all the volumetric flasks and sonicated for 15 min. The final volume was made to the mark with acetonitrile.

Working standard solutions (100 μ g/mL)

Working standard solutions of impurity A, impurity C and impurity D were prepared by accurately transferring 1 mL each of the impurity from their respective stock standard solutions (1,000 μ g/mL) into 3 separate 10 mL volumetric flasks. Final volume to the mark was made with acetonitrile to get 100 μ g/mL working solutions of each component.

Selection of the detection wavelength for the method

Individual spectra scan was carried out for mefenamic acid and all its specified impurities in the range of 190 nm to 400 nm. From the overlays of the spectra's the wavelength that showed sufficient ab-

sorbance of all the components was selected for the study. Thus wavelength of 225 nm was selected as the detection wavelength for the proposed method.

Method validation

The developed method for related specified impurities of mefenamic acid was validated as per ICH guidelines ²⁴.

Specificity

The specificity of the method was evaluated by application of the developed method to a mixture containing different concentration of mefenamic acid, impurity A, impurity C and impurity D. Further, specificity was confirmed by calculating system suitability testing parameters such as resolution, peak asymmetry, and no of theoretical plates, for the separated chromatographic peaks.

Linearity and range

Accurately measured volumes equivalent to 10-100 µg/mL of mefenamic acid were separately transferred from its stock standard solution (1,000 µg/mL) into 10 mL volumetric flask and the volume was made up to the mark with mobile phase. For impurities, accurately measured volumes equivalent to 0.5-10 µg/mL of impurity A, 1-10 µg/mL of impurity C, and 1-10 µg/mL of impurity D were separately transferred from their respective working standard solutions (100 µg/mL) into 10 mL volumetric flasks. The volume was completed to the mark with the mobile phase. Six 10 µL injections were given of each concentration. The peak areas were recorded and the calibration curves were plotted.

Precision

Intraday repeatability of the method was evaluated by analyzing three concentrations of mefenamic acid (30, 50, and 70 µg/mL), and three concentration of impurity A, impurity C and impurity D (3, 5, and 7 µg/mL). Three different sets were prepared each containing mefenamic acid, impurity A, impurity C, and impurity D. The first set contained 30 µg/mL of mefenamic acid and 3 µg/mL of each impurity. The second set contained 50 µg/mL of mefenamic acid and 5 µg/mL of each impurity and the third set contained 70 µg/mL of mefenamic acid and 7 µg/mL of each impurity. Interday precision was evaluated by assaying the three chosen sets of mefenamic acid, impurity A, impurity C and impurity D in triplicates on two successive days using the same procedure stated under chromatographic conditions. The % RSD values were then calculated.

Accuracy

Accuracy of the proposed method was determined by recovery studies using standard addition method. The percentage recovery studies of mefenamic acid,

impurity A, impurity C, and impurity D was carried out in triplicate at 3 different levels: 80%, 100%, and 120%, by spiking standard mefenamic acid, impurity A, impurity C, and impurity D.

Sensitivity

The sensitivity of the developed method was determined by calculating the limit of detection (LOD) and limit of quantification (LOQ) for mefenamic acid and its three impurities. LOD and LOQ were calculated for mefenamic acid and all its impurities as follows: $LOD = 3.3 \times \text{standard deviation of the response} / \text{slope of the calibration curve}$ and $LOQ = 10 \times \text{standard deviation of the response} / \text{slope of the calibration curve}$.

Robustness

The robustness was tested by checking the effect of small deliberate changes in the chromatographic conditions. Changes in the flow rate of the mobile phase (± 0.1 unit) and the proportion of organic phase in mobile phase ($\pm 2\%$ units) on the developed method were studied.

RESULT AND DISCUSSION

Development and optimization of HPLC method

The RP HPLC method mentioned in BP were carried out on the available column using the chromatographic conditions as specified in the monograph of mefenamic acid ³. Mobile phase comprising of 50 mM phosphate buffer (pH adjusted to 5.0 with dilute ammonia): acetonitrile: THF (40:46:14 v/v) is used with flow rate of 1 mL/min, injection volume of 10 µL and detection wavelength of 254 nm, on a C18 column (4.6 × 250 mm, 5 µm) ³. Solution containing 50 µg/mL of mefenamic acid and 5 µg/mL of each impurity were injected and the chromatogram obtained as shown in Fig. 3.

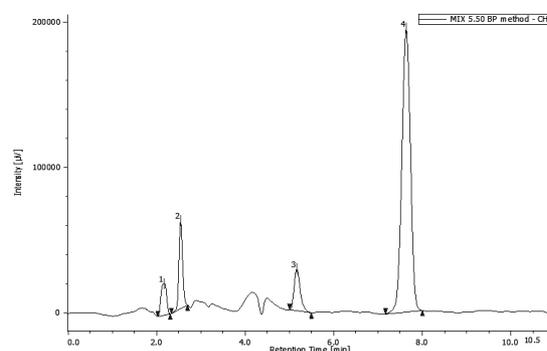


Figure 3. Representative chromatogram (BP method) of mefenamic acid (peak 4) with impurity A (peak 3), impurity C (peak 1), and impurity D (peak 2).

The obtained chromatogram showed several noise peaks in the vicinity of impurity peaks. Base line stabilization was not satisfactory and unconventional sol-

vent like THF is used in the method. Hence the proposed method was developed to separate all three specified impurities from each other and from the drug using HPLC by avoiding use of THF as a component of the mobile phase. Also to minimize the noise peaks interfering with the peaks of impurities and drug.

Based on the pKa values of all the components Mefenamic acid (pKa 4.2), impurity A (pKa 4.70), impurity C (pKa 2.89), and impurity D (pKa 4.2), the preliminary trials were carried out using Sunfire BDS C18 (4.6 × 250 mm, 5 μm) column as stationary phase and acetonitrile and 10 mM ammonium dihydrogen

phosphate buffer with pH of the buffer varying from 3.2 to 6.2 as mobile phase. The flow rate was kept at 1 mL/min and the temp of the column was maintained at 40 °C. The analysis was carried out at detection wavelength of 225 nm.

Based on various trials conducted it was observed that all the impurities and drug were separated from each other when the pH of buffer was adjusted to 4 and the composition of mobile phase was in the ratio of 55:45%v/v of acetonitrile and buffer, respectively. The final optimized chromatographic condition used in the proposed method is listed in **Table 1**.

Mobile phase	Mixture of acetonitrile and 10 mM and ammonium di hydrogen phosphate buffer (pH adjusted to 4 with dilute acetic acid solution) in the ratio of 55:45v/v.
Column	Sunfire ODS C18 (4.6 × 250 mm, 5 μm) column
Injection Volume	10 μL
Flow Rate	1 mL/min
Column oven temperature	40 oC
Detection wavelength	225 nm

Table 1. Optimized chromatographic conditions of the proposed RP HPLC method.

The chromatogram obtained with the optimized chromatographic conditions of solution containing 50 μg/mL of mefenamic acid and 5 μg/mL of each impurity is shown in **Fig. 4**.

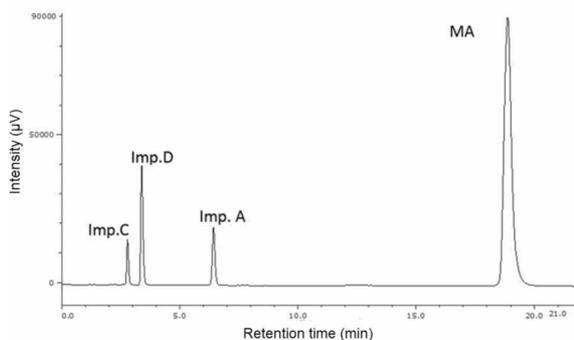


Figure 4. Optimized chromatogram showing separation of Mefenamic acid (MA) from the impurities and also from each other.

Validation of the developed method

The proposed method was validated with aspect of system suitability, specificity, linearity and range, accuracy, precision, LOD, LOQ, stability and robustness according to the ICH guidelines²⁴.

System suitability

The developed chromatographic method shows better separation between the impurities and Mefenamic acid from its impurities, where all system suitability parameters were very well within the limits. The chromatogram showing separation between drug and impurities is depicted in **Fig. 4**. The results of the system suitability parameters are presented in **Table 2**.

Sr. no	Components	RRT	Resolution	Peak asymmetry	Theoretical plate
1	Mefenamic acid	1	NA	1.28	17560
2	Impurity A (2,3-dimethyl aniline)	0.34	32.9	1.19	16490
3	Impurity C (2-dihlorobenzoic acid)	0.14	3.9	1.31	6301
4	Impurity D (benzoic acid)	0.18	16.6	1.34	6558

Table 2. System suitability testing parameters of the proposed RP HPLC method.

Linearity

The linearity of the proposed method was evaluated and found in the range of 10-100 µg/mL for mefenamic acid, 0.5-10 µg/mL for impurity A, and 1-10 µg/mL each for Impurity C and Impurity D. Re-

gression analysis was performed and correlation coefficient values ranged from 0.9982 to 1. Results of regression parameters are shown in Table 3. The results indicated that the method is linear over the studied concentration range.

Parameters	Mefenamic acid	Impurity A	Impurity C	Impurity D
Range(µg/mL)	10-100	0.5-10	1-10	1-10
Regression equation	$y = 52433x - 45446$	$y = 31772x - 761$	$y = 14321x - 946$	$y = 51500x - 1287$
Correlation coefficient	0.999	0.9997	0.9982	1
Intraday precision (%RSD)	0.41	0.9	0.92	0.3
Interday precision (%RSD)	0.37	0.78	0.99	0.45
LOD (µg/mL)	4.09	0.22	0.54	0.34
LOQ (µg/mL)	12.42	0.67	1.66	1.04

Table 3. Regression parameters of the developed RP HPLC method.

Precision and sensitivity

The developed method showed acceptable intraday and interday variation, revealing good precision of the method and its suitability for efficient separation of all the components. Results showed good values of % RSD. Results of precision studies and the values of LOD and LOQ obtained for Mefenamic acid and its 3

specified impurities are shown in Table 3.

Accuracy

Good percentage recoveries were obtained when MA and its specified impurities were subjected to standard addition method as shown in Table 4. The results are within the acceptance criteria of 95-105% indicating accuracy of the method.

Component	Amount of sample (µg)	Level of spiking (%)	Amount of standard spiked (µg)	Amount recovered (µg)	% Recovery
Mefenamic acid	40	80	32	32.38	101.18
		100	40	39.98	99.95
		120	48	48.13	100.27
2,3-Dimethyl aniline (Impurity A)	4	80	3.2	3.16	98.76
		100	4	3.96	99.04
		120	4.8	4.84	100.95
2-Chloro Benzoic acid (Impurity C)	4	80	3.2	3.28	102.57
		100	4	4.14	103.66
		120	4.8	4.99	103.99
Benzoic acid (Impurity D)	4	80	3.2	3.21	100.47
		100	4	4.05	101.45
		120	4.8	4.81	100.22

Table 4. Data of Accuracy studies for Mefenamic acid and its specified impurities.

Robustness

The developed method was found to be robust and any deliberate changes in the studied chromatographic condition did not show significant changes in the % RSD values of mefenamic acid and its impurities as shown in Table 5.

Component	Change in flow rate (± 0.1 mL)	Change in organic phase composition ($\pm 2\%$)	%RSD
Mefenamic acid	1.7	1.13	
Impurity A	1.64	1.73	
Impurity C	1.46	0.72	
Impurity D	1.5	0.9	

Table 5. Results of robustness studies.

CONCLUSION

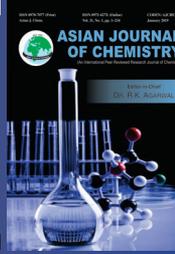
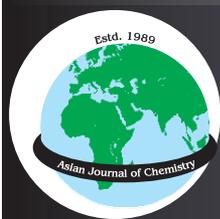
The developed RP HPLC method was found to be simple, specific, accurate, precise and robust for the determination of specified impurities of mefenamic acid. The developed method has advantage over the BP method in terms of baseline stabilization, better resolution of peaks and avoidance of THF in the mobile phase. As compared to other available method²³, the proposed method can simultaneously separate all the three specified impurities of mefenamic acid. Hence the proposed method can be useful for routine quality control of mefenamic acid samples.

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REFERENCES

- Merck Index (2001) *An encyclopedia of chemicals, drugs and biologicals*, 13th edition.
- Goodman and Gilman's (2001) *The pharmacological basis of therapeutics*. McGraw Hill Publication, pp. 703-8.
- British Pharmacopoeia (2013) 15th ed. Vol. I. United Kingdom: The HMSO Publication Centre.
- Alarfaj, N.A., S.A. Altamimi & L.Z. Almarshady (2009) *Asian J. Chem.* 21: 217-26.
- Singh, H., R. Kumar & P. Singh (2011) *Int. J. Pharm. Sci.* 3(2): 237-8.
- Subramanian, N., T. Devipriyadharshini, K. Venkateshwaran & P. Chandrasekar (2011) *Int. J. Pharm. Biomed. Res.* 1(20): 26-9.
- Kormosh, Zh. A., O. Yu. Matviichuk & Ya. R. Bazel (2014) *J. Anal. Chem.* 69(10): 960-4.
- Naveed, S. & F. Qamar (2014) *Int. J. Pharm. Sci. Res.* 5(7): 364-6.
- Al Abachi, M.Q. & H. Hadi (2014) *J. Anal. Chem.* 69(8): 769-76.
- Maron, N. & C. Wright (1990). *J. Pharm. Biomed. Anal.* 8: 101-5.
- Poirier, J.M., M. Lebot & G. Cheymol (1992) *Drug Monit.* 14: 322-6.
- Murali, K.R., M. Buela & A. Sahithi (2011) *Int. J. Chem. Sci.* 9: 1587-94.
- Rouini, M.R., A. Asadipour, Y.H. Ardakani & F. Aghdasi (2004) *J. Chromatogr. B.* 800:189-92.
- Sato, J., E. Owada, K. Ito, Y. Nidya, A. Wakamatsu & M. Umetsu (1989) *J. Chromatogr. Sci.* 493: 239-43.
- Padmalatha, H. & G. Vidyasagar (2011) *Int. J. Res. Pharm. Biomed. Sci.* 2: 1261-5.
- Wahab, S.U., F.P. Mohamed, H.S. Mohamed, M. Chandran & M. Kadher (2011) *Int. J. Pharm. Sci.* 3: 115-8.
- Al-Qaim, F.F., M.P. Abdullah, M.R. Othman & W.A. Khalik (2014) *Int. J. Chem. Sci.* 12(1): 62-72.
- Oswal, T., S. Bhosale & S. Naik (2014) *Int. J. Pharm. Sci. Res.* 5(6): 232-3.
- Prajapati, D. & H. Raj (2012) *Int. J. Pharm. Biosci.* 3: 611-25.
- Saleh, S.F., S.M. Dereaya & M.A. Omar (2014) *Int. J. Chem. Anal. Sci.* 5: 55-60.
- Satish Y., N. Gabhe, K Reddy, E.P. Sachin & R.M. Kakasaheb (2013) *Der Pharmacia Sinica* 4: 16-21.
- Duscim L.J. & L.Pm Hackett (1978) *J. Chromatogr. A* 161: 340-2.
- Morcoss, M.M., N.S. Abdelwahab, N.W. Ali & M.T. Elsaady (2017) *J. Chromatogr. Sci.* 55(7): 766-72.
- ICH (2005) Q2 (R1). *International conference on Harmonization, IFPMA, Geneva, Switzerland.*



Development and Validation of Stability Indicating RP-HPLC Assay Method for Mefenamic Acid

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The present research work was carried out to evaluate the stability behaviour of mefenamic acid under ICH Q1A (R2) recommended stress conditions. The drug was subjected to hydrolytic, oxidative, photolytic and thermal stress conditions. The drug was found susceptible to degradation under oxidative stress condition but was stable under hydrolytic, photolytic and thermal stress conditions. A total two degradation products were formed, which were separated using HPLC. The chromatographic separation was carried out on Sunfire ODS C-18 (250 × 4.6 mm, 5 μm) column. Optimum resolution was obtained using ammonium dihydrogen phosphate buffer (10 mM, pH 4) and acetonitrile programmed in isocratic elution mode in the ratio of 45:55 v/v at 225 nm using photodiode array detector at a flow rate of 1 mL/min. The designed method was validated as per ICH Q2 (R1) guidelines. The response of drug was linear in the concentration range of 10-100 μg/mL ($R^2 = 0.9998$). The method was found specific, precise and accurate. The mean accuracy was found to be 100.46 %. The developed method was successfully applied for the analysis of marketed formulation.

Keywords: HPLC, Mefenamic acid, Stability indicating, Stress conditions.

INTRODUCTION

A matter of great concern for pharmaceutical molecule is its chemical stability, which in turn determines the welfare and potency of the drug product. The importance of stability testing profiles of active pharmaceutical ingredients and drug products is held in high esteem by various regulatory bodies like FDA, ICH guidelines, so as to understand the behavior of drug under various stress conditions with respect to time. For filling in registration dossier for new drug moiety, performing stability studies has become mandatory [1,2]. It is very important that the developed stability indicating method should be able to estimate the percentage of unchanged drug during the process when applied for dosage forms. After subjecting the drug to various stress conditions, the samples of drug are utilized for developing the stability indicating method which can be further applied for the testing of drug samples [3,4]. Mefenamic acid, 2-(2,3-dimethyl phenyl)aminobenzoic acid (Fig. 1) is a powerful anti-inflammatory drug used as a effective analgesic and anti-inflammatory agent for various clinical conditions like nonarticular rheumatism, osteoarthritis, sport injuries and other sever musculoskeletal illnesses [5,6]. Various literatures on

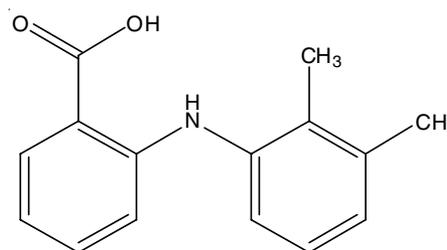


Fig. 1. Chemical structure of mefenamic acid

stability indicating methods for mefenamic acid individually [7,8] and in combination [9-12] with other drugs have been reported. However, few literatures revealed differences in stability studies data of mefenamic acid, indicating different behavior of mefenamic acid in different stress conditions [7,8]. Hence, the purpose of the work was to bring forth an alternative validated RP-HPLC stability indicating method for the estimation of mefenamic acid in pharmaceutical dosage forms.

EXPERIMENTAL

Mefenamic acid was obtained as a gift sample from Blue Cross Ltd. (Goa, India). Acetonitrile (HPLC grade), ammonium

dihydrogen phosphate, sodium hydroxide, hydrochloric acid and hydrogen peroxide were obtained from Merck (India). HPLC grade water was obtained from Bio-age water purification system. Mefenamic acid with brand name MEFTAL® -250 DT with a label claim of 250 mg drug was purchased commercially. All chemicals were of an analytical grade and used as received. The membrane filters (0.45 µ) were procured from Merck, India.

HPLC Instrument and analytical conditions: In current study, Jasco LC-4000 series HPLC system consisting of a quaternary solvent delivery system (PU-4180), an on-line degasser, an auto-sampler (AS-4050), a column temperature controller (CO-4061) and a diode array detector (MD-4010) were used. System control and data analysis were processed with Jasco Chrom NAV software. Other instruments used for this study include Bath sonicator (Citizon Ultrasonic cleaner), Digital Balance (Wensar Digital Electronic Balance) and pH meter (Labtronics, LT-10). The separation and quantification by RP HPLC were achieved on Sunfire ODS C18 column (4.6 × 250 mm, 5 µm) from Waters. The mobile phase used for study involved acetonitrile and 10mM ammonium dihydrogen phosphate buffer (pH adjusted to 4 with dilute acetic acid solution) in the ratio of 55:45 v/v, with flow rate of 1 mL/min. Studies were performed at 40 °C temperature, with the injected volume of 10 µL and detection wavelength of 225 nm.

Method development

Selection of mobile phase: The objective behind development of simple RP-HPLC method is to study degradation behaviour of mefenamic acid under ICH recommended stress conditions. Different trials were carried out using Sunfire ODS C18 (4.6 × 250 mm, 5 µm) column as stationary phase and acetonitrile and ammonium dihydrogen phosphate buffer (pH ranging from 2.5 to 6.5) as a mobile phase.

Preparation of standard stock solution: Stock solution of mefenamic acid was prepared by dissolving accurately weighed 100 mg of mefenamic acid in acetonitrile in 100 mL volumetric flask and making up the mark with acetonitrile. Solutions were filtered through a 0.45 µm membrane filter prior to injection in the system. Twenty tablets of MEFTAL® -250 DT were purchased from the local market, weighed and crushed to a fine powder. Powder equivalent to 50 mg of mefenamic acid was accurately weighed into a 25 mL volumetric flask, made up to volume with acetonitrile, sonicated for 30 min and filtered. The filtrate was diluted to the required concentration with mobile phase before injecting. The solutions were filtered through a 0.45 µm membrane filter before injections.

Calibration curve: Required aliquots were taken from stock solution in 10 mL volumetric flasks and diluted to the mark with mobile phase to get final concentrations of the drug in the range of 10 to 100 µg/mL. 10 µL of each concentration in triplicate were injected and the obtained chromatograms peak areas were recorded. Calibration curves were constructed by plotting the peak area on the y-axis and concentration of the drug on x-axis. The calibration curve was evaluated by its coefficient of determination (R^2).

Method validation: The developed method was validated for selectivity, linearity, accuracy, precision and robustness as per reported method [13].

Linearity: Accurately measured volumes equivalent to 10-100 µg/mL of mefenamic acid were separately transferred from its stock standard solution (1,000 µg/mL) into 10 mL volumetric flasks and the volumes was made up to the mark with mobile phase. Triplet 10 µL injections were given of each concentration. The peak areas were recorded and the calibration curve was plotted.

Accuracy: Accuracy of the proposed method was determined by recovery studies using standard addition method. The percentage recovery studies of mefenamic acid was carried out in triplicate at three different levels 80, 100 and 120 %.

Precision: Intraday repeatability of the method was evaluated by analyzing three concentration of mefenamic acid (30, 50 and 70 µg/mL). Interday precision was evaluated by assaying the chosen concentration of mefenamic acid in triplicates on two successive days using the same procedure stated under chromatographic conditions. The % RSD values were then calculated.

Robustness: The robustness was tested by checking the effect of small deliberate changes in the chromatographic conditions. Changes in the flow rate of mobile phase (± 0.1 unit) and the proportion of organic phase in mobile phase (± 2 % units) on the developed method were studied.

Forced degradation studies: To evaluate the stability indicating properties and specificity of the method, forced degradation studies were performed [14,15]. Drug solution used in stress studies were prepared from stock solution after dilutions and then diluted with mobile phase to give a final concentration of 10 µg/mL. The acidic and alkaline degradations of mefenamic acid were carried out in HCl (1 M) and in NaOH (1M) at 70 °C over a period of 6 h, respectively. The stressed samples were cooled to room temperature, neutralized and diluted with mobile phase. Neutral hydrolytic degradation was done by subjecting the drug in water for a period of 6 h at 70 °C. Oxidation of the drug was done by using 10 % H₂O₂ solution for period of 6 days. Photolysis was carried by exposing the drug to direct sunlight for 7 days, whereas thermal degradation was done by heating the drug in oven at 80 °C for 4 days. The stressed samples obtained in neutral, oxidation, photo and thermal stress conditions were cooled to room temperature and diluted with mobile phase.

RESULTS AND DISCUSSION

Chromatographic separation: Based on various trials conducted drug showed good symmetrical peak with system suitability parameters in acceptable limits when the pH of buffer was adjusted to 4 and the composition of mobile phase was in the ratio of 55:45 % v/v of acetonitrile and buffer, respectively as shown in Table-1. The final optimized chromatographic condition used in the proposed method is listed in Table-2 and the chromatogram obtained with the optimized chromatographic conditions of solution containing 10 µg/mL of mefenamic acid is shown in Fig. 2.

TABLE-1
SYSTEM SUITABILITY PARAMETERS OF
THE PROPOSED RP HPLC METHOD

Components	Rt	Area	Peak asymmetry	Theoretical plate
Mefenamic acid (50 µg/mL)	21.10	585330	1.28	17560

TABLE-2
OPTIMIZED CHROMATOGRAPHIC CONDITIONS OF
THE PROPOSED RP HPLC METHOD

Mobile phase	Mixture of acetonitrile and 10 mM ammonium dihydrogen phosphate buffer (pH adjusted to 4 with dilute acetic acid solution) in the ratio of 55:45v/v.
Column	Sunfire ODS C18 (250 × 4.6 mm, 5 μm) column
Injection Volume	10 μL
Flow Rate	1 mL/min
Column oven temperature	40 °C
Detection wavelength	225 nm

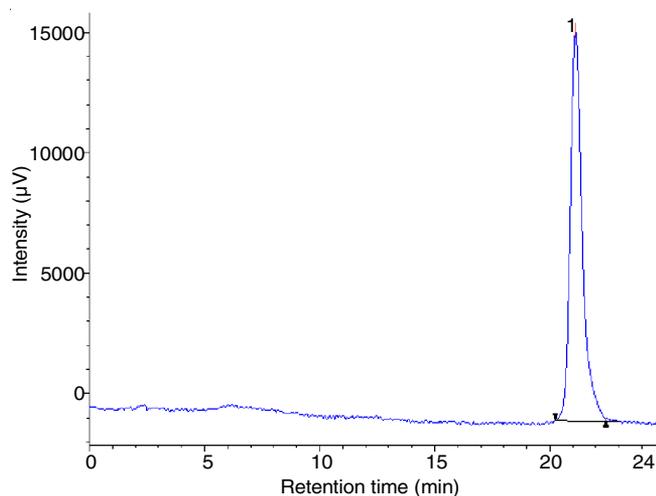


Fig. 2. Optimized chromatogram of mefenamic acid in mobile phase

Calibration curve of mefenamic acid: The correlation coefficient of determination (R^2), slope and intercept for mefenamic acid were 0.999, 52423 and 45446, respectively over the range of 10-100 μg/mL. The calibration curve of mefenamic acid is shown in Fig. 3.

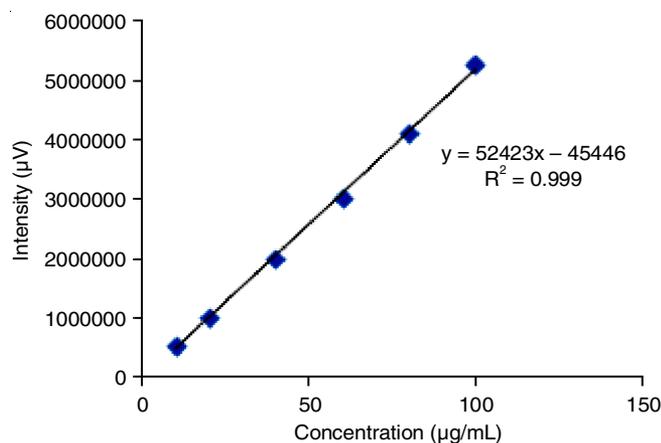


Fig. 3. Standard calibration curve of mefenamic acid

Method validation

Linearity, accuracy and precision: The results indicated that the method is linear over the studied concentration range of 10-100 μg/mL as per calibration curve. Intra and inter-day precision data of the RP-HPLC method for mefenamic acid is shown in Table-3. Results showed good values of % RSD which were within the limits.

The accuracy of the method is determined by recovery studies using standard addition method. The results of accuracy studies are shown in Table-4. The results are within the acceptance criteria of 95-105 % indicating accuracy of method.

TABLE-4
ACCURACY-RECOVERY STUDY OF MEFENAMIC
ACID BY STANDARD ADDITION METHOD

Sample No.	Spiked concentration (μg/mL)	Measured concentration (mg/mL)	Recovery (%)
1	32(80%)	32.38	101.18
2	40(100%)	39.98	99.95
3	48(120%)	48.13	100.27

Robustness: Robustness of the method was studied by deliberate variations of the analytical parameters such as flow rate (1 ± 0.1 mL/min) and change in organic phase composition of mobile phase (± 1 %). The results are given in Tables-5.

TABLE-5
RESULTS OF ROBUSTNESS STUDIES OF
THE PROPOSED METHOD

Parameter	Variation	Observed value		
		%RSD of area	Tailing factor	Theoretical plates
Flow rate	0.9 mL/min	0.77	1.021	15394
	1.1 mL/min	1.25	1.072	14511
% of organic phase in mobile phase	54 % acetonitrile	0.36	1.042	11590
	56 % acetonitrile	1.13	1.112	9850

Analysis of marketed product: The validated method was applied for the analysis of mefenamic acid tablet. The assay obtained was more than 99 %. Results are summarized in Table-6.

TABLE-6
ASSAY OF MEFENAMIC ACID
COMMERCIAL PRODUCT (TABLET)

Sample no.	Formulation	Conc. of sample solution (μg/mL)	Amount found (mg)	Recovery (%)
1	MEFTAL [®] -250 DT	40	39.60	99.02

TABLE-3
INTRA AND INTER-DAY PRECISION STUDIES OF MEFENAMIC ACID

Sample No.	Conc. (μg/mL)	Intra-day precision		Inter-day precision	
		Mean ^a ± SD	^a RSD (%)	Mean ^a ± SD	^a RSD (%)
1	30	1289450 ± 4572	0.35	1287460 ± 3236	0.25
2	50	2038633 ± 8437	0.41	2091966 ± 4785	0.22
3	70	2921366 ± 1236	0.42	2904700 ± 9793	0.33

^aMean of 3 replicates

Forced degradation studies

Degradation in acidic medium: During acid hydrolysis, drug was allowed to react with 1M HCl for 6 h. The acid hydrolysis was carried out at room temperature initially, followed by heating with 1 M HCl at 70 °C for 6 h using constant water bath. Negligible degradation of drug was observed. Chromatogram obtained by degradation of drug in acidic medium is shown in Fig. 4 and the percentage of degradation of drug is shown in Table-7.

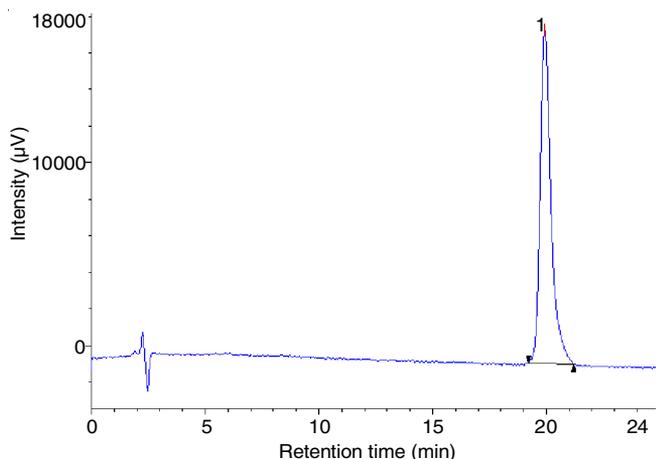


Fig. 4. Representative chromatogram of mefenamic acid on acidic degradation

TABLE-7 RESULTS OF FORCED DEGRADATION STUDIES OF MEFENAMIC ACID		
Stress conditions	Drug recovered* (%)	Drug decomposed* (%)
Standard drug	100.0	–
Acidic condition	90.04	9.96
Alkaline condition	92.64	7.36
Neutral condition	93.08	6.92
Oxidative condition	62.56	37.44
Photolytic condition	96.38	3.62
Thermal condition	89.61	10.39

*Mean of three replicates.

Degradation in alkaline medium: The drug was treated with 1 M NaOH initially at room temperature for 6 h, followed by further heating with 1 M NaOH at 70 °C for 6 h using constant waterbath. Negligible degradation of drug as shown in Table-7 was seen in the chromatogram (Fig. 5) representing degradation in basic medium.

Degradation in neutral medium: Neutral degradation of mefenamic acid was performed using distilled water. The drug was treated with water at room temperature for a period of 6 h followed by heating with water at 70 °C for 6 h using constant water bath. Table-7 gives the percentage of degradation of drug in neutral medium which showed that the drug is stable in neutral medium and the chromatogram obtained is shown in Fig. 6.

Oxidative degradation: For oxidation, the reagent chosen was hydrogen peroxide (10 %). The drug was made to react with 10 % H₂O₂, for 5 days. In 10 % H₂O₂, decrease in the peak area of the drug was seen from 2nd day onwards. Two degradants peaks were seen in the chromatogram of drug on 3rd day (Fig. 7).

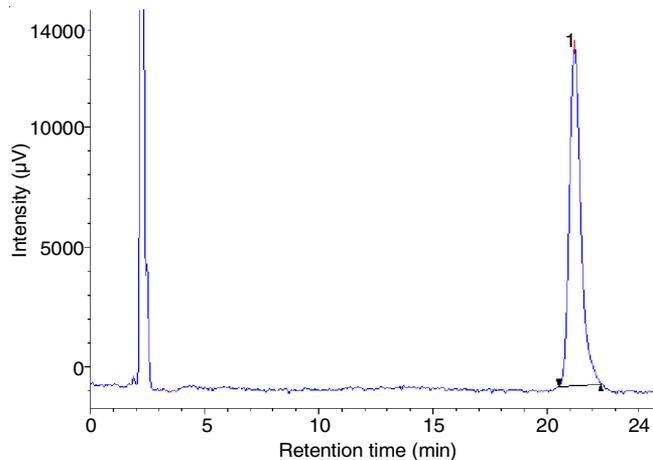


Fig. 5. Representative chromatogram of mefenamic acid on alkaline degradation

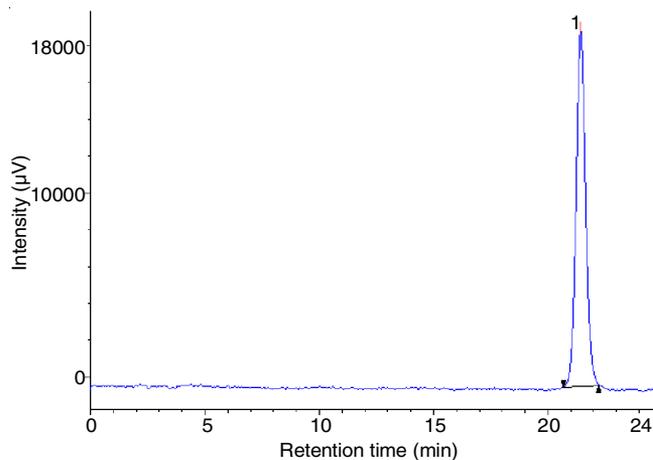


Fig. 6. Representative chromatogram of mefenamic acid on neutral degradation

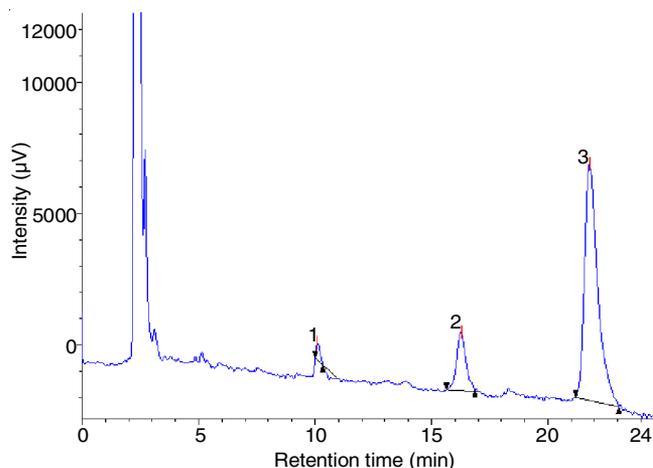


Fig. 7. Representative chromatogram of mefenamic acid on oxidative degradation

Photolytic degradation: The solid drug was exposed to direct sunlight for 7 days. The standard drug was placed in volumetric flask and exposed to sunlight. The chromatogram of sample subjected to sunlight is shown in Fig. 8 which shows that the percentage of degradation of drug is photo stable as listed in Table-7.

Thermal degradation: Thermal degradation was performed by placing mefenamic acid in volumetric flask in an oven at

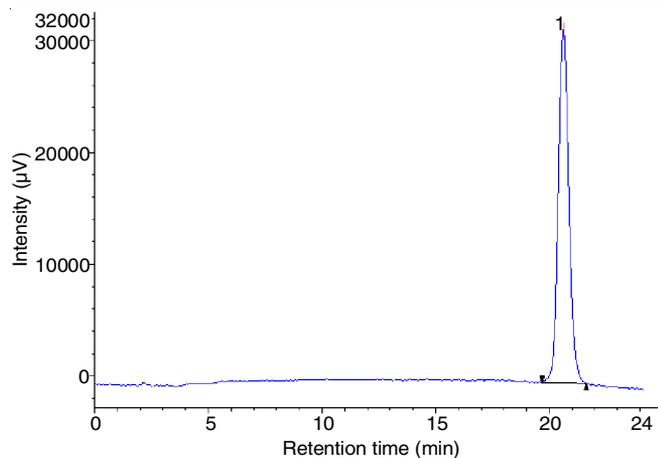


Fig. 8. Representative chromatogram of mefenamic acid on photolytic degradation

80 °C for 4 days. Significant degradation (> 10%) is shown in Table-7 without any appearance of additional peak on chromatogram (Fig. 9).

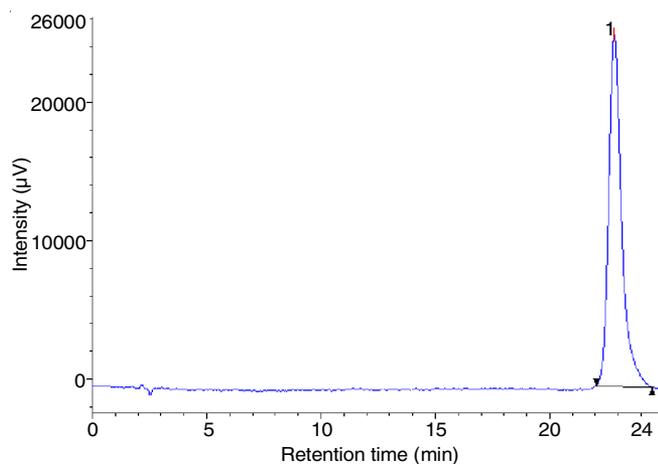


Fig. 9. Representative chromatogram of mefenamic acid on thermal degradation

Conclusion

The developed stability-indicating and validated RP-HPLC method is precise, accurate and robust, and can be applied for the determination of mefenamic acid in pharmaceutical dosage forms. The drug was found to be more degraded when exposed

to oxidation stress conditions as it degraded by 37.44 % with the appearance of two degradants peaks and least degraded when exposed to hydrolysis (acidic, basic and neutral), thermal and photo-stress conditions.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

REFERENCES

1. M. Blessy, R.D. Patel, P.N. Prajapati and Y.K. Agrawal, *J. Pharm. Anal.*, **4**, 159 (2014); <https://doi.org/10.1016/j.jpha.2013.09.003>.
2. ICH Guidelines, Q1A (R2): Stability Testing of New Drug Substances and Products (Revision 2), International Conference on Harmonization (2003).
3. D.W. Reynolds, K.L. Facchine and J.F. Mullaney, *Pharm. Technol.*, **26**, 48 (2002); <https://doi.org/10.1002/ardp.18280260113>.
4. H. Brummer, *Life Sci. Technol.*, **31**, 1 (2011); <https://doi.org/10.1093/benz/9780199773787.article.b00027698>.
5. H. Abdolmohammad-Zadeha, F. Morshedzadehb and E. Rahimpoura, *J. Pharm. Anal.*, **4**, 331 (2014); <https://doi.org/10.1002/ardp.18551320330>.
6. K.W. Parfitt, Martindale: The Complete Drug Reference, Pharmaceutical Press: London (1999).
7. B. Dhumal, K.Bhusari, M.Tajne, M. Ghante and N. Jain, *J. Appl. Pharm. Sci.*, **4**, 60 (2014); <https://doi.org/10.3109/13880209.2013.869232>.
8. S.F. Saleh, S.M. Dereaya and M.A.Omar, *Int. J. Chem. Anal. Sci.*, **5**, 55 (2014); <https://doi.org/10.22159/ijpps.2016v8i10.13298>.
9. P.A. Patil, A. Umarkar, M. Bari and S. Barhate, *Am. J. Adv. Drug Deliv.*, **3**, 110 (2015); <https://doi.org/10.4172/2167-1052.1000e135>.
10. R.S. Sakhare, S.S. Pekamwar, R.B. Kadam and S. Kanthale., *J. Pharm. BioSci.*, **5**, 1 (2017); <https://doi.org/10.7897/2230-8407.079109>.
11. Y.Gandhi, P. Deshpande, N. Deore and G. Sarowar, *J. Chem. Pharm. Res.*, **8**, 677 (2016); <https://doi.org/10.1002/ardp.201670033>.
12. P.R. Tiwari, A.G. Patel, S.V. Luhar and S. Narkhede., *Eur. J. Biomed. Pharm Sci.*, **4**, 377 (2017); <https://doi.org/10.1002/ardp.201770022>.
13. ICH, Guidelines Q2 (R1): Validation of Analytical Procedures: Text and Methodology, International conference on Harmonization, IFPMA, Geneva, Switzerland (2005).
14. M. Bakshi and S. Singh, *J. Pharm. Biomed. Anal.*, **28**, 1011 (2002); [https://doi.org/10.1016/S0731-7085\(02\)00047-X](https://doi.org/10.1016/S0731-7085(02)00047-X).
15. R.D. Patel, P.N. Prajapati and Y. Agrawal, *J. Pharm. Anal.*, **4**, 159 (2014); <https://doi.org/10.1016/j.jpha.2013.09.003>.

A VALIDATED STABILITY INDICATING RP-HPLC METHOD FOR ESTIMATION OF TOLFENAMIC ACID IN PRESENCE OF ITS PHARMACOPOEIAL IMPURITIES

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ABSTRACT

Objective: The proposed research work was conducted to develop a single reverse-phase high-performance chromatography (RP-HPLC) method capable of separating two Pharmacopoeial related impurities as well as degradation product of Tolfenamic acid (TA). The drug was subjected to various stress conditions recommended under ICH Q1A (R2) guidelines.

Methods: The desired separation of two Pharmacopoeial impurities and one degradant generated under oxidative stress was carried out using Sunfire ODS C-18 (250 x 4.6 mm, 5 μm) column maintained at 40 °C. Isocratic elution was carried out using acetonitrile and ammonium dihydrogen orthophosphate buffer (10 mmol, pH 2.5) in the ratio of 80:20 v/v. The detection was carried out at 205 nm using flow rate of 1 ml/min. The developed method was validated as per ICH Q2 (R1) guidelines for specificity, linearity, accuracy, precision, Limit of detection (LOD), Limit of Quantification (LOQ) and robustness.

Results: Linearity response of TA was found at a concentration range of 10-100 μg/ml, with a correlation coefficient of 0.9987. The Pharmacopoeial impurity A and impurity B showed linearity results at concentration of 0.1-1 μg/ml, with correlation coefficient of 0.9984 for Impurity A and 0.9989 for Impurity B. The % recovery during accuracy studies for TA and the two impurities were within the acceptance range of 95-105%. LOD and LOQ for TA were found to be 4.561 μg/ml and 133.771 μg/ml respectively. For impurity A, LOD and LOQ were found to be 0.035 μg/ml and 0.106 μg/ml and for Impurity B, LOD and LOQ were 0.042 μg/ml and 0.128 μg/ml. With slight variation of organic phase in mobile phase and flow rate the method exhibited good robustness. Under forced degradation studies the drug was found stable under hydrolytic, photolytic and thermal stress conditions, but was found susceptible for degradation under oxidative stress with appearance of a degradant peak. From on the RRT values of Pharmacopoeial impurities and the formed degradant it was inferred that the developed method is selective for the drug in the presence of impurities or degradants.

Conclusion: The developed stability-indicating method is found to be simple, rapid, accurate, precise and robust as compared to other proposed methods while determining TA in presence of its Pharmacopoeial impurities and degradation products. Hence the developed method can be used for analysis of stability samples of TA in presence of its related impurities.

Keywords: Tolfenamic acid, Related impurities, RP-HPLC, ICH Q2 (R1) guidelines, Stability indicating

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INTRODUCTION

Tolfenamic acid (TA) is 2-[(3-chloro-2-methylphenyl) amino] benzoic acid (fig. 1) [1]. British pharmacopoeia lists three impurities in the monograph of TA (fig. 1) [2]. TA belongs to the class of fenamates of the nonsteroidal anti-inflammatory drug (NSAID) and is used for treatment of inflammation and pain in humans as well as animals. Mostly used for the remedy of acute migraine attacks and for the relief of pain in conditions such as osteoarthritis, dysmenorrhea and rheumatoid arthritis [3-4]. TA has gained high popularity recently for its anticancer activity against different types of cancer [5-8]. The concentration of all the related substances associated with drug as per ICH guidelines must be less than 0.2% of the concentration of drug except for isopropyl ethers and monoester, whereas the limit should not exceed 0.2% at different storage concentrations [9].

Study on TA by various analytical methods has been reported in the literature. Few of them include titrimetry [10], spectrophotometry [11], spectrofluorimetry [12] and reversed phase high-performance liquid chromatography (RP-HPLC) methods [13-20]. A literature highlighted the separation of two related impurities of TA i.e. 2-chlorobenzoic acid (CBA) and 3-chloro-2-methylaniline (CMA) by HPLC and TLC densitometric method [21] without commenting on the stability-indicating potential of the method. Whereas minimum literature, till date is an available wherein stability-indicating method for TA by RP HPLC in presence of its related impurities, has been reported.

Hence the current work was undertaken to develop and validate a RP-HPLC method for the estimation of TA in the presence of its two

Pharmacopoeial impurities i.e. Impurity A (CBA) and Impurity B (CMA) and also perform as a stability-indicating method. The drug was subjected to various stress conditions of hydrolysis, oxidation, photolysis and thermal as per ICH guidelines [22]. The developed method is the first report of its kind which could be applied for effective separation of TA from its Pharmacopoeial impurities and other degradants formed under stress conditions.

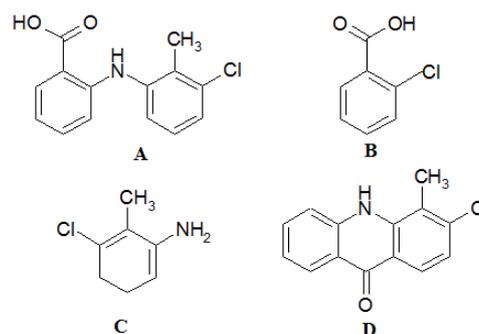


Fig. 1: A) Chemical structure of Tolfenamic acid, B) Chemical structure of 2-chlorobenzoic acid (Impurity A), C) Chemical structure of 3-chloro-2-methyl aniline (Impurity B) and D) Chemical structure of 3-chloro-4-methyl-9-oxo-9, 10-dihydroquinidine (Impurity C)

MATERIALS AND METHODS

Chemicals

Tolfenamic acid was obtained as a gift sample from Pure and Cure Healthcare Ltd (Uttarakhand, India). Impurities were purchased from Sigma-Aldrich Co., St. Louis, MO, USA. The Chemicals used in this work include, Acetonitrile (HPLC grade-Rankem, India), whereas ammonium dihydrogen orthophosphate (AR), orthophosphoric acid (AR), ammonia solution (AR), Hydrochloric acid(HCl)(AR), Sodium hydroxide(NaOH) (AR), and hydrogen peroxide (10%, v/v) (AR), were procured from Merck, India. HPLC grade water used in the study was obtained from Bio-age water purification system. All the solvents and sample solutions were filtered through 0.45 µm membrane filter before injecting into the HPLC system.

HPLC Instrument and chromatographic conditions

In the current research work, Jasco LC-4000 series HPLC system consisting of a quaternary solvent delivery system, an on-line degasser, an auto-sampler, a column temperature controller and a diode array detector was used. System control and data analysis were processed with Jasco Chrom NAV software. Other instruments utilized in the study include Bath sonicator (Citizon Ultrasonic cleaner); Digital Balance (Wensar Digital Electronic Balance) and pH meter (Labtronics, LT-10). Optimum separation and estimation were achieved on Sunfire ODS C18 column (250×4.6 mm, 5 µm) from Waters. The mobile phase used for the study involved acetonitrile and 10 mmol ammonium dihydrogen ortho phosphate buffer (pH adjusted to 2.5 with o-phosphoric acid) in the ratio of 80:20 v/v, with flow rate of 1 ml/min. Studies were performed with column temperature at 40 °C, and injecting volume as 10µl with detection wavelength of 205 nm.

Buffer solution

10 mmol ammonium dihydrogen ortho phosphate buffer was prepared by dissolving 1.15 g of ammonium dihydrogen ortho phosphate in HPLC water and the volume was made up to the mark in a standard 1 litre volumetric flask. The pH was adjusted to 2.5 using ortho phosphoric acid solution. The prepared buffer solution was filtered through 0.45 µm membrane filter.

Stock standard solutions (1,000 µg/ml)

Stock standard solutions of TA, CBA, and CMA were prepared by weighing separately and accurately 10 mg of pure powder of each into three separate 10 ml standard volumetric flasks. Sufficient quantity of acetonitrile was added in all the volumetric flasks and sonicated for 15 min to dissolve the substance. The final volumes were made to the mark with acetonitrile and mixed well.

Working standard solutions (100 µg/ml)

Working standard solutions of CBA and CMA were prepared by accurately transferring 1 ml each of CBA and CMA from their respective stock standard solutions (1,000 µg/ml) into 2 separate 10 ml volumetric flasks. Volume up to the mark was made with acetonitrile to get 100 µg/ml working standard of each component.

Selection of the detection wavelength for the method

Spectra scan of the three components in the range of 190 nm to 400 nm was run. From the Overlays of the spectra's the wavelength that showed sufficient absorbance of all the components was selected for the study. Thus Wavelength of 205 nm was selected as the detection wavelength for the current method.

Method validation

The developed RP HPLC method for separation of specified impurities and possible degradants obtained of TA was validated as per ICH guidelines Q2 (R1) [23].

Specificity

The specificity of the method was evaluated by application of the developed method to a mixture containing 10µg/ml concentration of TA, and 1µg/ml of CBA and CMA. Specificity of the method was also checked for the samples of TA subjected to various forced/stressed conditions as per ICH guideline. Specificity of the method was

confirmed by calculating system suitability testing parameters such as resolution, peak asymmetry, and no of theoretical plates, for the separated chromatographic peaks and the formed degradants peaks.

Linearity and range

Fixed volumes equivalent to 10–100 µg/ml of TA were separately transferred from the stock standard solution of TA (1,000 µg/ml) into 10 ml volumetric flask and the volume was made up to the mark with the mobile phase. For impurities, volumes equivalent to 0.1-1 µg/ml of CBA, and CMA were separately transferred from their respective working standard solutions (100 µg/ml) into 10 ml volumetric flasks. The volume was made to the mark with the mobile phase. Six 10 µl injections were given of each concentration. The calibration curves were plotted and the regression parameters were obtained.

Precision

Intraday repeatability and interday repeatability of the method was evaluated by analyzing 40 µg/ml concentration of and 0.4 µg/ml concentrations of each impurity. Interday precision was done on two successive days using the same procedure stated under chromatographic conditions. The percentage relative standard deviation (%RSD) values were then calculated.

Accuracy

Accuracy of the developed method was determined by recovery studies using standard addition method. The percentage recovery studies of TA, CBA, and CMA was carried out in triplicate at 3 different levels of 80%, 100%, 120%.

Sensitivity

The sensitivity of the developed method was determined by calculating LOD and LOQ for TA and its two impurities. LOD and LOQ were calculated for TA and its two impurities based on the Standard deviation of the Response and the Slope as mentioned below.

LOD = 3.3 X Standard deviation of the response/Slope of the calibration curve

LOQ = 10 X Standard deviation of the response/Slope of the calibration curve

Robustness

The robustness of the method was done to check the effect of small deliberate changes in the chromatographic conditions. Changes in the flow rate of the mobile phase (±0.2 unit) and the proportion of organic phase in the mobile phase (±2 units) on the developed method were studied. The system suitability parameters were checked for verifying the robustness of the method.

Forced degradation studies

To assess the stability-indicating power and specificity of the method, forced degradation studies were performed. Forced degradation of drug in acidic and basic medium was carried in 1N HCl and 1N NaOH respectively at 70 °C for a period of 6 h. The stressed samples were first cooled to room temperature, followed by neutralization and finally the volume was made up to mark by mobile phase. Degradation of drug in neutral medium was done by treating the drug with water for a period of 6 h at 70 °C.

Oxidative degradation of the drug was carried by subjecting the drug to react with 10% H₂O₂ solution for a period of 6 d. Photolysis of the drug was carried by exposing the drug to direct sunlight for 4 d, whereas for thermal degradation the drug was heated in an oven at 80 °C for 9 h. The stressed samples obtained in neutral and thermal stress conditions were cooled to room temperature and diluted with mobile phase. The oxidative and photolytic stressed samples were diluted with mobile phase. The samples of forced degradation studies were diluted with mobile phase to give a final concentration of 10 µg/ml before injecting in the system.

RESULTS AND DISCUSSION

Chromatographic separation

The method involved separation of TA from its two impurities and other possible degradants under stress conditions. For carrying out

efficient separation effect of various pH of the buffer along with the various composition of the mobile phase was studied. The mobile phase selected was acetonitrile as organic phase and ammonium dihydrogen ortho phosphate as buffer (10 mmol). Various trials were carried out with pH of buffer ranging from 2.5 to 6.5. Optimum separation of the drug from its specified impurities was obtained when the pH of the buffer was fixed at 2.5 and the composition of

acetonitrile and buffer was in the ratio of 80:20%v/v under isocratic elution. The following separation was performed on the available Sunfire ODS C-18 (250 x 4.6 mm, 5 μ m) column maintained at 40 °C. The detection was carried out at 205 nm using flow rate of 1 ml/min. The optimized chromatographic conditions used for the developed method are different from the available method [21] as shown in table 1.

Table 1: Optimized chromatographic conditions of the proposed RP HPLC method

Mobile phase	Mixture of acetonitrile and 10 mmol ammonium dihydrogen orthophosphate buffer (pH adjusted to 2.5 with orthophosphoric acid) in the ratio of 80:20 % v/v.
Column	Sunfire ODS C18 (4.6 x 250 mm, 5 μ m) column
Injection Volume	10 μ l
Flow Rate	1 ml/min
Column oven temperature	40 °C
Detection wavelength	205 nm

System suitability

The developed method at the optimized chromatographic conditions efficiently resolved TA from its 2 specified impurities as shown by a

representative chromatogram in fig. 2. The concentration of TA and the two impurities used for the system suitability is 50 μ g/ml and 5 μ g/ml respectively. The system suitability parameters of all the three components are listed in table 2.

Table 2: System suitability parameters of the proposed RP HPLC method

S. No.	Components	Rt	RRT	Area	Resolution	Peak asymmetry	Theoretical plate
1	Tolfenamic acid (50 μ g/ml)	6.650	-	3218433	NA	1.098	14400
2	2-Chlorobenzoic acid	3.017	0.453	533577	7.504	1.213	7515
3	3-chloro-2-methylaniline	4.108	0.617	829977	13.621	1.069	11607

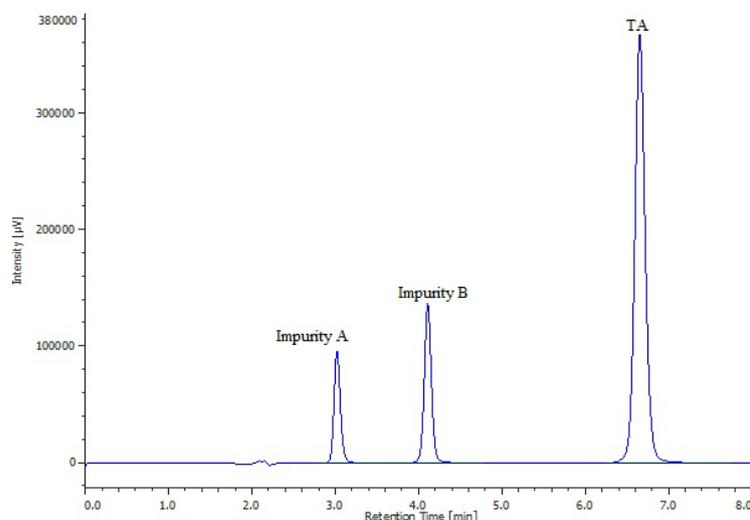


Fig. 2: Optimized chromatogram showing separation of tolfenamic acid (TA) from the impurities and also from each other

The obtained chromatogram showed tailing factor of less than 1.3 for all the peaks, a resolution of more than 2 for CBA and DMA with respect to TA. The number of theoretical plates, tailing factor and the resolution for the three components were within the limits of acceptance criteria (reference) specified in ICH guidelines.

Specificity

The chromatogram shown in fig. 2 represented the specificity of the developed method in efficiently separating the drug from its two impurities as seen from the RRT values and also thus satisfying the system suitability parameters. The method was further evaluated to

check its specificity for the samples obtained after subjecting to various stress conditions as compared to the available method which is not a stability-indicating method [21]. The results of the forced degradation studies are reported in table 3.

Acidic stress conditions

The drug after subjecting to react with 1N HCl at 70 °C for 6 h showed no significant decrease in the peak area of the drug indicating its stability in acidic condition as shown in fig. 3. The peaks seen at R_t from 2 to 3 are due to blank samples treated with HCl at the detection wavelength.

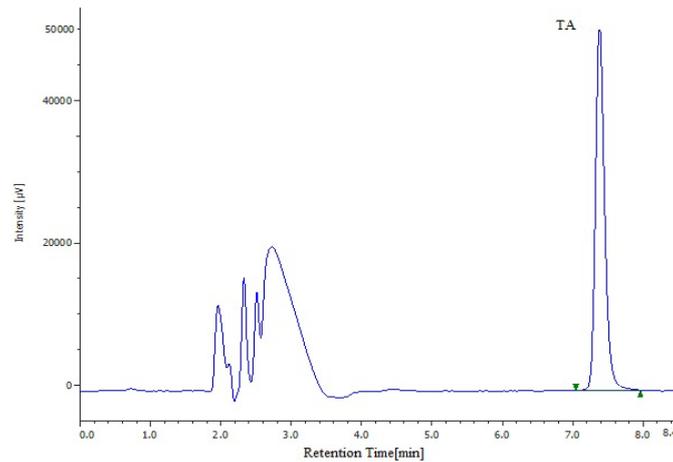


Fig. 2: Representative chromatogram of tolfenamic acid in acidic stress condition

Basic stress condition

Under the influence of 1N NaOH at 70 °C for 6 h the drug didn't show any degradation, further highlighting its stability in the basic medium as shown by fig. 3. The peaks seen at R_t from 2 to 3 are due to blank samples treated with NaOH at the detection wavelength.

Neutral stress condition

When the drug was made to react with water for 6 h at 70 °C it showed no degradation as shown by fig. 4. Hence the drug was found to be stable in neutral medium.

Thermal stress condition

No degradation of the drug was seen when the drug was kept in oven at a temperature of 80 °C for 96 h as confirmed by fig. 5. Thus indicating its stability in thermal stress condition.

Photolytic stress condition

After exposure of the drug to direct sunlight for 4 d the samples showed no degradation of the drug, confirming its stability in the applied condition as per fig. 6.

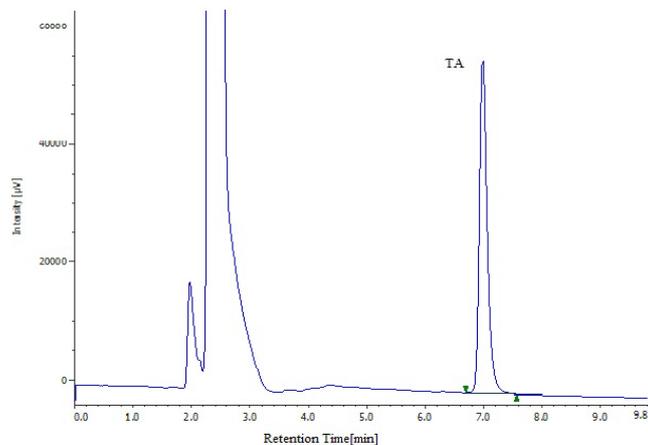


Fig. 3: Representative chromatogram of tolfenamic acid in basic stress condition

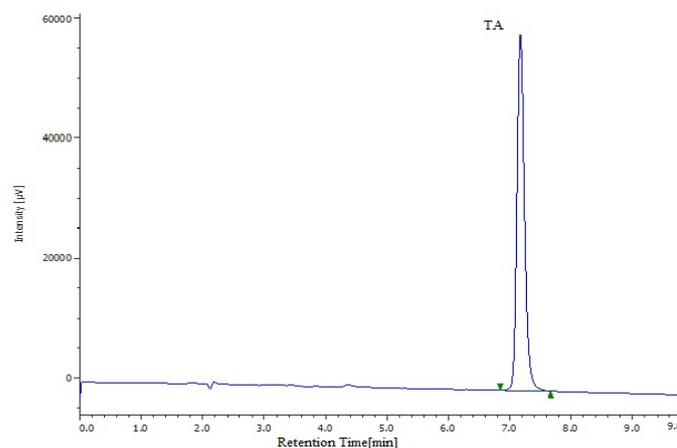


Fig. 4: Representative chromatogram of tolfenamic acid in neutral stress condition

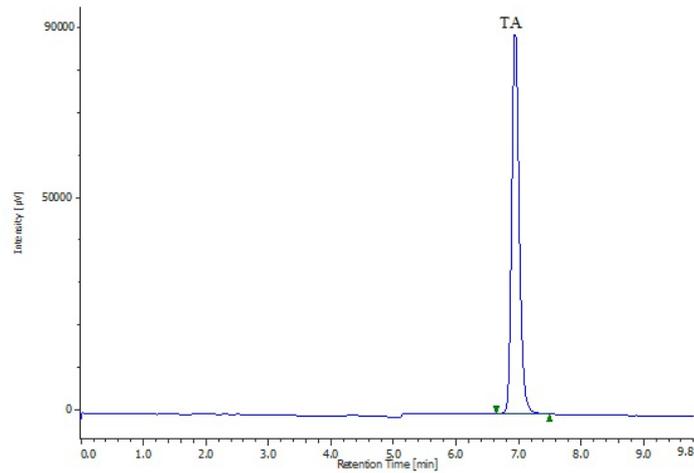


Fig. 5: Representative chromatogram of tolfenamic acid in thermal stress condition

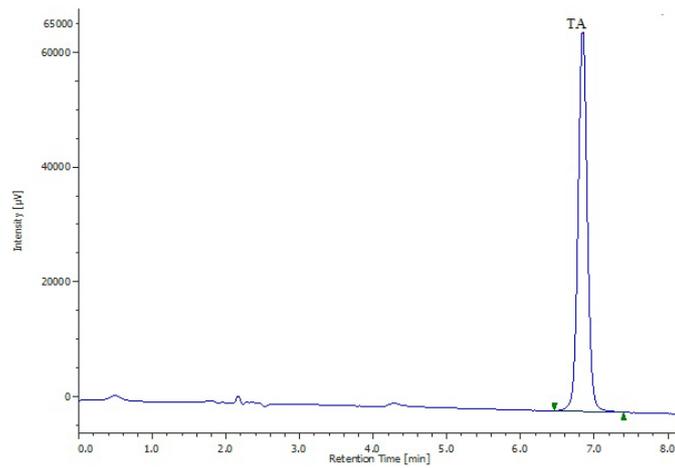


Fig. 6: Representative chromatogram of tolfenamic acid in photolytic stress condition

Oxidative stress condition

TA was found to be degraded in oxidative conditions, when treated with 10% H₂O₂ after 2 d. The obtained chromatogram on the sample (fig. 7) showed one peak of degradation product along with the peak of the drug. The RRT of the degradant formed is 0.54 which is

different as compared to the RRT value of the two impurities with respect to the drug. Hence the formed degradant can be easily separated along with the two impurities highlighting the methods specificity. The peak at R_t of 2.51 is due to the blank treated sample with H₂O₂ which is seen in all the samples subjected to oxidative stress condition.

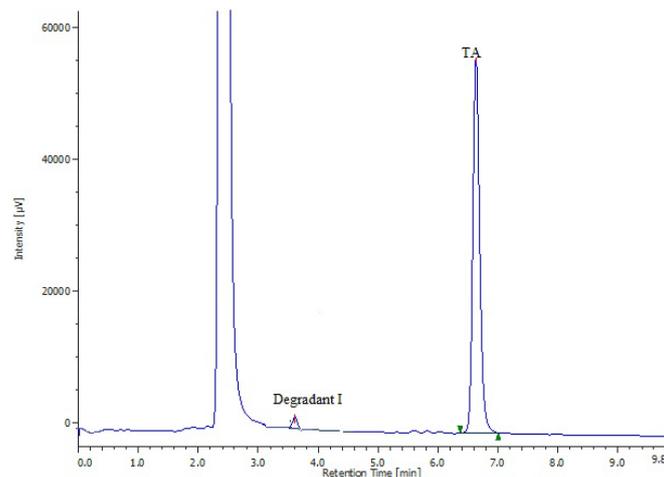


Fig. 7: Representative chromatogram of tolfenamic acid in oxidative stress condition

Table 3: Forced degradation studies of tolfenamic acid (n=2)

Stress condition	Drug recovered (%)	Drug decomposed (%)	RRT of the degradant
Standard drug	100	-	
Acidic hydrolysis	90.50	9.5	
Alkaline hydrolysis	90.50	9.5	
Neutral hydrolysis	98.47	1.53	
Oxidative degradation	85.61	14.39	0.54
Thermal degradation	92.93	7.07	
Photolytic degradation	95.34	4.66	

n=number of determinations for each condition

Linearity

Standard calibration curves were obtained for TA and its two impurities i.e. CBA and CMA. The data showed linearity in the range of 10-100µg/ml for TA and 0.1-1µg/ml for CBA and CMA, with correlation coefficients of ≥ 0.995 in all the cases which are within acceptable range i.e. more than 0.98. Table 4 highlights the linearity parameters of the calibration curves for TA and its two impurities.

Precision and sensitivity

Intraday and intraday precision results obtained for the method are listed in the table 4. Percentage RSD values obtained for TA and the impurities were less than 2, which were within the acceptance

criteria. Method sensitivity in terms of LOD and LOQ for TA and the impurities are as shown in table 4.

Accuracy

Satisfactory percentage recoveries of TA and the impurities were obtained at 80,100% and 120% of the test concentration. The accuracy results of the method were within the acceptable range of 95 % to 105% as shown in table 5.

Robustness

With deliberate changes in flow rate and in the proportion of organic phase in mobile phase, no significant changes in the system suitability parameters of the method for TA and for the impurities were seen as shown in table 6. Thus the method was found to be robust.

Table 4: Regression parameters of the developed method

Parameters	Tolfenamic acid	Impurity A	Impurity B
Range(µg/ml)	10-100	0.1-1	0.1-1
Regression equation	$Y=48849x+53707$	$Y=86129x+2217.4$	$Y=119746+49.33$
Correlation coefficient	0.9987	0.9984	0.9989
Intraday precision(n=3)			
Area (%RSD)	0.81	0.44	0.56
Interday precision(n=3)			
Area(%RSD)	0.86	0.22	0.82
LOD(µg/ml)	4.54	0.035	0.042
LOQ(µg/ml)	13.77	0.106	0.128

n= number of determinations

Table 5: Accuracy data of drug and its impurities (n=3)

Component	Amount of sample (µg)	Level of spiking (%)	Amount of spiked (µg)	Amount recovered (µg)	% recovery \pm SD	% RSD
Tolfenamic acid	40	80	32	30.70	95.96 \pm 0.88	0.91
		100	40	40.20	99.99 \pm 1.6	1.60
		120	48	47.09	98.11 \pm 0.78	0.79
2-chloro benzoic acid (Impurity A)	0.4	80	0.32	0.31	96.82 \pm 1.34	1.38
		100	0.4	0.41	103.1 \pm 0.65	0.63
		120	0.48	0.47	99.79 \pm 0.22	0.22
3-Chloro 2-methylaniline (Impurity B)	0.4	80	0.32	0.3204	100.12 \pm 0.70	0.70
		100	0.4	0.399	99.90 \pm 0.72	0.72
		120	0.48	0.47	97.14 \pm 1.76	1.81

n = number of determinations, SD= Standard Deviation, %RSD=% Relative Standard Deviation

Table 6: Results of robustness studies (n=3)

Variable	Tolfenamic acid area (%RSD)	Impurity A area (%RSD)	Impurity B area (%RSD)
Change in flow rate			
0.8 ml/min	0.35	0.39	1.77
1.2 ml/min	0.77	1.21	0.71
Change in composition of mobile phase (ACN: Buffer)			
78:22v/v	0.81	1.86	0.52
82:18 v/v	0.35	0.71	0.13

n= number of determinations, (%RSD) =% Relative Standard deviation

CONCLUSION

A RP HPLC method was developed and validated as per ICH guidelines for estimation of TA in the presence of its two Pharmacopoeial impurities as well as degradation products. The method was found to be stability-indicating from the results obtained after application of the method to the stress samples of TA. The drug TA showed stability in acidic, basic, neutral, photo and thermal stress conditions and indicated degradation in oxidative stress conditions. The developed stability-indicating method is found to be simple, rapid, accurate, precise and robust as compared to other proposed methods while determining TA in presence of specified impurities and degradation products. Hence, the proposed method can be used for routine and stability sample analysis of TA.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally

CONFLICT OF INTERESTS

Declared none

REFERENCES

1. The Merck Index. In: Budawari S. editor. 13th ed. Whitehouse Station, NJ: Merck and Co Inc; 2001.
2. British Pharmacopoeia. The department of Health. London. Stationary Office; 2009. p. 2244-5.
3. Martindale. The complete drug reference. In: Sweetman SC. editors. 34th ed. London: The Pharmaceutical Press; 2007.
4. Niopas I, Georganakakis M. Determination of tolfenamic acid in human plasma by HPLC. *J Liq Chromatogr* 1995;18:2675-82.
5. Colon J, Basha MR, Madero Visbal R, Kundari S, Baker CH, Herrera LJ, *et al.* Tolfenamic acid decreases c-Met expression through Sp proteins degradation and inhibits lung cancer cells growth and tumor formation in orthotopic mice. *Invest New Drugs* 2011;29:41-51.
6. Eslin D, Sankpal UT, Lee C, Sutphin RM, Maliakal P, Currier E, *et al.* Tolfenamic acid inhibits neuroblastoma cell proliferation and induces apoptosis: a novel therapeutic agent for neuroblastoma. *Mol Carcinog* 2013;52:377-86.
7. Kim JH, Jung JY, Shim JH, Kim J, Choi KH, Shin JA, *et al.* Apoptotic effect of tolfenamic acid in KB human oral cancer cells: possible involvement of the p38 MAPK pathway. *J Clin Biochem Nutr* 2010;47:74-80.
8. Liu X, Abdelrahim M, Abudayyeh A, Lei P, Safe S. The nonsteroidal anti-inflammatory drug tolfenamic acid inhibits BT474 and SKBR3 breast cancer cell and tumor growth by repressing erbB2 expression. *Mol Cancer Ther* 2009;8:1207-17.
9. Babu C, Devanna N, Suresh R. Validated gradient stability indicating RP-HPLC method for the simultaneous quantification of 11 related substances in the combined dosage forms of lamivudine and tenofovir disoproxil fumarate. *Int J Appl Pharm* 2017;9:61-8.
10. Shim JH, Shin JA, Jung JY, Choi KH, Choi ES, Cho NP. Chemopreventive effect of tolfenamic acid on KB human cervical cancer cells and tumor xenograft by downregulating specificity protein 1. *Eur J Cancer Prev* 2011;20:102-11.
11. Tsiliou S, Kefala LA, Perdih F, Turel I, Kessissoglou DP, Psomas G. Cobalt (II) complexes with non-steroidal anti-inflammatory drug tolfenamic acid: Structure and biological evaluation. *Eur J Med Chem* 2012;48:132-42.
12. Ioannou PC, Rusakova NV, Andrikopoulou DA, Glynou KM, Tzompanaki GM. Spectrofluorimetric determination of anthranilic acid derivatives based on terbium sensitized fluorescence. *Analyst* 1998;123:2839-43.
13. Papadoyannis I, Georganakakis M, Samanidou V, Zotou A. Rapid assay for the determination of tolfenamic acid in pharmaceutical preparations and biological fluids by high-performance liquid chromatography. *J Liq Chromatogr Relat Technol* 1991;14:2951-67.
14. Gallo P, Fabbrocino S, Dowling G, Salini M, Fiori M, Perretta G, *et al.* Confirmatory analysis of non-steroidal anti-inflammatory drugs in bovine milk by high-performance liquid chromatography with fluorescence detection. *J Chromatogr A* 2010;1217:2832-9.
15. Ibrahim H, Boyer A, Bouajila J, Couderc F, Nepveu F. Determination of non-steroidal anti-inflammatory drugs in pharmaceuticals and human serum by dual-mode gradient HPLC and fluorescence detection. *J Chromatogr B* 2007;857:59-6.
16. Gonzalez Barreiro C, Lores M, Casais MC, Cela R. Simultaneous determination of neutral and acidic pharmaceuticals in wastewater by high-performance liquid chromatography-post-column photochemically induced fluorimetry. *J Chromatogr A* 2003;993:29-37.
17. Mikami E, Goto T, Ohno T, Matsumoto H, Inagaki K, Ishihara H, *et al.* Simultaneous analysis of anthranilic acid derivatives in pharmaceuticals and human urine by high-performance liquid chromatography with isocratic elution. *J. Chromatogr B: Biomed Sci Appl* 2000;744:81-9.
18. Rozou S, Antoniadou Vyza E. An improved HPLC method overcoming Beer's law deviations arising from supramolecular interactions in tolfenamic acid and cyclodextrins complexes. *J Pharm Biomed Anal* 1998;18:899-905.
19. Niopas I, Georganakakis M. Determination of tolfenamic acid in human plasma by HPLC. *J Liq Chromatogr Relat Technol* 1995;18:2675-82.
20. Papadoyannis IN, Zotou AC, Samanidou VF. Simultaneous reversed-phase gradient-HPLC analysis of anthranilic acid derivatives in anti-inflammatory drugs and samples of biological interest. *Liq Chromatogr Relat Technol* 1992;15:1923-45.
21. Shinozuka T, Takei S, Kuroda N, Kurihara K, Yanagida J. Micro determination of anthranilic acid derivatives of anti-inflammatory drugs by high-performance liquid chromatography and its application to forensic chemistry. *Eisei Kagaku* 1991;37:461-6.
22. Abdelwahab NS, Ali NW, Zaki MM, Abdelkawy M. Validated chromatographic methods for simultaneous determination of tolfenamic acid and its major impurities. *J Chromatogr Sci* 2015;53:481-91.
23. International Conference on Harmonization (ICH), Stability testing of new drug substances and products, Q1A (R2); 2003.
24. International Conference on Harmonization (ICH) Harmonized Tripartite Guidelines, Validation of Analytical Procedures, Text, and Methodology, Q2 (R1), Parent Guidelines on Methodology Dated; 1996. p. 6.



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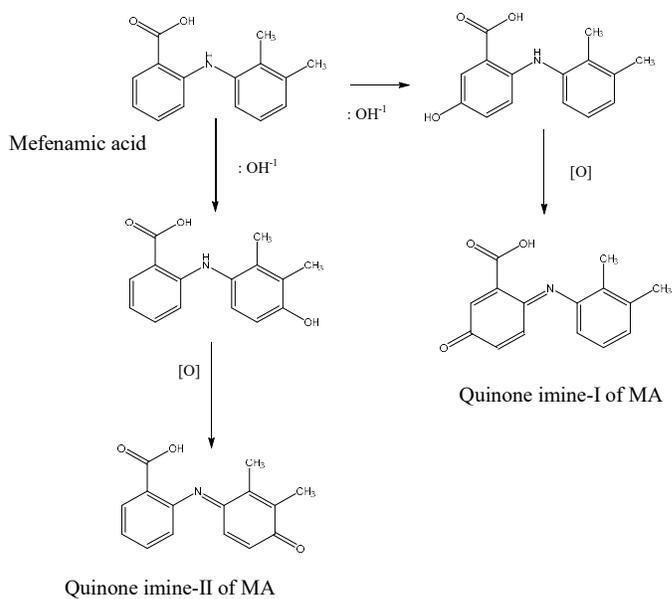
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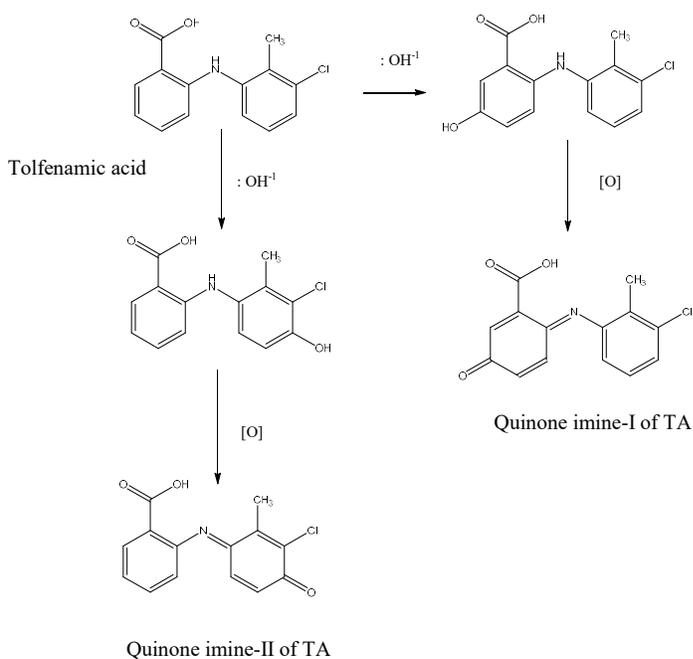
11.3 DEGRADATION PATHWAY OF MEFENAMIC ACID (MA) & TOLFENAMIC ACID (TA) UNDER CONDITIONS OF STRESS INDUCED OXIDATION

Schematic diagram showing possible formation of Quinone imines as degradation products under stress induced oxidation conditions for MA & TA is presented below.

Mefenamic Acid



Tolfenamic Acid



ERRATA

Sr. No	Page No	Paragraph No	Line No	Error	To be corrected as
1	6	2	1	form	from
2	8	3	4	ass	as
3	11	4	4	id	is
4	15	1	2	comlementary	complementary
5	78	1	2	form	from
6	202	2	7	trails	trials
7	208	2	1	indispensible	indispensable
8	216	3	7	regent	reagent

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