IMPURITY PROFILING AND QUANTIFICATION OF SELECTED ANTI-HIV DRUGS IN THEIR DOSAGE FORMS BY CHROMATOGRAPHY TECHNIQUE

A Thesis submitted to Gujarat Technological University

for the Award of

Doctor of Philosophy

in

Pharmacy

by
Nirav Rajendrakumar Soni
(189999901009)

under supervision of

Dr. Pragnesh Patani

GUJARAT TECHNOLOGICAL UNIVERSITY
AHMEDABAD

November-2025

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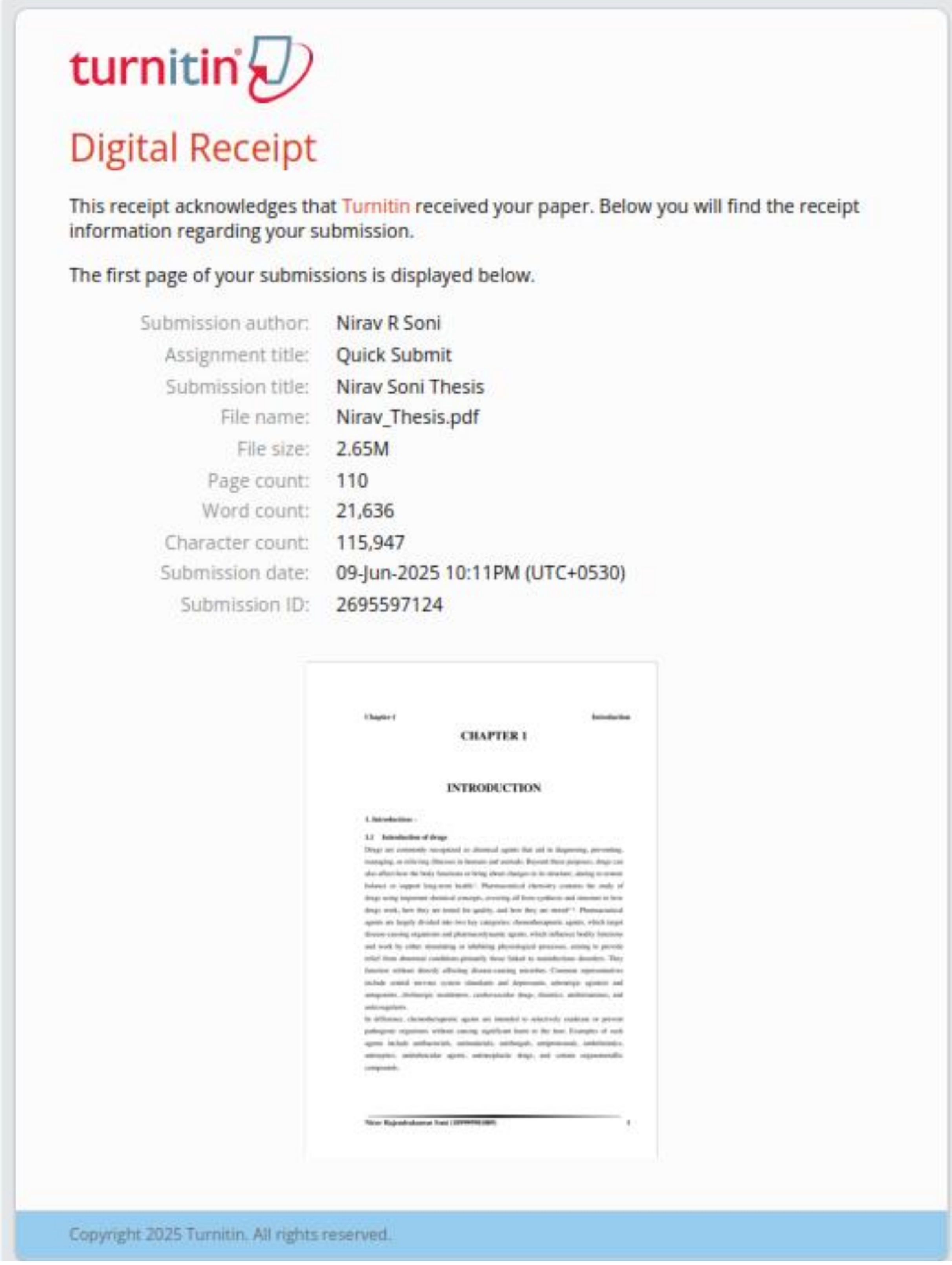

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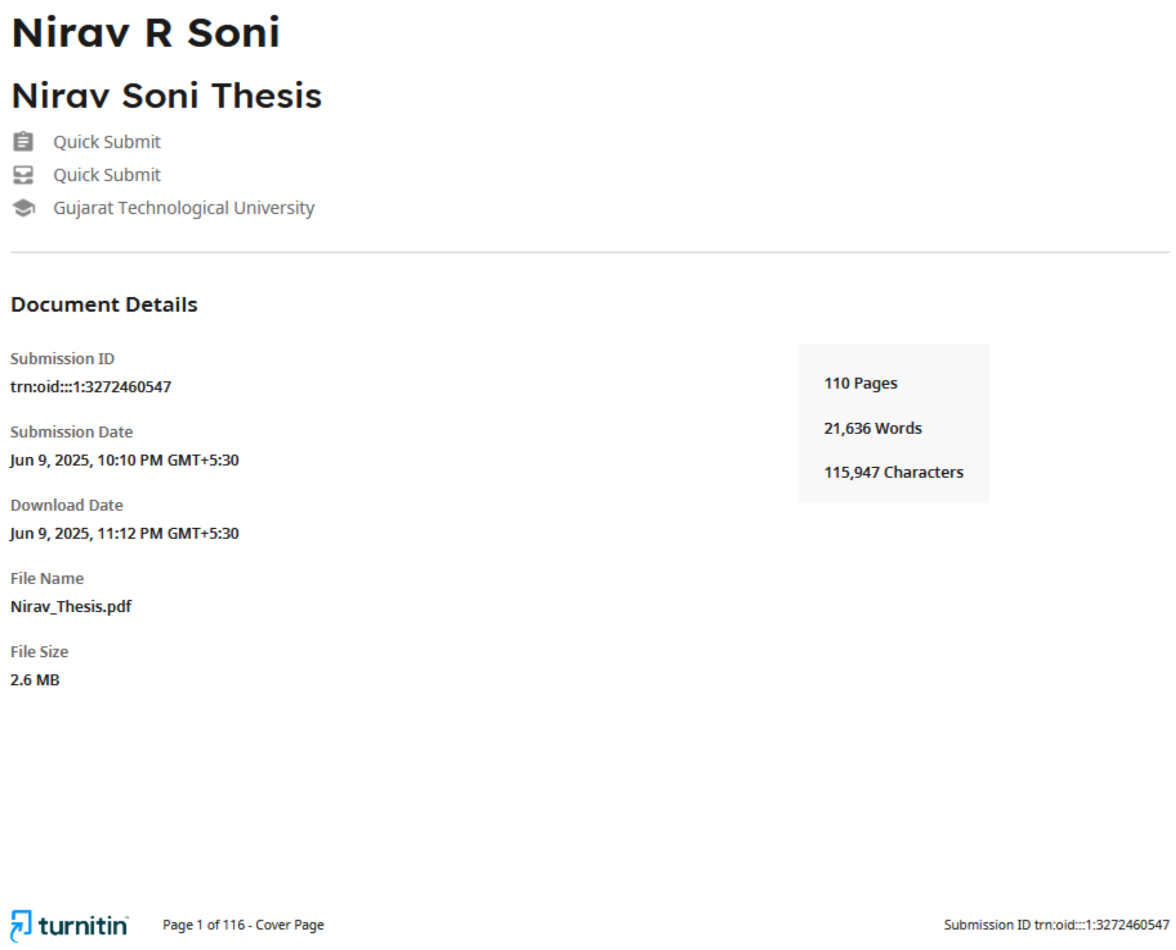

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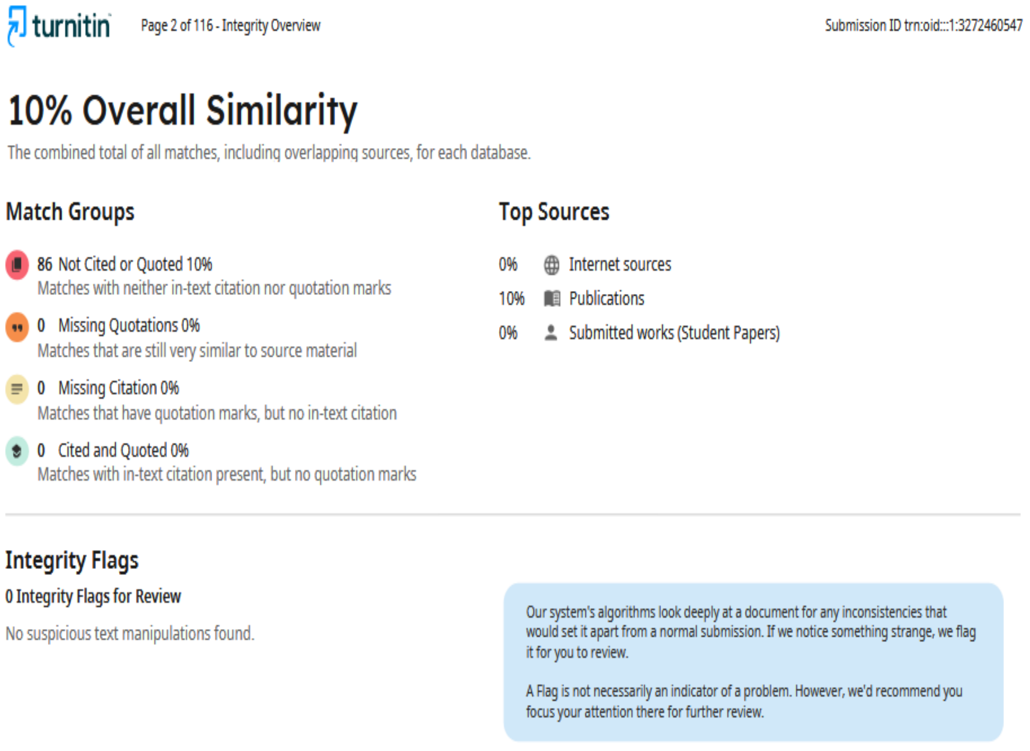

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ABSTRACT

1. Atazanavir sulphate (ATZ)

Analytical method was developed using HPLC Shimadzu [with power stream] gradient chromatographic technique. Data were passed through the spinchrom software. Separation was achieved on hypersil BDS C18 (250 x 4.6 mm, 5 µm) column using mobile phase composition of 0.05M potassium phosphate buffer: methanol (60:40 v/v), (15:85 v/v), (60:40 v/v), pH adjusted to 4.0 with 1% orthophosphoric acid (OPA). Make up volume with water. Flow rate was maintained at 1 ml/min with 225 nm UV detection. The retention time (RT) obtained for Atazanavir sulphate (ATZ), impurity A and impurity 5 was at 5.3 min, 6.23 min and 14.53 min respectively with injection volume 20 µl and the detection was made at 225 nm. Validation of the method was successfully established by performing various parameters such as accuracy, precision, specificity, linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), ruggedness, robustness, according to ICH guidelines.

2. Etravirine (ETR)

An accurate, precise, rapid, and economical RP- HPLC method has been developed and validated for the estimation of etravirine in pharmaceutical dosage forms, using PDA detector. Analytical method was developed using HPLC Shimadzu [with power stream] gradient chromatographic technique. Data were passed through the spinchrom software. Separation was achieved on Xselect HSS T3 (150 x 4.6 mm, 3.5 µm) column and using mobile phase A (Buffer) was used at pH 4.0 and mobile phase B [Methanol: Acetonitrile: Water (90:5:5 v/v)] by gradient programme. Flow rate was maintained at 1 ml/min with 310 nm PDA detection. The retention time (RT) obtained for ETR, impurity 1 and impurity 2 was at 15.813 min, 12.043 min and 17.704 min respectively with injection volume 10 µl and the detection was made at 310 nm. The % recovery of impurity-1 and impurity-2 observed was above 90% from LOQ level to 150%. The correlation coefficient r2 was 0.9993 for impurity-1 and 0.9997 for impurity-2. The method was found unaffected by change in method variance during the robustness study. Validation of the method was successfully established by performing various parameters such as accuracy, precision, specificity, linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), ruggedness, robustness, according to ICH guidelines.

Acknowledgement and / or Dedication

It is the moment of happiness for me and my family as the dreams seen come true with completion of the PhD degree course, key mile stone in my education. "Research is to see what everybody has seen but nobody has thought"

I express my heartfelt gratitude to the ***Ph.D. supervisor****,* ***Dr. Pragnesh Patani*** *whose expert guidance, unwavering support, and insightful suggestions have been instrumental throughout my doctoral research journey. I am deeply thankful for the encouragement, valuable time, and constructive feedback that significantly contributed to the successful completion of my Ph.D. work.*

I extend my sincere thanks to the Doctoral Progress Committee (DPC) members, Dr. C N. Patel and Dr. Dimalkumar A. Shah for their continuous monitoring, critical evaluation, and valuable inputs at various stages of my research progress. Their support played a vital role in shaping my research direction and academic growth.

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This academic milestone would not have been possible without the collective support and guidance of all those mentioned above.

*Finally, the words of thanks would be insufficient for the pacemaker of My wife-****Priti soni,*** *My lovely daughter-* ***Dhriti soni,*** *My mother-****Harshaben Soni,*** *My father-* ***Rajendrakumar soni*** *and my siter-* ***Jimisha*** *and all other family members. Their unending care, motivation and encouragement have helped me to complete the project successfully.*

TABLE OF CONTENTS

List Of Abbreviation

ACN -Acetonitrile

AE – Affinity Exchange

AF- Asymmetry factor

AIDS   Acquired Immunodeficiency Syndrome

AMV- Analytical Method Validation

API - Active Pharmaceutical Ingredient

ART- Antiretroviral therapies

ARV-Antiretroviral

ATC- Anatomical Therapeutic Chemical Classification System

ATZ-Atazanavir sulphate

BCS - Biopharmaceutical Classification System

BDS- Base Deactivated Silica

BP- British Pharmacopoeia

CAS - Chemical Abstracts Service

CD$_4$ - Cluster of Differentiation 4

cDNA -Complementary DNA

CDSCO - Central Drugs Standard Control Organization

CKD- Chronic Kidney Disease

CYP- Cytochrome P450 enzyme

DAPY – diarylpyrimidine

DMSO- Dimethyl sulfoxide

DNA -Deoxy Ribo Nucleic Acid

EP- European Pharmacopoeia

ETR- Etravirine

FDA - Food and Drug Administration

FTIR - Fourier Transform Infrared Spectroscopy

GAA- Glacial Acetic Acid

GAP- Good Analytical Practice

GFC - Gel Filtration Chromatography

GPC - Gel Permeation Chromatography

HAART- Highly Active Antiretroviral Therapy

HCl- Hydrochloric acid

HIV - Human Immunodeficiency virus

HPLC- High-Performance/Pressure Liquid Chromatography

HPTLC - High-Performance Thin-Layer Chromatography

HSS-High Strength Silica

ICH - International Council for Harmonization

IE-Ion Exchange Chromatography

IP -Indian Pharmacopoeia

IR- Infrared Spectroscopy

IS – Internal standard

IUPAC- The International Union of Pure and Applied Chemistry

KH_2PO_4 - Potassium Dihydrogen Orthophosphate

LC-MS/MS - Liquid Chromatography with tandem Mass Spectrometry

LOD-Limit of Detection

LOQ- Limit of Quantification

MD - Method Development

MS-Mass Spectrometry

MV- Method Validation

NaOH – Sodium Hydroxide

NMR - Nuclear Magnetic Resonance

NNRTI - Non-Nucleoside Reverse Transcriptase Inhibitor

NP-Normal Phase

ODS - Octadecylsilane

OPA- Ortho Phosphoric Acid (phosphoric(V) acid)

PDA - Photo Diode Array

PI- Protease inhibitor

QBD- Quality by Design

RNA-Ribo Nucleic Acid

RP-HPLC-Reverse Phase High-Performance Liquid Chromatography

RP-Reverse Phase

RP-Reversed Phase

RRT-Relative Retention Time

SEC- Size Exclusion Chromatography

SIAM – Stability Indicating Analytical Method

SST- System Suitability Test

TMS-Tetramethyl Silane

UHPLC-Ultra High-Performance Liquid Chromatography

UPLC-Ultra Performance Liquid Chromatography

USP-United State Pharmacopoeia

UV-Ultraviolet

WHO-World Health Organisation

List Of Symbols

µg – microgram

Avg.- Average

Conc.- Concentration

HZ - Hertz

Log P- Partition coefficient

mg- milligram

MHZ -Megahertz

min: Minute

ml- Milliliter

mV – millivolts

mV.S – millivolts. Second

ND- Not detected.

NLT- Not Less Than

NMT- Not More Than

°C – Degree Celsius

pH- Potential of Hydrogen

Pka- Acid dissociation constant

ppm: Parts per million

R^2 - Coefficient of determination

RSD – Relative Standard Deviation

Rt- Retention time

Rt- Retention Time

S/N: - Signal to Noise ratio

SD - Standard Deviation

Tmax- Time to Peak Drug Concentration

v/v - volume per volume

LIST OF FIGURES

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CHAPTER 1

INTRODUCTION

1. Introduction: -

1.1 Introduction of drugs

Drugs are commonly recognized as chemical agents that aid in diagnosing, preventing, managing, or relieving illnesses in humans and animals. Beyond these purposes, drugs can also affect how the body functions or bring about changes in its structure, aiming to restore balance or support long-term health.[1]. Pharmaceutical chemistry contains the study of drugs using important chemical concepts, covering all from synthesis and structure to how drugs work, how they are tested for quality, and how they are stored[2–6]. Pharmaceutical agents are largely divided into two key categories: chemotherapeutic agents, which target disease-causing organisms and pharmacodynamic agents, which influence bodily functions and work by either stimulating or inhibiting physiological processes, aiming to provide relief from abnormal conditions-primarily those linked to noninfectious disorders. They function without directly affecting disease-causing microbes. Common representatives include central nervous system stimulants and depressants, adrenergic agonists and antagonists, cholinergic modulators, cardiovascular drugs, diuretics, antihistamines, and anticoagulants.

In difference, chemotherapeutic agents are intended to selectively eradicate or prevent pathogenic organisms without causing significant harm to the host. Examples of such agents include antibacterials, antimalarials, antifungals, antiprotozoals, anthelmintics, antiseptics, antitubercular agents, antineoplastic drugs, and certain organometallic compounds.

1.1.1 Introduction of Anti-HIV Drugs: -

HIV is the retrovirus that causes AIDS. It belongs to the retrovirus subfamily lentivirus. HIV confers to cells with CD4 receptors (T_4 cells and macrophages). AIDS remains a danger because of the outlay and unapproachability of ART agents in the developing countries in which the ailment is most widespread. Moreover, the effectiveness of antiretroviral therapy (ART) has been challenged by the rise of viral strains that show resistance to multiple drugs.[1,5,7]

1.2 HIV Life Cycle[1,2]: -

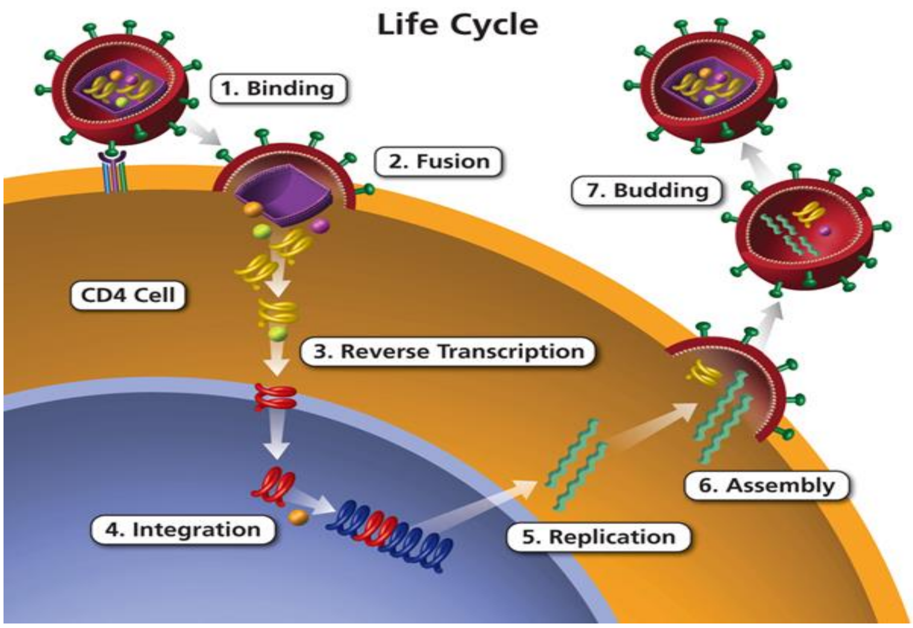


FIGURE 1 HIV life cycle

TABLE 1 Sequential Steps of HIV Infection and Replication

Step No.	Stage	Description
1.	**Fusion**	HIV initially attaches to the host cell by binding its surface glycoproteins to the CD4 receptors and co-receptors (CCR5 or CXCR4) present on the cell membrane. The envelope surrounding the virus fuses with the membrane of the host cell, allowing the viral core to move into the cytoplasm of the cell.

2.	**Reverse Transcription**	After entering the host cell, the viral RNA is converted into complementary DNA (cDNA) with the help of the enzyme reverse transcriptase.
3.	**Integration**	Once formed, the viral DNA enters the nucleus and becomes part of the host cell's genome through the action of the integrase enzyme
4.	**Transcription and translation**	Using the host cell's machinery, the integrated viral DNA is converted into RNA and subsequently synthesized into viral proteins required for new virus formation.
5.	**Assembly**	These RNA strands and proteins are accumulated into immature HIV within the host cell.
6.	**Maturation**	Once the virus leaves the host cell, it triggers an enzyme called protease. This enzyme refines the viral components, resulting in mature, infectious particles ready to infect additional cells.

1.3 Classification of Anti-HIV Drugs [3,4]: -

The following drugs are presently employed in clinical practice:

TABLE 2 Categories and Examples of Anti-HIV Drugs

Sr.No	Class	Drugs
1.	**Nucleoside reverse transcriptase inhibitors (NRTIs)**	Zalcitabine, Emtricitabine, Didanosine, Tenofovir disoproxil, Tenofovir alafenamide. , Zidovudine, Stavudine, Lamivudine, Abacavir,
2.	**Nonnucleoside reverse transcriptase inhibitors (NNRTIs)**	Delaviridine, Rilpivirine, **Etravirine**, Efavirenz, Nevirapine, Doravirine
3.	**Nucleotide reverse transcriptase**	Tenofovir

	inhibitors (NtRTIs)	
4.	**Protease inhibitors (PIs)**	Fosamprenavir, Ritonavir, Lopinavir, **Atazanavir,** Saquinavir, Indinavir, Nelfinavir, Amprenavir
5.	**Entry/Fusion inhibitors**	Abacavir, Zalcitabine, Emtricitabine, Didanosine, Enfuvirtide, Zidovudine, Stavudine, Lamivudine, Maraviroc
6.	**Integrase Strand Transfer Inhibitors (INSTIs)**	Elvitegravir, Raltegravir, Bictegravir, Dolutegravir,
7.	**Pharmacokinetic Enhancers (Boosters)**	Ritonavir, Cobicistat

1.4 Overview of Analytical Methods [5-8]

Classical liquid chromatography, originating from the Greek term "chromatography," meaning "color writing," was first demonstrated by the Russian botanist Mikhail Tswett in 1903. **Tswett** separated plant pigments using chalk (calcium carbonate, $CaCO_3$) packed into glass columns. During the 1930s, gravity-driven silica column chromatography emerged as a extensively used method for purifying organic compounds. Concurrently, ion-exchange resins became involved in separating ionic species, including radioactive isotopes. During the late 1960s, breakthroughs in analytical instrumentation gave rise to high-performance liquid chromatography, which enhanced the accuracy and efficiency of compound separation. This advancement was driven by the implementation of columns packed with fine particles, which required the application of high-pressure pumping systems for efficient operation. Early pioneers in this development, such as Horvath, Kirkland, and Huber, laid foundational groundwork in the 1960s. Subsequent commercial developments, including automated injectors and sensitive inline detectors, transformed HPLC into a robust, quantitative analytical technique, dramatically expanding its scope and practical applications.

By the 1980s, the versatility and accuracy of HPLC had established it as an indispensable analytical method within pharmaceuticals and numerous other industries. Currently, HPLC technology continues its evolution towards enhanced speed, precision, and sensitivity, propelled by increasing demands in pharmaceutical research and life sciences.

Figure 2A depicts traditional liquid chromatography, utilizing glass columns with gravity-based solvent flow and manual collection of separated fractions**.** In contrast, **Figure 2B** depicts contemporary, computer-controlled HPLC systems.

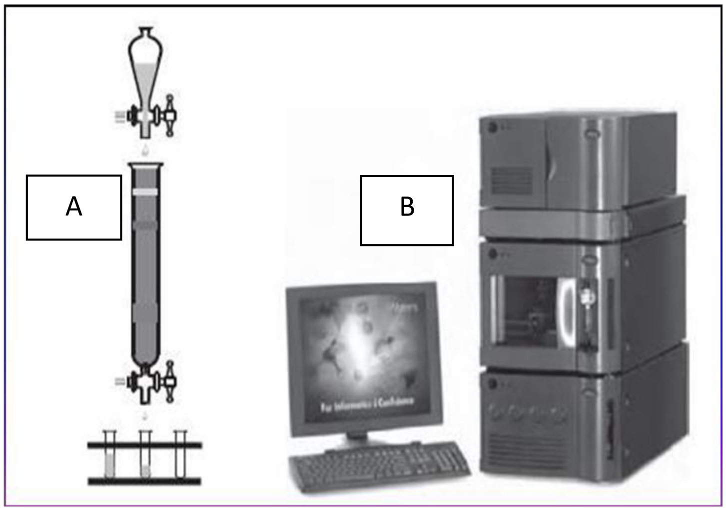


FIGURE 2 : Liquid Chromatography Instruments

A. The traditional liquid chromatography
B. A modern automated HPLC

1.5 Introduction to HPLC method [5-7,15-18]

Liquid chromatography (LC) is an analytical technique that operates in the liquid phase to separate mixtures into individual components. This separation is achieved through the differential distribution of analytes between a mobile phase typically a liquid solvent and a stationary phase composed of solid particles packed into a column. For instance, the mobile phase may consist of an organic solvent driven through the column under pressure, while the stationary phase often includes porous silica particles. Advances in this methodology have led to what is now known as HPLC, a modern form of LC that utilizes columns packed with fine particles and operates under high pressure to enhance separation efficiency and speed. HPLC relies on a solid stationary phase and a liquid mobile phase and is extensively used in both research and industry for precise analytical applications. The work out flow about HPLC is depicted in **Figure 3**.

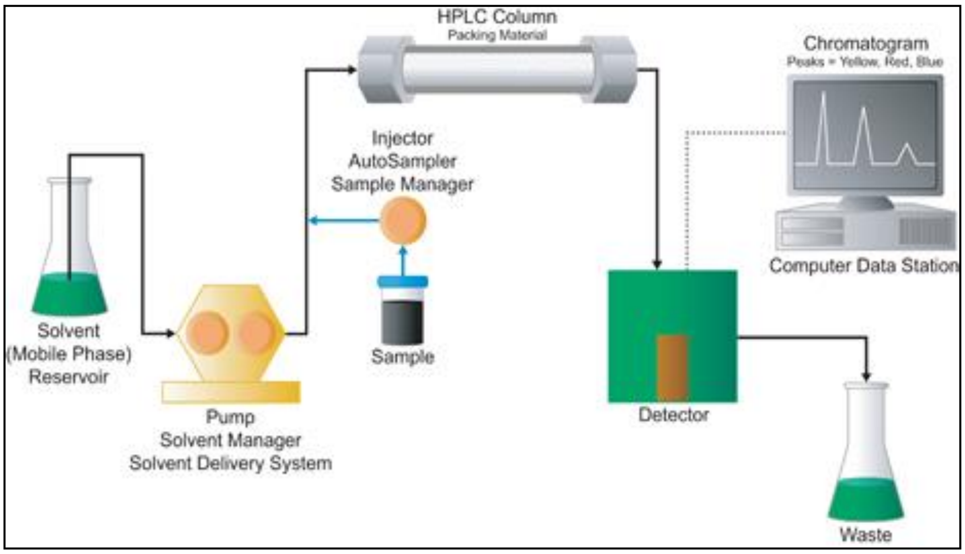


FIGURE 3 Block diagram of HPLC

1.5.1 Principle of separation [5-8]

In HPLC whether operating in normal-phase or reverse-phase mode, the separation of substances is primarily based on their varying degrees of interaction with the stationary phase. When a sample mixture is introduced into the column, each component travels at a different rate depending on how strongly it adheres to the stationary material. Substances with stronger affinity move more slowly, whereas those with weaker attraction pass through more rapidly. This difference in interaction allows each compound to be separated distinctly. As the analytes emerge from the column, a detector monitors their concentrations in real time, generating a graphical output known as a chromatogram. This process is visually represented in **Figures 4 and 5.** The efficiency of separation can be influenced by factors such as particle size of the stationary phase, temperature, flow rate, and the composition of the mobile phase. Proper optimization of these parameters ensures better resolution, faster analysis, and reliable quantification of each compound. The choice between normal-phase and reverse-phase HPLC depends on the polarity of the analytes and the nature of the separation required. In reverse-phase HPLC, non-polar compounds are retained longer, whereas in normal-phase, polar compounds exhibit stronger retention. The resulting chromatogram provides valuable information about the identity and purity of each component based on its retention time and peak characteristics.

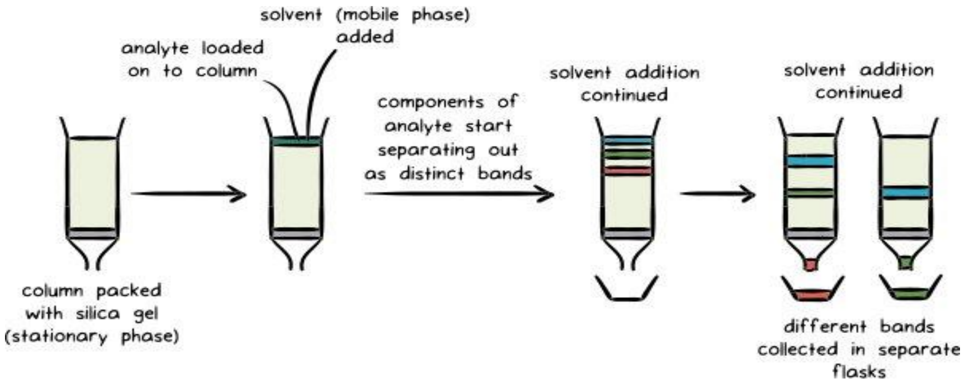


FIGURE 4 Representation of the chromatographic process

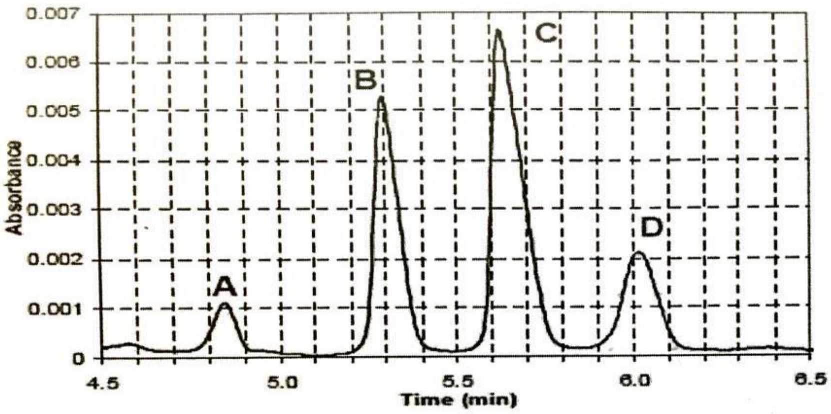


FIGURE 5 A chromatogram plotting the signal from a UV detector

1.5.2 Modes of separation in HPLC [5,14,15,17]

The main modes of separation in HPLC include:

1. **Normal-Phase (NP) HPLC**
2. **Reverse-Phase (RP) HPLC**
3. **Ion-Exchange (IE) HPLC**
4. **Size-Exclusion (Gel Permeation) (SE) HPLC**
5. **Affinity Chromatography (AC)**

1) **Normal phase:** Normal-phase A polar stationary phase and a non-polar mobile phase are used in HPLC for separation. Non-polar molecules elute faster in this mode due to their reduced interactions with the polar stationary phase. Likewise, **polar compounds show sturdier attraction** for the stationary surface and are therefore **retained longer** within the column. As a result, they require more time to pass through. This separation

mode is **less commonly employed in pharmaceutical analysis** because most drug molecules are polar and would consequently have prolonged retention times.

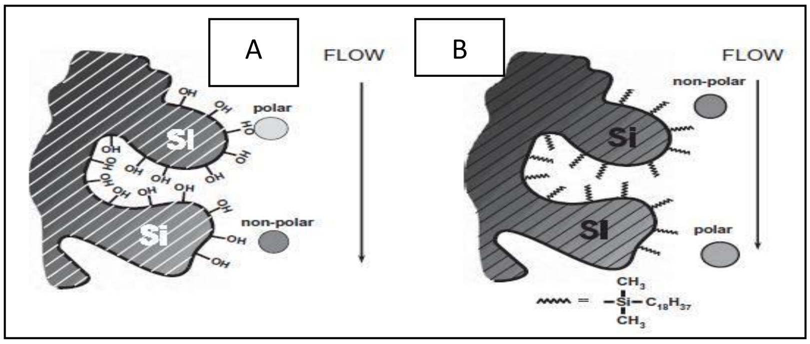


FIGURE 6 Schematic diagrams depicting separation modes

A. Normal-phase chromatography (NPC); B. Reversed-phase chromatography (RPC)

2) **Reversed Phase (RP) mode:** RP-HPLC is the most widely used technique for both analytical and preparative separations in the fields of chemistry, biology, pharmaceuticals, food science, and biomedical research. This technique employs silica gel functionalized with non-polar alkyl groups (C8 or C18) to form a hydrophobic stationary surface. The mobile phase is polar, often water-based, allowing the application of secondary solute equilibria such as ionization control, ion suppression, ion pairing, and complexation to fine-tune retention and selectivity. In RP-HPLC, polar compounds elute earlier, while non-polar compounds are retained longer due to stronger interactions with the non-polar stationary phase. Because most pharmaceutical compounds are polar, they generally elute more quickly, making RP-HPLC especially suitable for pharmaceutical analysis.

   Stationary phase polarity increases from C18 to C4, affecting retention time-shorter alkyl chains (C4) retain compounds for a shorter duration compared to longer ones (C18).

3) **Ion Exchange Chromatography (IEC):** In IEC, the stationary phase is functionalized with ionic groups such as quaternary ammonium (NR_3^+) for anion exchange or sulfonic acid (SO_3^-) for cation exchange. These charged groups interact with the oppositely charged functional groups of the sample molecules, enabling effective separation of ionic or charged species. This technique is particularly suitable for the separation of charged compounds, such as amino acids, peptides, proteins, and inorganic ions. The

retention behavior can be precisely controlled by adjusting the pH and salt concentration of the mobile phase, which affects the strength of ionic interactions between the analytes and the stationary phase.

4) **Size Exclusion Chromatography (SEC):** In SEC separates analytes by molecular dimensions, with larger species eluting first due to limited pore access, and smaller species eluting later as they diffuse into the porous matrix. The method is most suitable when the molecular weight difference among components is at least 10%. SEC is further categorized into two primary subtypes: Gel Permeation Chromatography (GPC) is typically performed using organic solvents and is widely applied in the characterization of synthetic polymers and plastic materials. In contrast, gel filtration chromatography (GFC) involves the use of a water-based mobile phase, primarily used to separate biological macromolecules, including proteins and enzymes.
5) **Affinity Chromatography (AC):** In AC relies on highly specific biological or biochemical interactions between the target molecule and the immobilized ligand on the stationary phase. Affinity ligands that recognise and hold onto analytes through shape and charge complementarity are added to the stationary surface. Proteins, enzymes, and antibodies are among the biomolecules that can be efficiently and specifically separated from complicated biological mixtures using this technique.

1.5.2 Parameters Affected by Chromatographic Conditions [5–8, 14, 15, 17]

The performance of chromatographic systems is significantly influenced by various operational conditions.

The key parameters impacted include:

1. **Resolution (Rs)**
2. **Column Efficiency / Number of Theoretical Plates (N)**
3. **Capacity Factor (k')**
4. **Peak Asymmetry Factor (As)**

1. Resolution (Rs):- A chromatographic system's resolution is its ability to distinguish between neighbouring peaks. The ratio of the retention times of two neighbouring peaks to the average of their peak widths is its definition.

$$Rs = \frac{t_{R2} - t_{R1}}{0.5 \times (W_1 + W_2)}$$

Where;

t_{R1} and t_{R2}: Retention times of the first and second peaks

W_1 and W_2: - Corresponding peaks widths (At base) in minutes

Ideal Value: Rs should be **greater than 2** for acceptable separation between closely eluting compounds.

Purpose: - Measures separation between peaks

2. Column Efficiency / Number of Theoretical Plates (N):- Column efficiency is quantified by the number of theoretical plates (N), which reflects the sharpness of the chromatographic peak. Higher values suggest better column performance and minimal band broadening.

$$N = \frac{16 \times t_R^2}{W^2}$$

Where; t_R: Retention time of the analyte **and** W: Peak width at baseline

Typical Range: 5,000 to 100,000 plates/meter, depending on column type and particle size

Factors affecting column efficiency: Column age and usage history**,** Injector malfunction or damaged tubing (band broadening)**,** Improper detector settings **and** variations in pump flow rate or temperature

Purpose: - Reflects column efficiency and band broadening

3. Capacity Factor (k'):- The capacity factor (k') indicates how long an analyte is retained on the column relative to an unretained species. It reflects the analyte's interaction with the stationary phase.

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

Where; tR: - Retention time of the analyte, **t_0 :-**Time taken by an unretained compound (dead time)

Ideal Range: 2 to 10 for optimal chromatographic performance

Purpose: - Indicates analyte retention on the column

4. Peak Asymmetry Factor (As) / Tailing Factor: - The peak asymmetry factor assesses the symmetry of a chromatographic peak. It is especially important for evaluating column packing and analyte-stationary phase interaction.

$$As = \frac{b}{a}$$

Where; a: Front half of the peak width at 10% peak height; b: Rear half of the peak width at 10% peak height.

Ideal Values: 0.9 to 1.1 for symmetric peaks and **As ≤ 2.0** is acceptable in most analytical applications.

Purpose: - Describes peak shape and column packing quality.

1.6 Analytical Method Validation (AMV) [5,7]

AMV is the systematic process of confirming that a given analytical procedure is **appropriate for its intended purpose**. It ensures that the method produces results that are **accurate, reliable, and consistent** under defined conditions, thereby supporting the **credibility and quality** of analytical data. Method validation is a fundamental component of **good analytical practices**.

Analytical procedures must be validated or revalidated in the following scenarios:

- **Prior to implementation** in routine analysis.
- **When modifications occur** in conditions for which the method was originally validated (e.g., change in instrumentation, matrix variations, etc.).
- **When the method undergoes modifications** that extend beyond its original scope.

Guidelines for validating analytical methods, particularly for compound evaluation, have been established by the **United States Pharmacopeia (USP)** and other regulatory agencies.

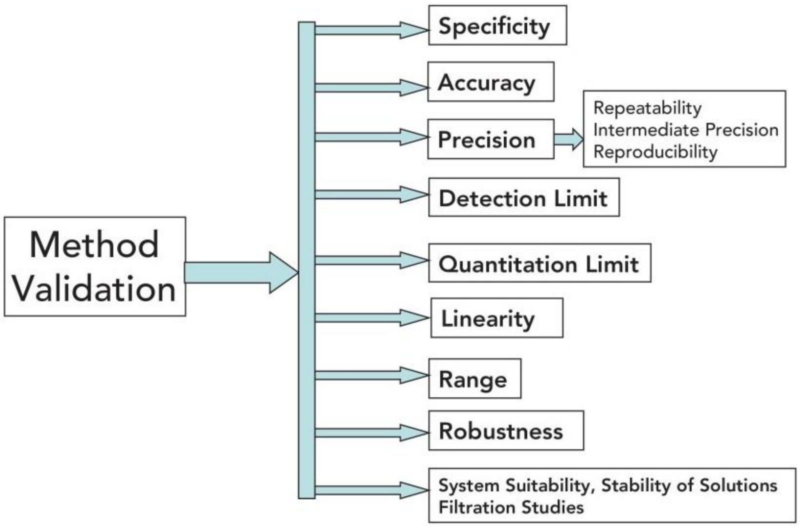


FIGURE 7 Analytical Method Validation Parameters

1) Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

The accuracy of an analytical method should be established across its range. In the case of the assay of a drug in a formulated product, accuracy may be determined by application of the analytical method to synthetic mixtures of the drug product components to which known amount of analyte have been added within the range of the method. Minimum of test concentrations from 50 % to 120 % are normally used, for establishment of accuracy in assay of drug substance (or a finished product). Average recovery should be 98 to 102 % of drug at each level.

2) Precision

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

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

The precision of an analytical procedure is usually expressed as the variance, standard deviation, or coefficient of variation of a series of measurements. In the precision results of all samples should not have RSD > 2%.

3) Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

Determination of Reproducibility:

Reproducibility can be assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance, for inclusion of procedures in pharmacopoeias.

4) Specificity

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

Identification: to ensure the identity of an analyte.

Purity Tests: to ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content, etc.

Assay: To provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample.

Determination of specificity:

ICH document state that when chromatographic procedure used, representative chromatograms should be used to demonstrate specificity and individual components should be appropriately detected. Peak purity tests may be useful to show that the analyte chromatographic peak is not attributable to more than one component (e.g., diode array, mass spectrometry).

5) Limit of Detection (LOD)

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

Determination of limit of detection:

For instrumental and non-instrumental methods detection limit is generally determined by the analysis of samples of known concentration of analyte and by establishing the minimum level at which the analyte can be reliability detected.

The limit of detection (LOD) may be expressed as:

$$\mathbf{LOD = 3.3\ \sigma/s}$$

Where, σ = the standard deviation of the response.

S = the slope of the calibration curve.

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

6) Limit of Quantitation (LOQ)

The limit of quantitation of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The limit of quantitation is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

Determination of limit of quantitation:

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

The limit of quantitation (LOQ) may be expressed as:

$$\mathbf{LOQ = 10\ \sigma/s}$$

Where, σ = the standard deviation of the response.

S = the slope of the calibration curve.

7) Linearity and Range

Linearity refers to the ability of an analytical method to produce results that are directly proportional to the concentration of the analyte within a specified range. It confirms that the response of the method remains consistent across varying levels of analyte concentrations. Range is defined as the concentration interval-from the lowest to the highest analyte level, including both limits-over which the method has been proven to maintain acceptable precision, accuracy, and linearity.

Determination of Linearity and Range:

For the determination of linearity, a minimum of 5 concentrations is recommended. Linearity can be determined by a series of sample whose concentrations span 80-120% of the expected concentration range. Linearity is evaluated by graphically.

8) Ruggedness

Ruggedness refers to the degree of reproducibility of test results obtained from the same sample when subjected to varying experimental conditions, such as differences in analysts, instruments, laboratories, or environmental factors. It indicates how well an analytical method withstands typical variations expected during routine use. A rugged method demonstrates minimal influence from operational or environmental changes, ensuring reliable performance across different settings. Thus, ruggedness is a key indicator of the method’s consistency and reliability under inter-laboratory or inter-analyst conditions.

Determination of Ruggedness:

By analysis of aliquots from homogenous lots in different laboratory, by different instrument and using operational and environmental condition that may differ but still with the specified parameters of the assay. Degree of reproducibility of test results is then determined as a function of the assay variables.

- Different operator in same laboratory, Different equipment in same laboratory.
- Different source of segment and solution, Different source of column.

9) Robustness

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

Determination of robustness:

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters.

Examples of typical variations are: Stability of analytical solutions & Extraction time.

In the case of liquid chromatography, examples of typical variations are: Influence of variations of pH in a mobile phase, Influence of variations in mobile phase composition, Different columns (different lots and/or suppliers) and Temperature and flow rate.

Applications and Advantages

- An ideal method for separation of various compounds in plant extracts which resemble in structure and thus demand specific and very sensitive method.
- A premier separation technique capable of multi component analysis of real-life samples and complex mixtures.
- This method is used for ascertaining of various pharmaceuticals. The analysis of the various degradation products can be done and thus stability indicating HPLC systems and method has developed.
- Highly automated, using sophisticated auto-samplers and data systems for unattended analysis and report generation. Few techniques can match its versatility and precision of ±0.5% RSD.
- A host of highly sensitive and specific detectors extend detection limits to nanogram, picogram, and even femtogram levels. As a preparative technique, it provides quantitative recovery of many labile components in milligram to kilogram quantities.
- Having Rapid and precise quantitative analysis. Quantitative sample recovery and amenable to diverse samples. Most importantly, It is amenable to 60% to 80% of all existing compounds.

TABLE 3 Validation Criteria for Impurity Profiling (as per ICH Q2(R1))

Parameter	Purpose	Acceptance Criteria	Notes
Specificity	Ability to detect impurities without interference from main peak, excipients, or degradants	No interference at retention time of impurity and main peak	Peak purity by PDA or MS
Linearity	Measure response is directly proportional to concentration	Correlation coefficient (r^2) ≥ 0.999	Typically for 5–6 concentration levels
Accuracy	% of known added impurity	98–102% (for known	Tested at 50%,

(Recovery)	recovered	impurities) For low levels (LOQ), 80–120%	100%, 150% of expected impurity level
Precision	Repeatability of results	%RSD ≤ 2%	Intra-day and inter-day (intermediate precision)
LOD (Limit of Detection)	Minimum impurity concentration detectable but not quantifiable	Signal-to-noise ratio ~3:1	Required only for known & toxic impurities
LOQ (Limit of Quantitation)	Lowest impurity concentration that can be reliably quantified	Signal-to-noise ratio ~10:1 %RSD ≤ 10%	Critical for low-level impurity detection
Robustness	Reliability under small variations in method parameters (pH, flow, temp)	No significant change in system suitability or impurity profile	Flow rate ±10%, temp ±5°C, pH ±0.2
System Suitability	Ensure system performance is adequate	- %RSD < 2% for peak area - Resolution > 2 between critical peaks - Tailing factor < 2	Evaluated before batch run

1.7 Introduction to impurity profiles [7-10]

Pharmaceutical drug stated as medicine or medication, officially termed as a medicinal product. “Pharmaceutical” derives from the Greek pharmakeutikos. A pharmaceutical drug is any chemical substance which may be used or administrated to diagnose, treat, cure or to prevent the disease or the other abnormal conditions (www.pharmacistspharmajournal.org, 2010). As per regulatory guidelines, pharmaceutical drug defined as it is any chemical substance that is recognized by any regulatory body or official pharmacopoeia proposed to diagnose, treat, cure or to prevent the disease or the other abnormal conditions in man or animals (www.fda.com, US Federal Food, Drug, and Cosmetic Act, 1938).

For many centuries, human civilization has been developing and consuming drugs, but it is only in last century that enormous and systematic research has been done on development of pharmaceutical drugs. The average life span of human is increased by the use of drugs by curing and preventing diseases. These drugs are manufactured in bulk and formulated into convenient dosage forms for their desired therapeutic use.

These formulations should be stable, nontoxic and in acceptable state, confirming safety, quality, and efficacy. (Gorog, 2000; Ahuja, 1998; Singh et al., 2012). Safety, quality and efficacy of drug substances are the fundamental concerns in drug therapy. Safety and quality of pharmaceutical substance is monitored by its pharmacological profile and/or toxicological profile and the adverse effects caused by its impurities. The drug should be safe, i.e. it should have acceptably low risk of adverse effects with doses of drug which provide the wanted therapeutic effects. Thus, quality of drug is directly related to safety.

The quality as well as safety of a drug is guaranteed by screening it using suitable analytical techniques. Therefore, the analytical techniques and related events about drug impurities are most main subject in pharmaceutical analysis (Ahuja, 2007, Smith and Webb, 2007). Until 1990s, there was no precise definition for ‘impurity’ in the pharmaceutical world, because of the apparent negativity attached to this word. The definition for impurity in Webster’s dictionary is ‘‘something that is impure or makes something else impure’’. In the pharmaceutical industry, it is the latter meaning that is frequently attached to the meaning of an impurity. A simple definition of impurity has been offered: impurity is a material which disturbs the purity of the material of target, viz., drug substance or drug product.

1.7.1 Impurity and its definition[8-11]

1.7.1.1 Impurity

An impurity is any constituent present in excipient, drug substance (Active Pharmaceutical Ingredient or bulk material) or drug product (Dosage form or Finished product) that is not an excipient, active drug substance, formulated drug product. This definition of impurity is broad enough to include degradation products as impurities. The term degradation product (DP) is defined in ICH as follows: (www.ich.org, ICH guidelines, 2012).

1.7.1.2 Degradation Products (DP)

"A molecule resultant from a modification in the active drug substance or formulated drug product brought over time."

1.7.2 Source of impurities [9]

Pharmaceutical development consists of two primary stages: the synthesis of the active pharmaceutical ingredient (API) and the formulation of the final drug product. Impurities can originate at both stages during API synthesis through process-related pathways or chemical degradation, and during formulation due to degradation, drug–excipient interactions, contamination, or leaching from packaging.

Types of impurities reported include crystallization-related, stereoisomeric, process-related by-products, residual solvents, inorganic impurities, and degradation products. Among these, degradation is a major contributor and may be triggered by factors such as heat, moisture, oxidation, light, pH shifts, or exposure to solvents. It can also occur during manufacturing steps like purification, drying, and storage, or due to excipient interactions.

The chemical stability of a drug-largely influenced by its functional groups plays a critical role in impurity control. Understanding these stability characteristics is essential for minimizing impurity formation and ensuring product safety and efficacy.

1.7.3 Classification of Impurities [9,14-22]

Theoretically possible (potential) impurities are classified **(Figure 8)** as following types (Guidance for industry, 1998).

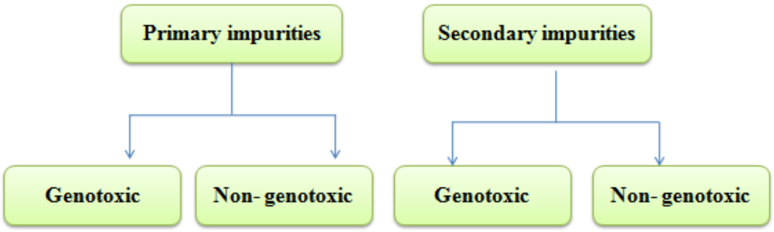


FIGURE 8 Types of impurities

1.7.3.1 Primary Impurities

These impurities are generated along with development of a wanted product, like by products, deposits of starting ingredients and intermediates.

1.7.3.2 Secondary Impurities

These impurities are deposits of impurities of starting ingredients, degradants of primary impurities, yields of reactions among impurities etc.

1.7.3.3 Non genotoxic Impurities

Non genotoxic impurities are ordinary impurities those don’t have any harmful effect on genetic material.

1.7.3.4 Genotoxic Impurities

Genotoxic impurities harm the organism by damaging its genetic material (DNA).

Based on the source of impurities they are classified (**Figure 9**) as follows:

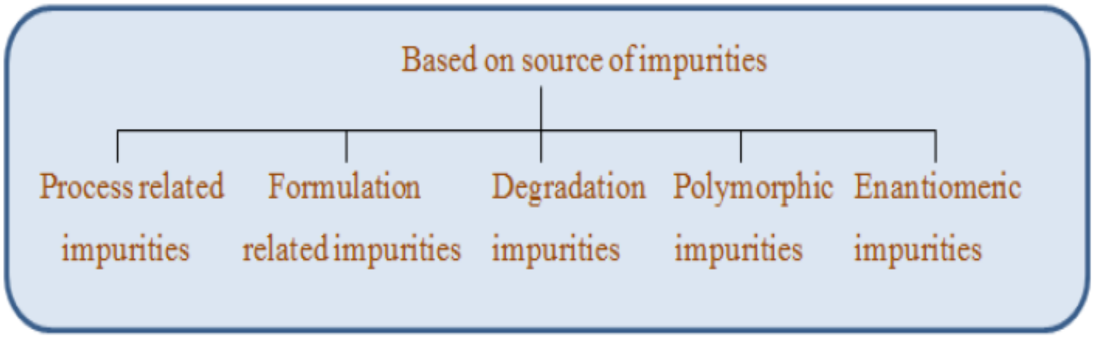


FIGURE 9 Classification of Impurities based on their source

1.7.4 Designation of impurities [9,14-25]

Impurities have been titled differently by various groups of scientists who deal with them. Commonly used terms are displayed in (**Figure 10)** and those terms have been found acceptable by ICH and various regulatory bodies.

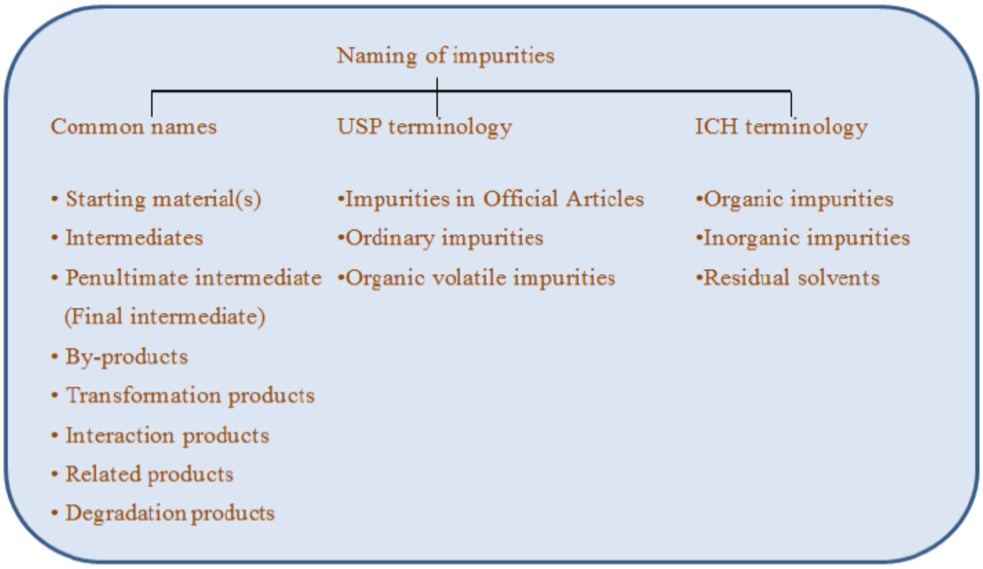


FIGURE 10 Designation of Impurities

1.7.5 Identification of impurities [9]: -

Identification and characterization of impurities is an important analytical step to determine the **structure** and **origin** of unknown impurities in drug substances. Due to the **complex nature** of impurities, the strategy used depends on the specific situation. However, a **general approach** includes the rational use of **analytical techniques** such as chromatography and spectroscopy.

The impurity profile includes:

- Key impurities in drug substances, intermediates, and starting materials
- Process-related impurities and their formation mechanisms
- Residual solvents and inorganic impurities

Steps in impurity profiling:

Detection – Unknown impurities are first detected during process control or final analysis.

Evaluation – Their source is analysed based on when and where they appear in the process.

Identification – Done using: Reference standard method, Separation or isolation techniques and Spectroscopic and hyphenated techniques (like LC-MS, NMR). In the **reference standard method**, the impurity is confirmed by comparing the sample, standard, and a spiked sample using chromatographic or spectroscopic tools.

The schematic use of the methods for impurity profiling of drug substances is shown in Figure 11.

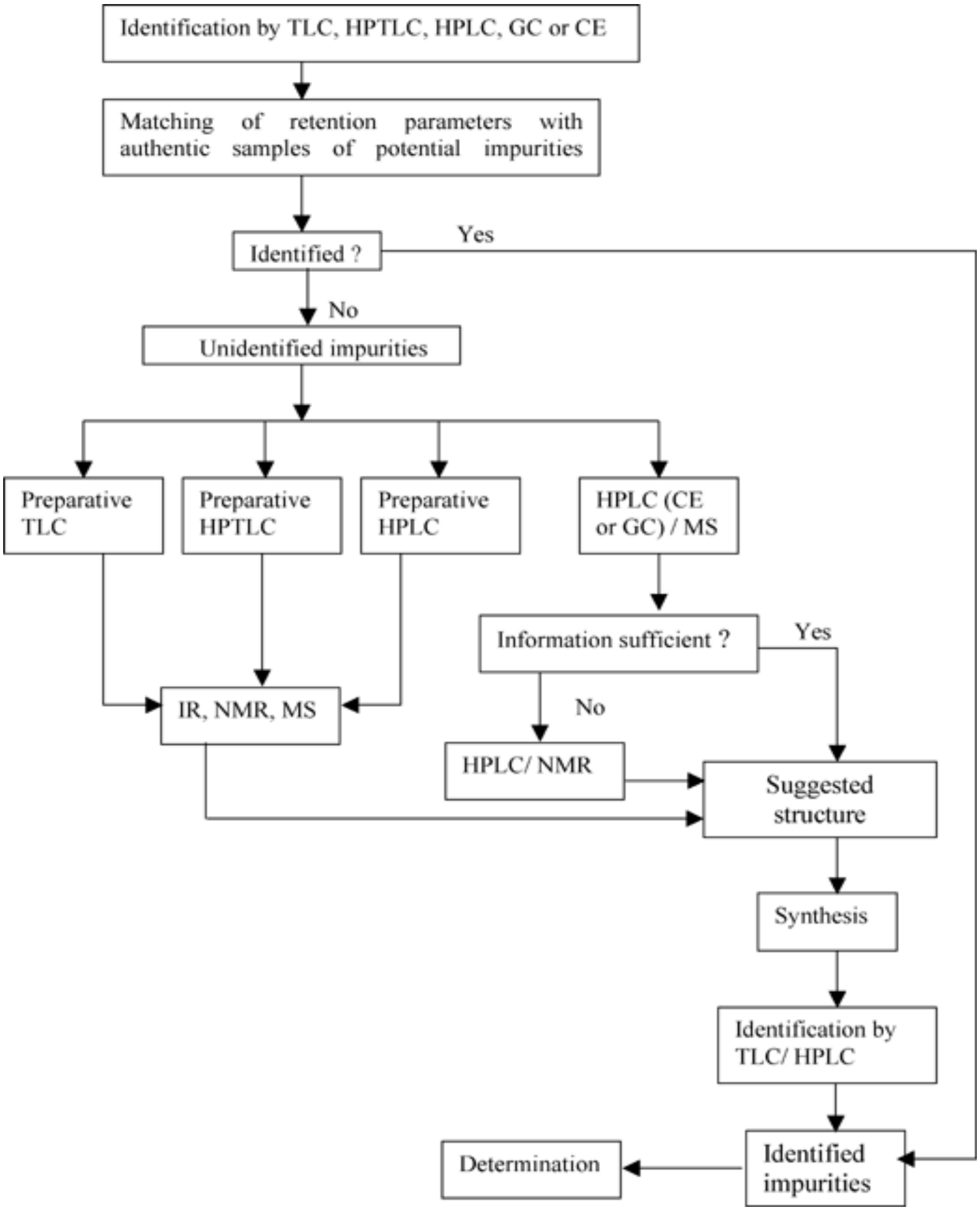


FIGURE 11 Scheme for impurities profile study

TABLE 4 Impurity Profiling and Method Validation – Summary Table

Aspect	**Impurity Profiling**	**Method Validation**
Purpose	Identify, quantify, and characterize known and unknown impurities in APIs and drug products	Confirm analytical method's suitability for intended purpose
Types of Impurities	Organic (process-related or degradation), inorganic, residual solvents	Not applicable directly, but method must detect all types accurately
Techniques Used	RP-HPLC, LC-MS/MS, GC, UV, NMR, FTIR	RP-HPLC, UV, LC-MS/MS, depending on specificity
Stages	Stress studies (acid/base/oxidation/heat/light) Degradation product identification - Threshold determination	-System suitability -Specificity -Linearity -Accuracy -Precision -LOD/LOQ -Robustness - Range
ICH Guidelines	Q3A(R2), Q3B(R2), Q3C, M7	Q2(R1) (Analytical Method Validation)
Acceptance Criteria	- Identified & quantified >0.1% or ICH threshold - Structure elucidated for unknown impurities	- %RSD < 2% (Precision) - Recovery 98–102% (Accuracy) - Correlation coefficient > 0.999 (Linearity)
Documentation	Chromatograms, degradation pathways, impurity tables	Validation report with statistical data & graphs

*** Even impurities below 0.1% must be evaluated if they are genotoxic (as per ICH M7), as they may pose significant safety concerns. ICH Q3A/B specifies that any impurity above 0.1% or threshold limit (whichever is lower) must be identified and qualified—even if it is unknown.**

1.7.6 Quantitation of Impurities[5,7,9]: -

Impurity profiling ensures product safety by tracking degradation and synthesis by products. Validation ensures that the method used can consistently detect and quantify impurities at trace levels. Forced degradation helps prove stability-indicating capability. Both are regulatory requirements for filing NDAs or ANDAs. For unknown impurities, the qualification threshold is usually 0.1% or 1.0 mg/day intake. Stability-indicating methods must separate API and all degradants. Validation must be performed using spiked impurities at expected and threshold levels.

The following techniques are being regularly used for the quantitation of impurities and degradation products: High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC), Capillary Electrophoresis (CE), Supercritical Fluid Chromatography (SFC), and Gel Permeation Chromatography (GPC). Recently Ultra Performance Liquid Chromatography (UPLC) is emerging as a fast separation liquid chromatographic technique. Since, we have used only HPLC in our present work.

CHAPTER-2

LITERATURE REVIEW

2.0 Literature review [26-84]: -

A thorough review of the literature revealed multiple investigations related to the chemical synthesis, structural characterization, and development of stability-indicating methods for selected classes of anti-HIV agents.

MD and **MV** processes have also been extensively reported for individual drug substances. Furthermore, some studies have addressed the simultaneous estimation and validation of analytical techniques applied to combinations of antiretroviral drugs.

From a pharmacological viewpoint, numerous studies have discovered the pharmacokinetic profiles of these agents, including drug–drug interactions and pharmacokinetic interactions. Several reports also highlight the use of LC-MS/MS-based methods for the quantitative determination of antiretroviral drugs in human plasma, underscoring their importance in therapeutic monitoring and bioanalytical research.

From a pharmaceutics perspective, various studies have focused on improving solubility using techniques like spray drying, crystal engineering, and lyophilization (freeze drying).

ATZ has shown **clinical efficacy** but also **high changeability** in its degradation behaviour, making it an **ideal applicant** for developing a sensitive and precise analytical method that ensures **safety, efficacy, and regulatory compliance**. Certain synthetic impurities are considered potentially genotoxic, necessitating highly sensitive and accurate detection even at trace levels. However, only a limited number of validated chromatographic methods are currently available for impurity profiling and quantification in fixed-dose combination drug products.

ETR (brand name: *Intelence*), established by Tibotec Pharmaceuticals Ltd., was granted its **initial approval from the USFDA on 18 January, 2008** (Application No.: 022187). On **26 March, 2012**, the indication was extended to include **treatment-experienced paediatric patients aged 6-18 years**, weighing **at least 16 kg**. ETR is to be used **only in combination with other anti-retroviral agents**.

This therapeutic agent is authorized for use in managing HIV infection among both adults and children aged two years and above, provided they weigh at least 10 kgs and have previously received antiretroviral treatment.

In **2009**, post-marketing surveillance reported cases of **Toxic Epidermal Necrolysis (TEN)**, **Stevens–Johnson Syndrome (SJS)**, **Erythema Multiforme**, and other **hypersensitivity reactions**, prompting revisions to the drug's "Warnings and Precautions" and healthcare provider communications.

Further updates to the **product monograph** were made in **2013** following reports of **autoimmune disorders**, including **Guillain–Barré syndrome**, **Graves' disease**, **polymyositis** and within the context of **immune reconstitution**. Additional safety data regarding the **development of dermatological reactions** also contributed to the modification.

Currently, **no monographs for ETR exist in the IP, BP, or EP.** However, a **new analytical monograph is under review**, as recently proposed in the **USP–Draft**.

Additionally, comprehensive drug information for ETR is available on professional resources such as Drugs.com, which includes details on usage, dosage, warnings, interactions, and pharmacology

However, despite the therapeutic importance of these agents, there remains a significant gap in reported analytical methods specifically dedicated to the **impurity profiling and quantification of selected anti-HIV drugs using chromatographic techniques. This highlights the need for a well-validated, sensitive, and reliable method to address impurity-related concerns in these formulations**. Only a limited number of analytical approaches have been documented for the identification and quantification of impurities in specific anti-HIV drugs using chromatographic techniques.

TABLE 5 Official method Of ATZ in pharmacopoeia [26]

Sr. No	Drugs	Parameters	Methods
1.	**Draft Proposal of ATZ sulphate In I.P**	**<u>Related substances</u>** **Mode: -** Gradient **Column: -** Zobrax Rx- C8 (5µm ,25	**HPLC [26]**

	(7^{th} Feb-2024) **Tentative effective date of monograph revisions July, 2026**	cm × 4.6 mm) **Column temperature: -** 45 ^{0}C **Mobile phase: -** The mobile phase was composed of 60 parts of buffer (1.6 gm NH_4HCOO in 1000 ml water, pH adjusted to 4.0 with HCOOH, 50 ml methanol, and 40 parts ACN. **Flow rate: -** 1.2 ml/min **Volume of injection**: - 10 µl **Wavelength: -** 254 nm	

Sr. No	Drugs	Parameters	Methods
2.	**ATZ**	**Related substances** **Mode: -** Gradient **Column: -** Intersil ODS-3 (5µm, 25 cm × 4.6 mm) **Column temperature: -** 45 ^{0}C **Mobile phase: -** $NaH_2PO_4{\cdot}H_2O$ (pH 2.5) and ACN **Flow rate: -** 1.0 ml/min **Volume of injection**: - 20 µl **Wavelength: -** 210 nm	**HPLC** [26]

TABLE 6 Reported method for ATZ and its combination [27-29]

Sr. No	Drugs	Parameters	Methods
1.	**ATZ**	**Mode: -** Gradient **Column: -** Ascentis, Express C8, (150 mm × 4.6 mm, 2.7 µm)	**RP-HPLC** [27]

		Column temperature: - 30 ^{0}C **Mobile phase: -** KH_2PO_4 (pH 3.5, 0.02 M) and ACN **Flow rate: -** 1.0 ml/min **Injection volume: -** 20 µl **Detector: -** PDA **Wavelength: -** 250 nm.	
2.	**ATZ**	**Mode: -** Gradient **Column: -** Express C8 (150 mm × 4.6 mm, 2.7 µm) **Column temperature: -**30 ^{0}C **Mobile phase: -** KH_2PO_4 (pH 3.5, 0.02M) and ACN **Flow rate: -** 1.0 ml/min **Detector: -** PDA **Wavelength: -** 250 nm	**RP-HPLC** [28]
3.	**ATZ+ Ritonavir**	**Mode: -** Gradient **Column: -** Acquity BEH C18 (100 mm × 2.1mm), 1.7 µm **Column temperature: -**30 ^{0}C **Mobile phase:** 0.01M KH_2PO_4 adjusted the pH to 3.6 and ACN **Flow rate: -** 0.4 ml/min **Run time: -** 18 min **Detector: -** PDA **Wavelength: -** 240 nm	**RP-UPLC** [29]

TABLE 7 USP Draft-2023 Reported Methods for ETR[75]

Sr. No	Drug	Parameters	Method
1.	**ETR**	**Mode: -** Gradient **Column: -** 3.0 mm × 15 cm; 5 µm packing	**RP-HPLC** [75]

		L1 **Column temperature: -** 35 °C **Mobile Phase: - Buffer**: - 10 mM NH_4HCOO in water **Diluent: -** ACN and Buffer (10:9 v/v) **Solution A: -** 50 mM HCOOH in buffer **Solution B: -** ACN **Solution C: -** Methanol **Flow rate: -** 0.6 ml/min **Injection volume: -** 10 µl **Wavelength: -** 310 nm **Detector: -** UV	

TABLE 8 Reported Methods of ETR [76-84]

Sr. No	Drug	Parameters	Method
1.	**ETR**	**<u>Stability</u>** **Mode: -** Gradient **Column: -** YMC's Ultra HT Pro C18 50 x 3.0 mm, 2.0 µm column **Column Temperature: -** 40 °C **Mobile Phase: -** 0.1 % HCOOH in water and Acetonitrile **Elution Time: -** 7.0 min **Flow rate: -** 0.8 ml/min **Detector: -** UV	**RP-HPLC** [76]

		Wavelength: - 310 and 250 nm	
2.	ETR	**Mode: -** Isocratic **Column: -** C8, 150 x 4.6mm, 5µm **Mobile Phase:** - CH_3COONH_4 Buffer solution and diluted with GAA PH 6.0 ± 0.05 and Methanol (35:65)	**RP-HPLC** [77]
3.	ETR	**Mobile Phase: -** Ethyl acetate: toluene (6:4 v/v) HPTLC plates with silica gel with fluorescent indicator with layer thickness (0.2 mm) aluminum (E-Merck-KgaA) as the stationary phase. **Wavelength: -**254 nm	**HPTLC** [78]
4.	ETR	**Column: -** Phenomex Luna C18 column (100 x 4.6 mm, 5µm) **Mobile Phase: -** KH_2PO_4 pH 3.45 with 0.2 ml Triethylamine ($C_6H_{15}N$) and ACN in the ration of 25:75 % v/v **Flow rate: -** 1 ml/min **Detector: -** UV **Wavelength:** 271 nm	**HPLC** [79]
5.	ETR	**Human Plasma** **Internal standard (IS) :-**fenofibrate **Column: -** C18 (250 x 4.6 mm, 5 µm) **Mobile Phase: -** ACN: Water (85:15 v/v) **Flow rate: -** 1 ml/min **Retention time: -** ETR 5.32 (± 0.1) and fenofibrate 3.58 (± 0.1) minutes	**HPLC** [80]

		Detector: - UV **Wavelength: -** 308 nm	
6.	**ETR**	**Mobile Phase: -** Acetonitrile (ACN) **Column: -** Hypersil ODS C18 (150*4.6 mm), 5 µm **Column temperature: -** 35°C **Flow rate: -** 1 ml/minute **Run time: -** 5 minutes **Retention time (Rt): -** 1.8 minutes **Injection volume: -** 10 µl **Detector: -** UV **Wavelength: -** 271 nm	**RP-HPLC** [81]
7.	**ETR**	**Mobile Phase: -** MeOH: ACN (60:40 v/v) **Injection Volume: -** 10 µl **Column: -** Phenomenex Kinetex C18 (250 x 4.6 mm, 5µm) **Column oven temperature: -** 25 ± 5 ºC **Flow rate: -** 1.0 ml/min, **Run time: -** 10 min **Detector:-** PDA **Wavelength: -** 311 nm	**RP-UFLC**[82]
8.	**ETR**	**Column: -** RP Inertsil ODS-3V C-18, 250x4.6 mm, 5µm **Mobile Phase: -** 0.03 M KH_2PO_4 in water pH-3.2 with orthophosphoric acid and ACN in the ratio of 30:70 v/v was used	**RP-HPLC** [83]

		Flow rate: - 1.0 ml/min **Injection Volume: -** 10 µl **Retention time (Rt): -** 9.11 min **Detector: -** PDA Detector **Wavelength: -** 309 nm	
9.	**ETR with Anti-retroviral agents**	**Human Plasma** **Internal standard (IS): -**Deuterium 8 **Column: -** C18 HPLC column (Waters Sunfire 100 × 2.1 mm, 3.5 µm **Flow rate: -** 0.3 ml/min	**LC-MS/MS** [84]

TABLE 9 Reported method for other class of anti-HIV Drugs [30-57]

Sr. No	Drugs	Types of research	Methods
1.	**Lopinavir + Ritonavir**	Bulk drug and pharmaceutical formulation with its impurities	**UPLC** [30]
2.	**Emtricitabine**	Related degradation substances	**HPLC** [31]
3.	**Darunavir**	Unknown impurities	**RP-HPLC** [32]
4.	**Zidovudine**:	A stability-indicating method for identification, characterization, and toxicity prediction of two major acid degradation products	**LC-MS/MS** [33]
5.	**Doravirine**	Characterization of impurities of	**UHPLC** [34]
6.	**Liponavir**	Impurities study	**HPLC** [35]
7.	**Tenofovir disoproxil fumarate**	Synthesis and characterization	**LC-MS and NMR Spectroscopy** [36]
8.	**ATZ sulphate**	MD and MV	**RP-HPLC** [37]

9.	**ATZ sulphate**	MD and MV	**RP-HPLC**[38]
10.	**ATZ + Cobicistat**	SIAM	**RP-HPLC**[39]
11.	**Raltegravir**	MD and MV	**RP-HPLC**[40]
12.	**Raltegravir**	SIAM	**RP-HPLC**[41]
13.	**Darunavir + Raltegravir**	Simultaneous determination	**UV-HPLC**[42]
14.	**Raltegravir**	MD and MV for the quantification	**LC-MS/MS**[43]
15.	**Raltegravir potassium + Rilpivirine HCl**	MD and MV	**HPLC AND HPTLC**[44]
16.	**Raltegravir**	Forced degradation studies and characterization of degradants	**RP-HPLC and LC-MS/MS**[45]
17.	**Maraviroc + Raltegravir**	Simultaneous determination in human plasma	**HPLC-UV**[46]
18.	**Lamivudine and Raltegravir**	Simultaneous estimation binary mixture by using design of experiment	**RP-HPLC**[47]
19.	**Raltegravir**	MD and MV in blood plasma	**HPLC**[48]
20.	**Darunavir Ethanolate**	SIAM	**HPLC Method**[49]
21.	**Darunavir Ethanolate**	SIAM	**HPLC**[50]
22.	**Darunavir**	Simultaneous determination of six process related impurities	**UPLC-MS/MS**[51]
23.	**Ritonavir**	Determination of three phenol impurities	**UPLC-MS/MS**[52]
24.	**ETR**	SIAM	**UPLC**[53]
25.	**Efavirenz**	Synthesis, isolation, and characterization and in process impurities in the presence of tetrahydrofuran solvent	**RP-HPLC**[54]
26.	**Cobicistat +**	MD for the assay and Degradation study	**RP-HPLC**[55]

	ATZ		
27.	**ATZ + cobicistat**	SIAM simultaneous estimation	**RP-HPLC** [56]
28.	**Darunavir**	MD and MV	**RP-HPLC** [57]

CHAPTER-3

AIMS AND OBJECTIVES, SCOPE OF THE WORK AND RATIONALE

3.0 Definition of the problem

Through a detailed literature review, it was found that while many studies have focused on stability-indicating methods, synthesis, characterization, and method development for anti-HIV drugs, few have specifically addressed impurity profiling and quantification using chromatographic techniques. This highlights a critical gap, especially given the therapeutic importance of these drugs. The present research aims to develop and validate a simple, rapid, precise, and robust RP-HPLC method for the simultaneous separation and quantification of known and unknown impurities in fixed-dose formulations of ATZ and ETR. As both are single-molecule drugs, structurally related impurities are expected in the formulations, making it essential to achieve effective resolution of specified and unspecified impurities alongside the active ingredients.

Both ATZ and ETR are PIs and NNRTIs respectively, used in HAART. Even minor impurities in such drugs can lead to toxicity or therapeutic failure, hence the critical need for high-resolution analytical methods like RP-HPLC.

The developed method is optimized for sensitivity to detect impurities at trace levels, ensuring the safety, efficacy, and regulatory compliance of the drug products. Addressing the major challenge posed by impurities in APIs, this research contributes to pharmaceutical quality by fulfilling the stringent demands of current regulatory guidelines.

3.1 Aims and Objectives

3.1.1 Aims

An extensive literature review indicates that only a limited number of chromatographic methods have been described for the determination of ATZ and ETR both in combination with other antiretroviral agents in biological matrices. Additionally, a few analytical methods report the separation, identification, and quantification of impurities in the API form of ATZ and ETR.

The present research focuses on developing a simple, rapid, precise, and robust analytical method for the simultaneous separation and quantification of impurities present in fixed-dose formulations of ATZ and ETR individually. As both are a single-molecule drug, structurally similar impurities are expected to be present in the final formulation.

Hence, the developed method critically aims to resolve both specified and unspecified impurities alongside the API with adequate resolution and reduced runtime. Furthermore, the method was optimized to exhibit sufficient sensitivity to detect and quantify impurities at trace levels, thereby ensuring the safety and efficacy of the drug product. The method development was guided by these objectives to fulfill regulatory and pharmaceutical quality requirements.

3.1.2 Objectives

The main objective of the present research work is outlined as follows:

1. **To develop a simple, robust, and reproducible RP-HPLC method** for the effective separation of known and unknown impurities present in the marketed formulations of ATZ and ETR following **ICH Q3A/Q3B and Q2(R1)** guidelines.

   To identify and characterize impurities in ATZ and ETR formulations, including degradation products and structurally related substances.

2. **To optimize chromatographic conditions** to achieve satisfactory resolution between APIs and co-eluting impurities within a reduced analytical runtime.

3. **To validate the developed RP-HPLC method** as per ICH guidelines (Q2(R1)), ensuring parameters such as specificity, linearity, accuracy, precision, robustness, LOD, and LOQ are met.

4. **To apply the validated method to routine quality control and regulatory compliance** for fixed-dose combination formulations of ATZ and ETR.

5. **To ensure the method is capable of trace-level detection**, thus supporting impurity profiling in accordance with regulatory standards for the safety and efficacy of antiretroviral drugs.

3.2 Scope of the work

The scope of this work includes the development of a simple, rapid, and reliable analytical method capable of resolving both specified and unspecified impurities in ATZ and ETR dosage forms. The method will be optimized to detect known impurities at trace levels, meet regulatory requirements, and ensure the safety, efficacy, and quality of the drug product. It also addresses the need to analyses structurally similar impurities commonly present in single-molecule.

3.3. Rationale

The increasing global burden of HIV infection necessitates the continued development, monitoring, and quality assurance of ARTs. Among these, ATZ and ETR are widely used antiretroviral agents in various treatment regimens, including fixed-dose combinations. However, despite their therapeutic significance, limited analytical methods have been reported for the impurity profiling and quantification of these drugs, particularly using chromatographic techniques.

Impurities, whether known or unknown, can arise during synthesis, formulation, or storage, potentially compromising the safety and efficacy of pharmaceutical products. Regulatory guidelines (e.g., ICH Q3A/B and Q2(R1)) emphasize the need for robust analytical methods to detect, identify, and quantify such impurities at trace levels. Given the structural similarities among degradation products and related substances in ATZ and ETR formulations, there exists a significant analytical challenge in achieving selective and sensitive resolution of these impurities.

Therefore, it becomes crucial to develop and validate a RP-HPLC method that can resolve these challenges. A validated method will support regulatory compliance, product quality control, and risk mitigation by ensuring that impurities remain within acceptable limits.

This research addresses a critical gap in pharmaceutical analysis by establishing a validated RP-HPLC method for the separation, identification, and quantification of impurities in marketed formulations of ATZ and ETR. The method is designed to be accurate, precise, sensitive, and time-efficient, aligning with industry requirements for routine quality control and regulatory submissions.

3.4. Original contribution by the thesis

This thesis outlines the development and validation of a novel, robust RP-HPLC method designed for the simultaneous separation and quantification of both known and unknown impurities in ATZ and ETR formulations-an area with limited prior research. **Unlike previously reported methods that primarily emphasize assay, stability studies, or method development for individual or combined drugs, this work focuses on impurity profiling with enhanced sensitivity, precision, and reduced runtime**.

From a pharmacological research perspective, most existing literature centers on pharmacokinetic studies, drug-drug interactions, and quantitative analysis in human plasma using LC-MS/MS. From a pharmaceutical sciences viewpoint, published studies have explored solubility enhancement through techniques such as spray drying, crystal engineering, and freeze drying. This research fills a critical gap by establishing a validated analytical approach capable of detecting trace-level impurities. It significantly enhances drug safety, efficacy, and regulatory compliance, offering a powerful tool for quality control in the manufacturing of anti-HIV formulations.

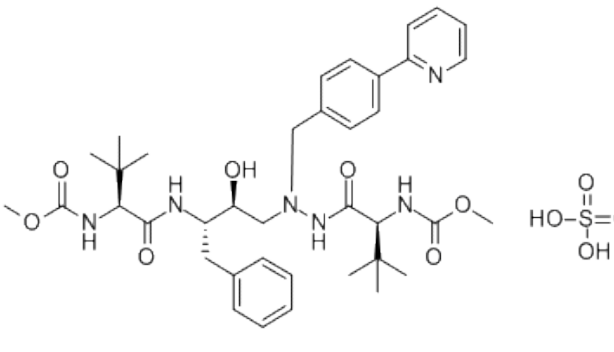
CHAPTER-4

IMPURITY PROFILING OF ATAZANAVIR SULPHATE (ATZ)

4. Introduction to drug profile [58-68]

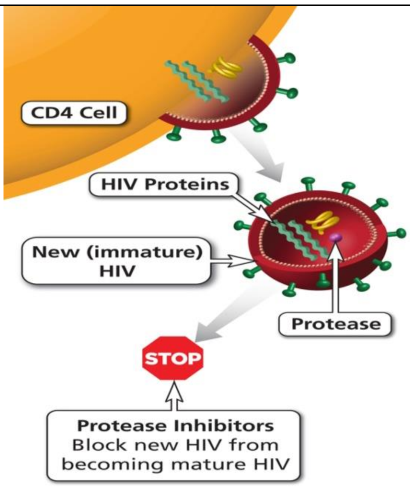
4.1 Drug profile of ATZ

TABLE 10 Drug profile of ATZ

Physicochemical Properties	
Name	**Atazanavir Sulphate**
Official in	IP, BP, EP, USP
Physical form/Appearance	It is a white to pale yellow crystalline powder with sulfate salt.
Description	ATZ is an aza-dipeptide analogue with a bis-aryl substituent on the (hydroxethyl) hydrazine moiety with activity against both wild type and mutant forms of HIV protease. ATZ does not elevate serum lipids, a common problem with other PI.
Structure	
Chemical class	Phenethylamines
BCS Class	Class II (high permeability, low solubility)
Categories/Therapeutic class	Antiretroviral, HIV (PI)

Chemical Formula	$C_{38}H_{54}N_6O_{11}S$
State	Solid
Melting point	195-209 °C
Experimental properties	**Log P:-** 4.36 **Pka (Strong Acidic) :-** 5.4 **Pka (Strong Basic) :-** 4.33
Mol. Weight	**Average:** 802.934 g/mol **Monoisotopic:** 802.35712729
IUPAC Name	(Methyl N-[(2S)-1-[[(2S,3S)-3-hydroxy-4-[[[(2S)-2-(methoxy carbonylamino)-3,3-dimethylbutanoyl]amino]-[(4-pyridin-2- yl phenyl)methyl]amino]-1-phenylbutan-2-l]amino]-3,3-dimethyl-1-oxobutan-2-yl]carbamate
Solubility	**ATZ** exhibits **limited water solubility**, with approximately **4–5 mg/ml (free base equivalent)** dissolving in water. The **pH of its saturated aqueous solution** is around **1.9 at 24 ± 3 °C**. Its solubility in various solvents is as follows: • **Dimethyl sulfoxide (DMSO):** ~104 mg/mL • **Water (H_2O):** < 1 mg/mL • **Ethanol (EtOH):** ~20 mg/mL
Approval	The U.S. FDA: - June 20, 2003 and CDSCO,2006
ATC Classification	J05AE08 (WHO)
CAS NO.	229975-97-7
ATZ** ***yellowish color and photosensitivity demand careful sample handling during analysis to avoid degradation errors. Most orally active drugs follow **Lipinski's Rule of 5**, which suggests Log P should be **≤ 5**. ATZ (Log P- 4.36) meets this, indicating **good oral drug-likeness and** A **Log P of 4.36** means the drug is **highly lipophilic**, which helps in **membrane penetration** but may result in **low aqueous solubility**, influencing its **formulation and bioavailability**.	
Pharmacological Profile	
Indications	It is administered in combination with other antiretroviral agents for the management of HIV-1 infection. Additionally, it is used as part of post-exposure

	prophylaxis (PEP) in individuals who have experienced occupational or non-occupational exposure to potentially infectious body fluids from a confirmed HIV-positive source, where such exposure carries a significant risk of HIV transmission.
Classes	HIV PI; Azapeptide inhibitor of HIV-1 protease
Pharmacodynamic	**ATZ** is an **azapeptide-based HIV-1 PI** with potent activity against **HIV-1**. The **HIV-1 protease enzyme** plays a critical role in the **cleavage of viral polyprotein precursors** into functional proteins essential for the assembly of infectious viral particles. **ATZ binds to the active site** of the protease enzyme, thereby **inhibiting its catalytic function**. This inhibition blocks the processing of polyproteins, leading to the production of **immature, non-infectious viral particles**. PI including ATZ, are typically administered in **combination with at least two other antiretroviral agents** as part of **HAART**. Although **pharmacologically related** to other protease inhibitors, **ATZ has a distinct chemical structure** compared to other PIs and currently available antiretroviral drugs.
Mechanism of action	HIV protease is a key viral enzyme that mediates the cleavage of precursor polyproteins into essential structural components and enzymes necessary for the maturation and assembly of infectious virions. PIs function by binding to this enzyme, thereby blocking the proper processing of viral proteins. As a result, the formation and release of mature, infectious HIV particles from host cells is effectively inhibited.

	 **FIGURE 12 Mechanism of PI**
Side effects	Fever, nausea, vomiting, abdominal discomfort, diarrhea, headache, and muscle pain. Some individuals may also experience mood disturbances such as depression, along with sleep-related issues like insomnia. Neurological symptoms can include numbness, tingling sensations, or burning pain in the hands and feet. Additionally, redistribution or abnormal accumulation of body fat may occur, affecting areas such as the arms, legs, face, neck, breasts, and waist.
Adverse drug effects	Possible adverse effects may include a mild skin rash characterized by redness and itching, CKD,kidney stone formation, and gallbladder-related disorders. Metabolic disturbances such as diabetes mellitus and elevated blood glucose levels (hyperglycemia) may also occur. In rare cases, serious complications like lactic acidosis, severe liver enlargement (hepatomegaly), and fatty liver infiltration (hepatic steatosis) can develop.
Contraindication	Underlying or associated conditions may include chronic hepatitis B and C infections, diabetes mellitus, and metabolic acidosis resulting from elevated lactic acid levels (lactic acidosis). Other potential concerns include hemophilia, atrioventricular block—a cardiac conduction disorder characterized by a slow heart rhythm—and the

	presence of gallstones (cholelithiasis).
Dose	A dosage of 300 mg is administered once daily, in combination with a pharmacokinetic booster—ritonavir at a reduced dose of 100 mg once daily.
Pharmacokinetic Profile	
Absorption	Its absorption is significantly influenced by gastric pH levels.
Protein binding	Approximately 86% of the drug binds to human serum proteins, primarily α1-acid glycoprotein, and albumin. This protein binding remains consistent and does not vary with concentration.
Distribution	The drug exhibits 86% binding to human serum proteins, and this binding remains constant across varying concentrations.
Metabolism	**ATZ** undergoes **extensive hepatic metabolism**, primarily mediated by the **cytochrome P450 3A4 (CYP3A4) enzyme**. The predominant metabolic pathways include **mono-oxygenation and di-oxygenation** reactions. Additionally, **minor biotransformation routes** involve **glucuronidation, N-dealkylation, hydrolysis**, and **oxygenation accompanied by dehydrogenation**. **In vitro studies** using **human liver microsomes** confirm that **CYP3A enzymes**, particularly **CYP3A4**, play a significant role in the metabolic processing of ATZ.
Excretion	Approximately **20% of the administered dose of Atazanavir** is excreted as **unchanged drug in the feces**, while around **7% is recovered unchanged in the urine**.
Excretion half life	The **elimination half-life of Atazanavir** in **healthy adults and HIV-infected patients** is approximately **7 hours** when administered as a **400 mg dose with a light meal**. In individuals with **hepatic impairment**, the half-life is **prolonged to around 12.1 hours** following a single 400 mg dose.

Tmax	2.5 Hours
Bioavailability	60-68%

4.2 List of marketed formulation of ATZ and its combination or combined dosage forms [60,69]

TABLE 11 List of marketed formulation

Brand name of the dosage form	Name of the company	Dosage form	Dose
Atazor	Emcure (ARV)	Capsule	100 mg, 150 mg, 200 mg, 300 mg
Atavir	Cipla	Capsule	100 mg, 150 mg, 200 mg, 300 mg
Virataz	Hetero HC (GenX)	Capsule	300 mg
Reyataz	Bristol-Myers Squibb	Capsule	150 mg, 200 mg, 300 mg

TABLE 12 List of combination formulations of ATZ

Brand name of the dosage form	Name of the company	Dosage form	Strength of the dosage form
Synthivan	Cipla	Tablet	ATZ 300 mg, Ritonavir 100mg
Virataz – R	Hetero HC (GenX)	Tablet	ATZ 300 mg, Ritonavir 100 mg
Aztanavir-R	**Cipla**	Tablet	ATZ 300 mg + Ritonavir 100 mg

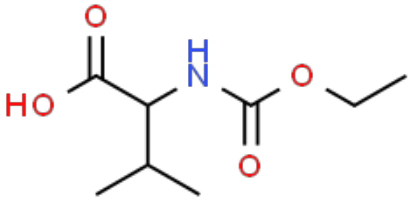
4.3 List of known impurities profile [70,71]

A wide range of **process-related and degradation impurities** have been identified in **Atazanavir (ATZ)**, including **diastereomers, isomers, synthetic intermediates, and related analogues**. These impurities can arise from synthetic pathways, degradation, or stereoisomeric variations. Key impurities associated with ATZ include:

- **Structural Isomers & Diastereomers**: ATZ S,S,R,S-Diastereomer, ATZ R,S,S,R-Diastereomer, ATZ R,S,S,S-Diastereomer, ATZ R,R,R,R Isomer, ATZ S,S,S,R-Diastereomer
- **Synthetic Analogues & Degradation Products**: ATZ Di-tert-butyl Analogue, Dealkyl ATZ, ATZ Diol Impurity, ATZ Hydrazine Analog Trihydrochloride (including RS form), ATZ Benzylidenehydrazine Carbamate (RSS), ATZ Benzylidenehydrazine Analogue (RS), ATZ-D5
- **Officially Designated Impurities** (based on pharmacopeial or industrial references): ATZ Impurity 1, ATZ Impurity 5, ATZ Impurity 6 (4R,5S-Diastereomer of DIBOC), ATZ Impurity 7, ATZ Impurity 8, ATZ Impurity 9 (4R,5R-Diastereomer of DIBOC), ATZ Impurity 10 (4S,5R-Diastereomer of DIBOC), ATZ Impurity 14, ATZ Impurity 16, ATZ Impurity 17 (RSSSS), ATZ Impurity 18 (SRS), ATZ Impurity 19
- **Mixtures and Racemic Forms**: rac-ATZ Impurity, Rs8 + Rs9 (Mixture), ATZ RS3, RS8, RS9, RS12 Impurities
- **Process-Related Aldehyde Impurity**: 5-(Hydroxymethyl)furan-2-carbaldehyde

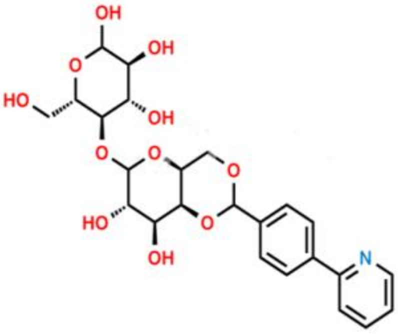
4.3.1 Impurities used in this project

TABLE 13 ATZ related compound A (Impurity 1)

ATZ related compound A		
1.	**CAS no.**	162537-11-3
2.	**Molecular structure**	

3.	**Molecular formula**	$C_8H_{15}NO_4$
4.	**Molecular weight**	189.21
5.	**CAT no.**	SZ-A009003
6.	**Category**	USP standards

TABLE 14 ATZ Impurity 5 (Impurity 2)

ATZ Impurity 5		
1.	**CAS no.**	857900-54-0
2.	**Molecular structure**	
3.	**Molecular formula**	$C_{24}H_{29}NO_{11}$
4.	**Molecular weight**	507.5
5.	**CAT no.**	SZ-A009D01
6.	**Category**	USP standards

4.4 Materials and Instrumentals Specification

4.4.1 Apparatus and Instruments

TABLE 15 Apparatus and Instruments List

Sr. No.	**Name**	**Description**
1.	UV Spectrophotometer	Systronics 119
2.	HPLC	**Shimadzu [with power stream]** **Column**: - C18 Hypersil BDS (250 mm × 4.6 mm , 5µm) **Pump**: - LC-20 AT **Syringe**: - Rheodyne injector valve with 20.0 µl loop **Detector: -** PDA 600 UV Detector

		Software: - Spinchrom
3.	pH Meter	Systronics PH361
4.	Digital Balance	Mettler Toledo ML 204
5.	Glass wares	Borosile
6.	Ultrasonicator	Toshcon
7.	Melting Point Apparatus	Veego VMP-01

4.5 Reagents and Materials

All chemicals and reagents used in the present study were of high analytical quality to ensure accurate and reliable chromatographic analysis. The materials employed for the experimental work are listed below **Table 16**

TABLE 16 List of Reagents and Materials of ATZ

Sr. No.	Name	Description
1.	Water	HPLC Grade
2.	Methanol	HPLC Grade
3.	ACN	HPLC Grade
4.	KH_2PO_4	HPLC Grade
5.	NaOH	AR Grade
6.	HCl	AR Grade

4.6 Detail of Drug Procurement

ATZ standards procured from Emcure pharmaceuticals limited, Ahmedabad and it’s both impurities were obtained from Medvin pharma, Ahmedabad

TABLE 17 Drug Procurement Detail of ATZ

Sr. No.	Name	Source
1.	**ATZ**	Emcure pharmaceuticals limited, Ahmedabad
2.	**Impurities A**	Medvin pharmaceutical limited, Ahmedabad
3.	**Impurities 5**	Medvin pharma, Ahmedabad

4.7. Identification of Drugs

4.7.1 Identification by Melting Point

Procedure: - Finely powder the ATZ sample using a mortar and pestle to ensure uniform heating. Dry it if moisture is suspected. Take a clean, thin-walled capillary tube (sealed at one end) and fill 2–3 mm of the powder into the bottom by tapping. Place the capillary tube in a **melting point apparatus** (e.g., **Thiele tube**, **digital melting point apparatus**, or **hot-stage microscope**). Start heating slowly (2–3°C/min) near the expected melting point (ATZ melts around **205–210°C**, depending on crystal form). Observe when the powder **starts melting** and when it **completely liquefies**. Melting point observed and compared with the reference in the given below **Table 18.**

TABLE 18 Melting Point comparison of ATZ

Sr. No.	Drug	Reported Melting Point	Observed Melting Point
1.	**ATZ**	195-209°C [60]	194 – 207 °C

4.7.2 Identification by Solubility

Procedure: - Take sample of ATZ. Add 10 mL of the test solvent like water, methanol, 0.1 N HCl and 0.1 N NaOH in a different test tube respectively. Shake manually or stir with a magnetic stirrer at room temperature for 15–30 minutes. Observe visually: clear solution, turbidity, or undissolved residue. Use USP/IP solubility terms given in **Table 19**.

- **Note if the test is done at 25°C or 37°C.**

TABLE 19 Solubility Determination of ATZ

Solvent	Solubility	Criteria (Parts of solvent per 1 part solute)
Water	Slightly soluble	100 to 1000
0.1 N NaOH	Slightly soluble	100 to 1000
ACN	Slightly soluble	100 to 1000
Methanol	Soluble	10 to 30
Ethanol	Soluble	10 to 30
0.1 N HCl	Insoluble	More than 10,000

4.7.3 Identification by IR Spectra

Accurately weigh about 1-2 mg of ATZ and add ~100-200 mg of dry, IR-grade KBr to the sample. Grind finely and mix the sample-KBr mixture to form a uniform powder. Compress the mixture in a hydraulic press at ~7 to 10 tons pressure for 2-3 minutes to form a transparent pellet/disc. Place the pellet in the IR sample holder and scan the sample from

1800 cm^{-1} to 600 cm^{-1} using an FTIR spectrometer. This disc was scanned in the region of 1800-600 cm^{-1} in FTIR and obtained IR spectrum was compared with the reference spectrum of ATZ. Record the spectrum and compare it with the reference spectrum or standard monograph. Following peaks were observed. Interpretation of functional groups such as- NH, –OH, C=O, C–N, and C–H by their characteristic absorption peaks in the given **Figure 13.**

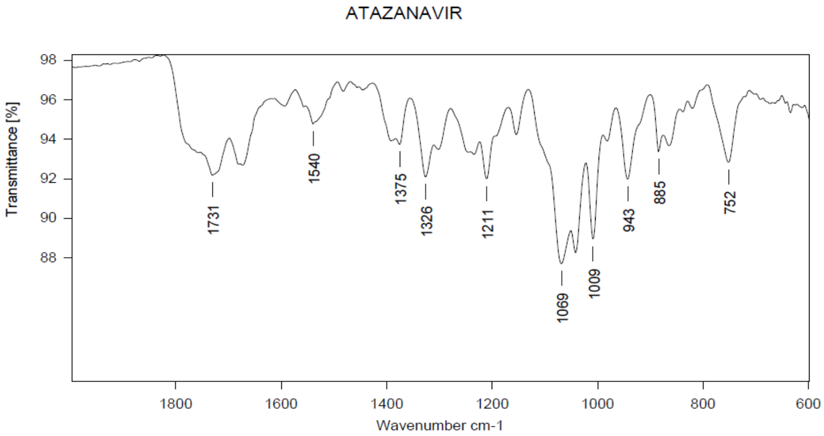


FIGURE 13 Interpretation of Infrared (IR) Spectral Data for ATZ Sample

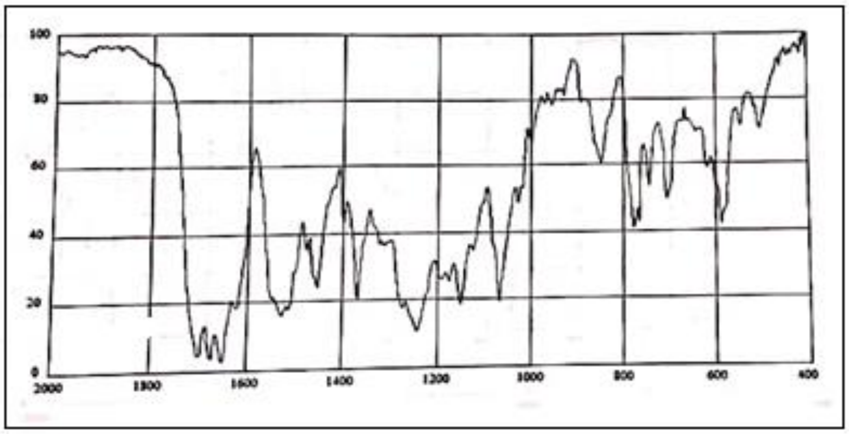


FIGURE 14 Interpretation from IR spectra of standard ATZ [72]

TABLE 20 Interpretation from IR spectra of sample ATZ

Type of vibration	Observed frequency value (cm^{-1})	Standard Value (cm^{-1}) [72]
C=O stretching	1731	1870-1540
C=C stretching	1540	1670-1600
N-H bending	1540	1650-1580
C-O Stretching	1069	1400-1000

S=O / C–N	1009	1060-1080

4.8 Selection of wavelength

- Turn on UV-Vis spectrophotometer and allow warm-up (~15 minutes). Use blank solvent to baseline the instrument.
- A standard stock solution of ATZ was prepared by dissolving 50 mg of ATZ in 100 ml of methanol to obtain a concentration of 500 µg/ml. From this, 1 ml was further diluted with methanol to achieve a working solution of 50 µg/ml.
- The prepared solution was transferred into a clean quartz cuvette, and the spectrum was scanned over the range of 200–400 nm using a UV–Visible spectrophotometer. The wavelength of maximum absorbance (λmax) was recorded for further analysis. Repeat the scan separately for Impurity A and Impurity B.
- Record λmax of each impurity. Compare spectra for overlapping or distinct peaks. (Wavelength was selected from the overlay spectra of above solutions).
- ATZ and its impurities A and B both show reasonably good response at 225 nm in Methanol. 225 nm is found as λmax. Hence, 225 nm wavelength has been chosen **Figure 15** for quantification of impurities due to satisfactory sensitivity and optimum response.

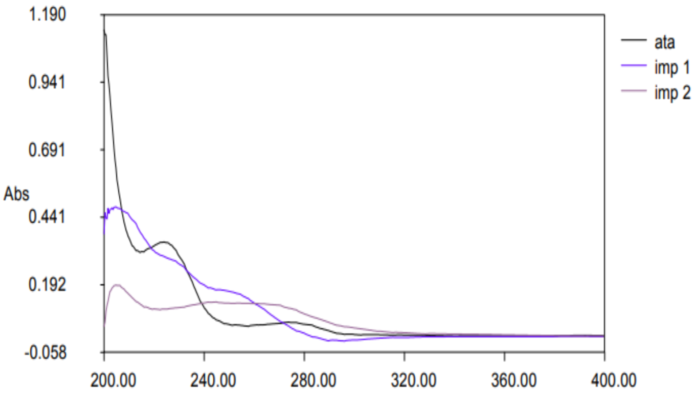


FIGURE 15 ATZ and its impurities for selection of wavelength

4.9 Selection of Impurities [30-57,74]

In previously reported analytical methods, **potential impurities of ATZ** were **not effectively separated from the main analyte**, particularly in **fixed-dose formulations**, even though these impurities are commonly present and have shown **genotoxic potential**.

This represents a significant limitation in ensuring the safety and regulatory compliance of ATZ-based products.

To date, there are **no comprehensive liquid chromatographic methods (HPLC or UPLC)** specifically reported for the **separation and quantification of ATZ impurities** in such fixed-dose combinations.

For this study, a **RP-HPLC** system was selected due to its advantages in terms of **sensitivity, selectivity, speed of analysis**, and **eco-friendliness** resulting from **lower solvent consumption**. RP-HPLC is also well-suited for routine pharmaceutical quality control.

In this research, **two specific impurities were selected for profiling** and quantification:

- **Impurity A (Designated as Impurity 1)**
- **Impurity 5 (Designated as Impurity 2)**

The development and validation of an RP-HPLC method targeting these impurities aim to overcome existing analytical challenges and contribute to safer pharmaceutical product monitoring.

4.10 Identification of impurity of ATZ-A

4.10.1 Mass Spectroscopy

The MS was performed on the **SCIEX Triple Quad 5500+** mass spectrometer instrument with the ESI method in a positive ion mode with turbo ion spray as an interface. Different parameters like IS (Ion Source) =55,00 V, DP (Declustering Potential) = 60 V, and EP (Entrance Potential) = 10 V, were set along with the pressure of 50 and 60 psi of air and nitrogen as nebulizer and curtain gas to acquired mass spectra. **The obtained MS spectrum was recorded and compared (M+) peak with molecular weight.**

This Given image displays a mass spectrometry (MS) match plot for a sample labelled "**ATZ impurity-A**” Here is a breakdown of the key elements in the plot:

1. Mass-to-Charge Ratio (m/z):

- The x-axis represents the mass-to-charge ratio (m/z), which is a critical parameter in MS for identifying compounds.
- The primary peaks observed are at **m/z 189.09** and **m/z 147.07**.

    - **Base peak: - m/z 189.09 is the base peak,** indicating it is the most intense ion detected and likely corresponds to the primary fragment or impurity of interest.
    - **Molecular Ion Peak: -m/z 147.07** represents a secondary fragment or impurity.

2. Intensity:

- **The y-axis denotes the intensity of the detected ions.**
- The base peak (m/z 189.09) shows the highest intensity, suggesting it is the **most abundant ion in the sample.**

3. Molecular Structure:

- The chemical structure displayed corresponds to the compound or impurity detected at the base peak.
- **It includes functional groups such as:**
    - **Hydroxyl group (OH)**
    - **Amide linkage (NH)**
    - **Ester group (COO)**
- These functional groups are typical in pharmaceutical compounds and can indicate potential degradation products or synthesis-related impurities.

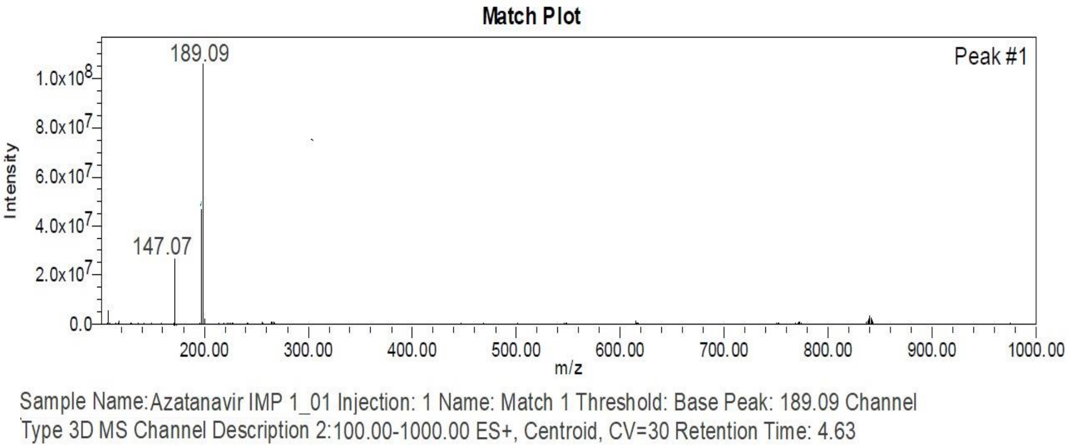
4. Interpretation (Figure 16):

- The presence of a significant peak at m/z 189.09 suggests a primary impurity or degradation product in the ATZ sample while 147.07 (M+) was an impurity-A
- The structure provided helps in understanding the possible pathways of impurity formation, such as esterification, amide hydrolysis, or oxidation.

5. Applications:

- This type of analysis is crucial for impurity profiling and quantification, ensuring drug safety and efficacy, especially in anti-HIV medications like ATZ.

- Such analysis is critical in impurity profiling for pharmaceuticals to meet regulatory guidelines (like ICH Q3A/B for impurities).
- The identified structure aids in understanding the origin of the impurity, whether from degradation, side reactions, or residual solvents.



Sample Name: Azatanavir IMP 1_01 Injection: 1 Name: Match 1 Threshold: Base Peak: 189.09 Channel Type 3D MS Channel Description 2:100.00-1000.00 ES+, Centroid, CV=30 Retention Time: 4.63

FIGURE 16 Mass spectrum of ATZ and its impurity-A

4.10.2 NMR Spectroscopy

NMR Spectroscopy: The **^{1}H and ^{13}C NMR spectra** of the impurities were recorded using nuclear magnetic resonance (NMR) spectroscopy. The **chemical shifts** obtained from both experiments were expressed on the **δ scale (ppm)**, using **tetramethylsilane (TMS)** as the internal reference standard.

1. Acquisition Parameters:

- **Instrument:** AvanceNeo Ascend 400 MHz (NMR spectrometer).
- **Solvent:** Methanol-d4 (MeOD), which eliminates interference from exchangeable protons (e.g., -OH or -NH).
- **NS (Number of Scans):** 32, which ensures adequate signal-to-noise ratio.
- **Temperature:** Not explicitly stated but typically ambient unless specified.
- **Processing Parameters:**
    - **LB (Line Broadening):** 0.30 Hz, ensuring good resolution for the peaks.
    - **SF (Spectrometer Frequency):** 400 MHz, suitable for routine analysis.

2. Interpretation:

- The spectrum provides detailed information about the proton environment in the impurity molecule.
- Peaks at **0.92–0.99 ppm** correspond to aliphatic CH_3 or CH_2 groups in the structure.
- Peaks between **3.14 and 4.29 ppm** indicate protons in close proximity to electronegative atoms like oxygen (e.g., -CH-O) or amide nitrogen (-NH).

3. Chemical Structure:

- The structure of the impurity is shown at the top left. Key functional groups include:
    - **Carboxylic acid (-COOH)**
    - **Amide (-CONH-)**
    - **Ester (-COOEt)**

These groups suggest that this impurity is likely a by-product or degradation product of ATZ

4. NMR Spectrum:

- **X-Axis (ppm):**
    - The chemical shifts range from 0 to ~16 ppm.
    - **Key peaks are observed at:**
        - **0.92 to 0.99 ppm:** These are likely due to **aliphatic protons** (e.g., methyl or methylene groups).
        - **3.14 to 4.29 ppm:** These correspond to protons attached to electronegative atoms (e.g., -CH groups near oxygen or nitrogen).
    - The absence of peaks in the downfield region (~6-12 ppm) indicates no aromatic or strongly deshielded protons.
- **Y-Axis:**
    - The intensity of signals indicates the abundance of corresponding protons.

5. Applications:

- **Impurity Profiling:** Identifying and quantifying impurities is crucial for ensuring drug safety and compliance with regulatory standards (e.g., ICH guidelines).
- **Structural Confirmation:** The NMR spectrum confirms the structure of Impurity-1 and helps in understanding its formation pathway during synthesis or storage.

Quality Control: This analysis can be used to monitor the levels of Impurity-1 during production and stability studies.

4.10.3 FT-IR Spectroscopy

1. X-Axis: Wavenumber (cm^{-1}):-The x-axis represents the **wavenumber**, which corresponds to the frequency of vibrational modes in the molecule. It ranges from **4000 cm^{-1} to ~400 cm^{-1}**, covering the typical IR region.

2. Y-Axis: Transmittance (%):-The y-axis represents **transmittance**, which measures how much infrared light passes through the sample.

Peaks represent regions where the molecule absorbs IR radiation due to bond vibrations.

FTIR spectrum and chemical structure of an impurity related to ATZ. **Figure 17** Characterized using FTIR with functional group correlation based on structure. **Spectrum shows key absorptions at ~3330.4 cm^{-1} (O–H/N–H), ~2916 cm^{-1} (C–H), ~1688.50 cm^{-1} (C=O), and ~1030 cm^{-1} (C–O), supporting carboxylic acid and amide presence.**

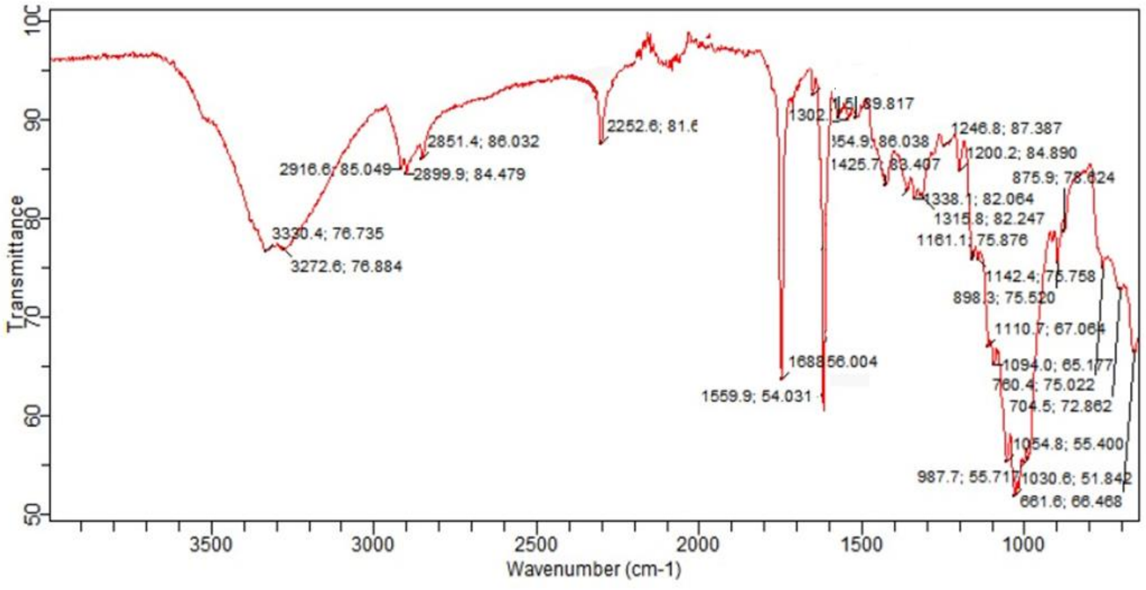
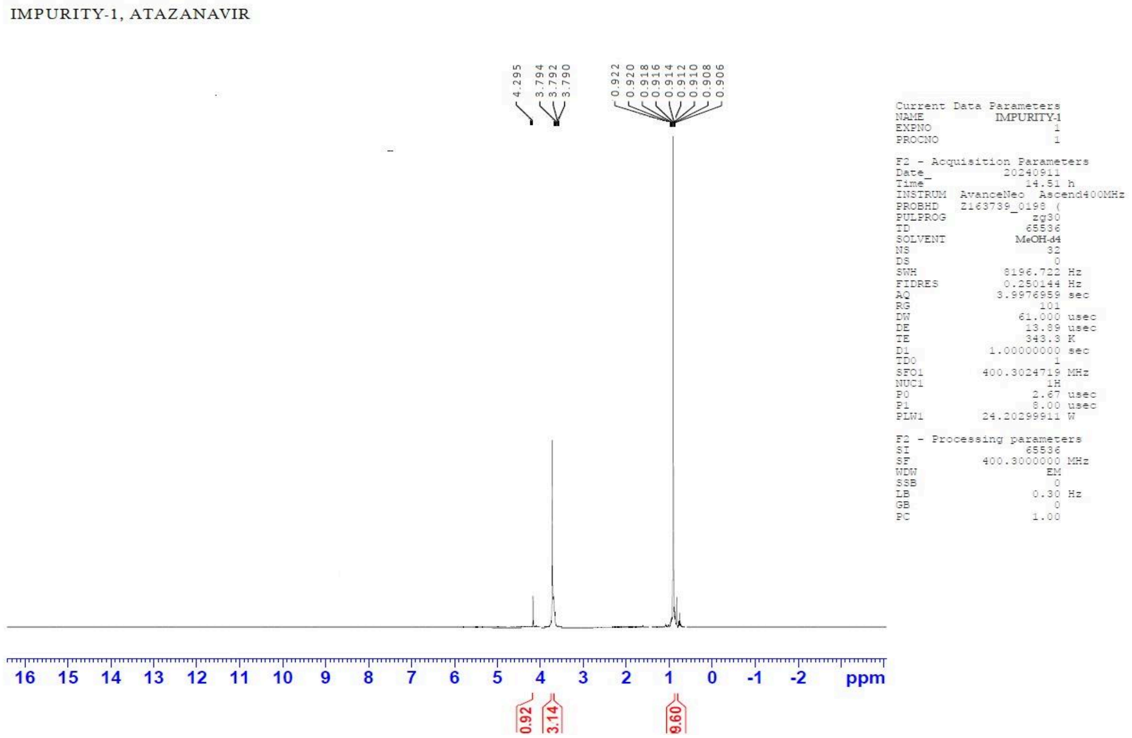


FIGURE 17 FT-IR of ATZ Impurity-A



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Current Data Parameters
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EXPNO                    1
PROCNO                   1

F2 - Acquisition Parameters
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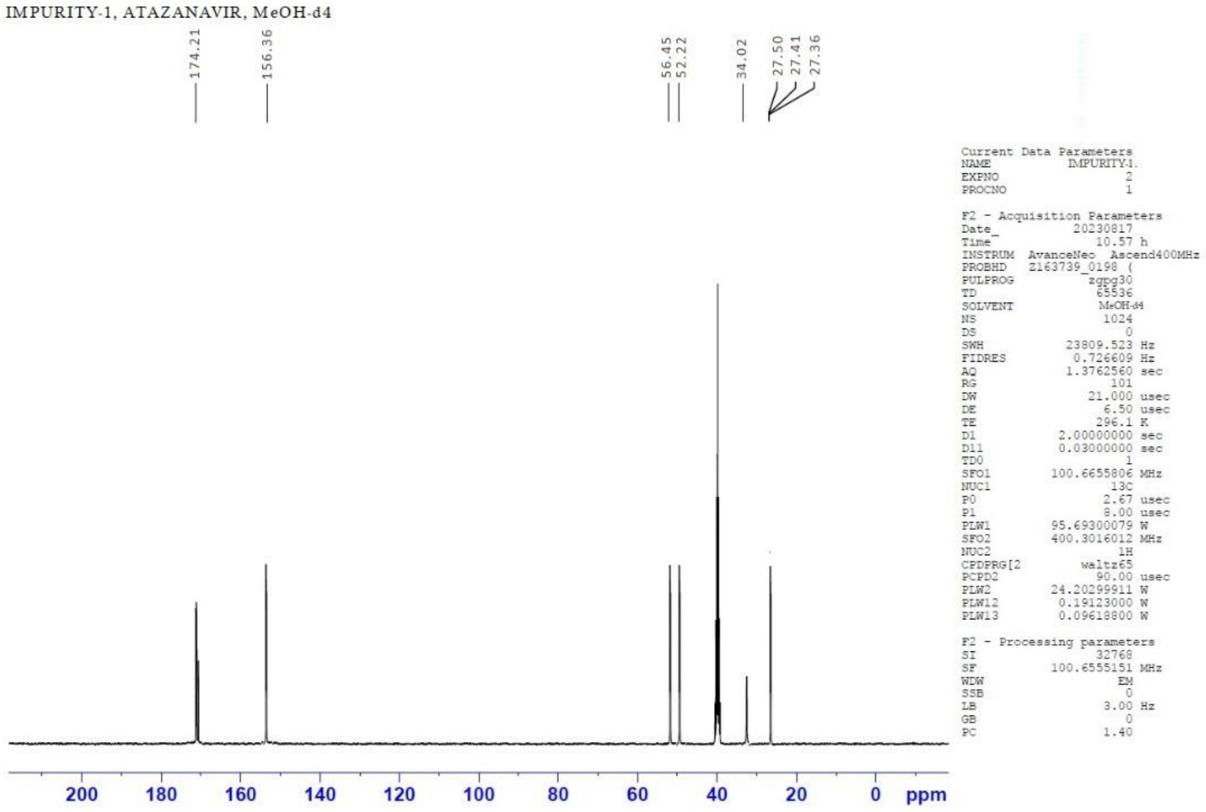
Time               14.51 h
INSTRUM   AvanceNeo  Ascend400MHz
PROBHD    Z163739_0198 (
PULPROG               zg30
TD                   65536
SOLVENT            MeOH-d4
NS                      32
DS                       0
SWH               8196.722 Hz
FIDRES            0.250144 Hz
AQ               3.9976959 sec
RG                     101
DW                  61.000 usec
DE                   13.89 usec
TE                   343.3 K
D1              1.00000000 sec
TD0                      1
SFO1           400.3024719 MHz
NUC1                    1H
P0                    2.67 usec
P1                    8.00 usec
PLW1           24.20299911 W

F2 - Processing parameters
SI                   65536
SF             400.3000000 MHz
WDW                     EM
SSB                      0
LB                    0.30 Hz
GB                       0
PC                    1.00
```

FIGURE 18 ATZ Impurity-A H1 NMR



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EXPNO                    2
PROCNO                   1

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PROBHD    Z163739_0198 (
PULPROG             zgpg30
TD                   65536
SOLVENT            MeOH-d4
NS                    1024
DS                       0
SWH              23809.523 Hz
FIDRES            0.726609 Hz
AQ               1.3762560 sec
RG                     101
DW                  21.000 usec
DE                    6.50 usec
TE                   296.1 K
D1              2.00000000 sec
D11             0.03000000 sec
TD0                      1
SFO1           100.6655806 MHz
NUC1                   13C
P0                    2.67 usec
P1                    8.00 usec
PLW1           95.69300079 W
SFO2           400.3016012 MHz
NUC2                    1H
CPDPRG[2           waltz65
PCPD2                90.00 usec
PLW2           24.20299911 W
PLW12           0.19123000 W
PLW13           0.09618800 W

F2 - Processing parameters
SI                   32768
SF             100.6555151 MHz
WDW                     EM
SSB                      0
LB                    3.00 Hz
GB                       0
PC                    1.40
```

FIGURE 19 ATZ Impurity-A C13 NMR

TABLE 21 Interpretation of ATZ Impurity-A

Assigned Group	Observed frequency (cm^{-1})	Reference frequency (cm^{-1})
N-H/ O-H stretching	**3330.4, 3272.6**	3200-3600
Secondary amine N-H stretch	**3272.6**	3300
C-H aliphatic stretch	**2851.4, 2916**	2850-2960
C-O stretching	**1688.50**	1600-1750
C-H Bending	**1425.7**	1350-1500
C-O ester	**1246**	1000-1300
C-O stretch or C-O bending	**1030**	1000-1100

4.11 Method developments (MD) of ATZ

4.11.1 Development and optimization of the RP-HPLC method

MD and optimization were carried out systematically by assessing various factors that impact chromatographic separation. Critical parameters-including buffer pH, stationary phase (column chemistry), type of organic solvent, and other chromatographic conditions were thoughtfully chosen and refined to achieve optimal results. A summary of these parameters is provided below.

4.11.2 Selection of buffer strength and pH

To achieve reproducible separation of impurities, the **buffer strength** was optimized by maintaining an appropriate concentration. A **0.05 M KH_2PO_4** buffer was selected for the initial trials, owing to its wide buffering capacity and compatibility with the analytes. Considering the presence of **amine functional groups** in both drug components, the buffer pH was initially adjusted to **4.0 ± 0.05**. Among the conditions tested, **pH 4.0** yielded improved resolution and better separation of all target peaks. Therefore, this pH was finalized to enhance **column performance** under mildly acidic conditions.

To attain **baseline resolution** and facilitate the elution of **late-eluting non-polar impurities**, **methanol** was chosen as the organic modifier. This combination resulted in satisfactory separation with **shorter retention times (Rt)**, sharp peaks, and good detector responses. Based on these findings, a **mobile phase** consisting of **methanol and 0.05 M KH_2PO_4 buffer (pH 4.0)** was selected as optimal for further analysis.

4.11.3 Evaluation of stationary phase

The selection of a suitable stationary phase plays a crucial role in achieving effective separation of impurities along with the primary drug components. Given the differences in polarity between ATZ and its related impurities, it was essential to use a column that could provide **satisfactory resolution** across all analytes. Various reverse-phase HPLC column chemistries were evaluated, including **HSS** and **Hypersil BDS** columns. Among these, the **Hypersil BDS C18 column (250 × 4.6 mm, 5 µm)** demonstrated superior performance, offering optimal separation of all critical peaks. This column features **trifunctional C18 ligand bonding**, which imparts **enhanced low-pH stability** and **ultra-low column bleed**. In addition, the **base-deactivated silica (BDS)** particle provides **high pH stability**, allowing operation over a **broad pH range**. The column also incorporates a **proprietary end-capping technique**, resulting in **excellent peak shape**, particularly for basic compounds. Based on these advantages, the Hypersil BDS C18 column was selected for the entire method development and validation process.

4.11.4 Optimization of gradient program

Due to the broad polarity range of impurities associated with Atazanavir (ATZ), **gradient elution** was selected over **isocratic elution** to achieve efficient separation. Initial method trials involved varying the composition of **buffer and methanol** while maintaining a constant **flow rate of 1.0 mL/min**. Among the various conditions tested, a suitable **gradient program** was identified that provided satisfactory separation of all target analytes, including the main drug and its related impurities. This gradient approach ensured **improved resolution**, peak symmetry, and retention time control across compounds of varying polarity.

4.12 Gradient program

TABLE 22: - Gradient program of ATZ method

Time (min)	0-7	7-20	20-25

Flow rate (ml/min)	1	1	1
% Buffer	60	15	60
% Methanol	40	85	40

4.13 Evaluation of flow rate and column oven temperature

For optimum Rts of all impurities, flow rate of 1 ml/min was adopted. To have symmetric peak shapes and optimum resolution between the impurities, column oven temperature set at 45°C**.**

4.14 Selection of detector wavelength

Impurities of ATZ show spectral absorption maxima at ~210 nm. Hence, wavelength of 210 nm has been chosen for quantification of impurities due to satisfactory sensitivity and optimum responses.

4.15 Selection of sample concentration and injection volume

Considering the solubility of ATZ in methanol, mixture of methanol and buffer (pH 4.0) in the ratio set by gradient technique at different interval time. It was set as diluents and found satisfactory solubility for impurities of ATZ. Sufficient responses were observed for impurities at 20 µl injection volume hence the same was finalized to attain reproducible area counts. In the finalized conditions, standard solutions were injected to check the system suitability.

4.16 Optimized chromatographic conditions for proposed method

TABLE 23 ATZ Proposal optimizing chromatographic method

Parameter	**Method**
Types of chromatography	Reverse phase (RP)
Mode of operation	Gradient
Stationary phase (Column)	Hypersil BDS C18 column (250 mm × 4.6 ;5 µm)
Mobile phase	Methanol: 0.05 M potassium phosphate buffer (pH 4.0)
Flow rate (ml/min)	1.0
Run time (Minutes)	15

Column temperature (^{O} C)	40
Volume of injection loop (µl)	20
Detection wavelength (nm)	210
Diluent	Methanol
Detector	PDA

4.17. Selection of Mobile phase

TABLE 24 Trials of ATZ method development and remarks

Sr. No	Mobile Phase A and B	Flow Rate (ml/min)	Ratio	Retention Time (min) ATZ	Remarks	Figure.
	Trials for ATZ by using isocratic Method					
1.	Water: Methanol	1	30:70	7.88	Single peak observed at 7.88 along with tailing	20
2.	Water: Methanol	1	20:80	7.88	Single peak observed at 7.88 along with tailing	21
3.	Water: Methanol	1	10:90	6.30	Rt reduced along with tailing	22
4.	Buffer (pH 7.0): Methanol	1	20:80	7.88	Single peak observed at 7.88 along with tailing	23
5.	Buffer (pH 6.0): Methanol	1	20:80	9.223	Single peak observed at 9.23 along with tailing	24
6.	Buffer (pH 5.0): Methanol	1	20:80	7.473	Single peak observed at 7.47 and Rt reduced	25
7.	Buffer (pH 5.0):	1	10:90	4.393	Rt reduced.	26

	Methanol					
Trials for ATZ and its impurities by using isocratic methods						
8.	Buffer (pH 5.0): Methanol	1	60:40	4.393 & 4.567	Two merge peaks observed (ATZ + Impurity 1)	27
9.	Buffer (pH 5.0): Methanol	1	60:40	4.393	Only one peak observed (ATZ). Impurity 2 peak is not observed	28
10.	Buffer (pH 5.0): Methanol	1	60:40	4.567	Only ATZ peaks detected.	29
11	Buffer (pH 5.0): Methanol	1	40:60	2.457 and 15.703	AZT Two peaks are separated and observed (ATZ + Impurity 2	30

Here above listed all the trials given in **Table 24,** we can conclude that three peaks are not separated by using isocratic method. Therefore, another option for going to take trial for separation of ATZ and its impurities peaks by gradient method.

TABLE 25 Trials for ATZ and its impurities 1 & 2 separated by using gradient method

Sr. No	**Mobile phase A: B**	**Flow Rate (ml/min)**	**Time and Mobile phase ratio**	**Retention Time (min)**	**Remarks**	**Figure.**
Gradient-1						
12.	Buffer (pH 5.0): Methanol	1	**1) 0-10 min: - 40:60** **2)10-25 min: - 25:75**	**ATZ: - 4.370** **Impurity 1: - 4.540** **Impurity 2: - 18.443**	ATZ and Impurity 1 peaks merged and impurity 2 separated at	31

					delayed.	
Gradient-2						
13.	Buffer (pH 5.0): Methanol	1	**1) 0-10 min: -50:50** **2)10-25min: - 15:85**	**ATZ: - 5.863** **Impurity 1: - 6.250** **Impurity 2: - 17.337**	ATZ and Impurity 1 peaks merged but resolution increased and impurity 2 separated at delayed	32
Gradient-3						
14.	Buffer (pH 5.0): Methanol		**1) 0-7 min: - 60:40** **2) 7-20min: - 15:85** **3) 20-25 min: - 60:40**	**ATZ: -6.320** **Impurity 1: - 7.410** **Impurity 2: - 17.807**	ATZ and Impurity 1 and impurity 2 peaks are separated. Rt is so long for impurity 2	33
	Gradient-4					
15.	Buffer (pH 4.0): Methanol	**1**	**1) 0-7 min: - 60:40** **2) 7-20min: - 15:85** **3) 20-25 min: - 60:40**	**ATZ: -5.313** **Impurity 1: - 6.230** **Impurity 2: - 14.537**	Due to change of buffer pH. Three peaks are valid as per SST parameter.	34

Gradient-5						
16.	Buffer (pH 4.0): Methanol	**1**	**1) 0-7 min: - 70:30** **2) 7-20min: - 15:85** **3) 20-25 min: - 60:40**	**ATZ: -4.807**	Rt is less than the #14 trial but ATZ and impurity 1 peak merged.	35
				Impurity 1: - 5.253		
				Impurity 2: - 13.123		

Following the evaluation of multiple mobile phase combinations, a gradient RP-HPLC method using Buffer: Methanol was finalized. The selected buffer consisted of 0.05 M KH_2PO_4, prepared by dissolving 6.8 g of KH_2PO_4 in 800 ml of water, adjusting the pH to 4.0 with 0.1 N NaOH, and diluting to 1000 ml with water. This mobile phase composition provided well-defined peak shapes and satisfactory resolution of all analytes.

4.18 System Suitability Test (SST)

SST is a crucial component of any chromatographic method. These tests confirm that the system's resolution and reproducibility meet the necessary criteria for reliable analysis. The principle behind system suitability lies in evaluating the complete analytical setup including instruments, software, operational procedures, and sample characteristics as a unified system. By conducting these tests, one ensures that the method can deliver accurate and precise results for its intended analytical application

SST is an essential part of the analytical procedure.

The below mentioned system suitability criteria were adopted from standard solution:

A. The column efficiency as determined from ATZ and its impurities peaks is not < 2000 plate count.
B. The Tailing factor for ATZ and its impurities are not more than 2.0.
C. RSD for peak areas of six injections of the standard solution is NMT 2.0%

4.19. Experimental works of ATZ

4.19.1 Preparation of stock solution

Accurately weigh and finely powder 20 capsules. Transfer an amount equivalent to approximately 20 mg of ATZ into a 100 ml volumetric flask. Add 60 ml of methanol and sonicate the mixture for 15 minutes to ensure complete dispersion. After sonication, dilute the solution to volume with methanol. Filter the resulting solution using Whatman filter paper No. 1 to obtain a final concentration of 200 µg/ml of ATZ.

4.19.2 Preparation of standard working solution

Pipette 1 ml of the stock solution into a 10 ml volumetric flask and dilute to volume with the mobile phase to obtain a final concentration of 20 µg/ml of ATZ.

4.19.3 Preparation of mobile phase

Mobile Phase A: 0.05 M KH_2PO_4 (pH-4.0)

Weigh 6.8 g of KH_2PO_4 and transfer it into a 1000 ml beaker. Add approximately 800 ml of distilled water and stir until completely dissolved. Adjust the pH to 4.0 using 1% OPA, then dilute to the final volume with distilled water.

Mobile phase B: Methanol

Preparation of working sample solution

Take 1ml from sample stock solution into a 10 ml and make up with **mobile phase**. (ATZ-20 µg/ml)

4.20 Results: - Chromatograms of mobile phase selection trials

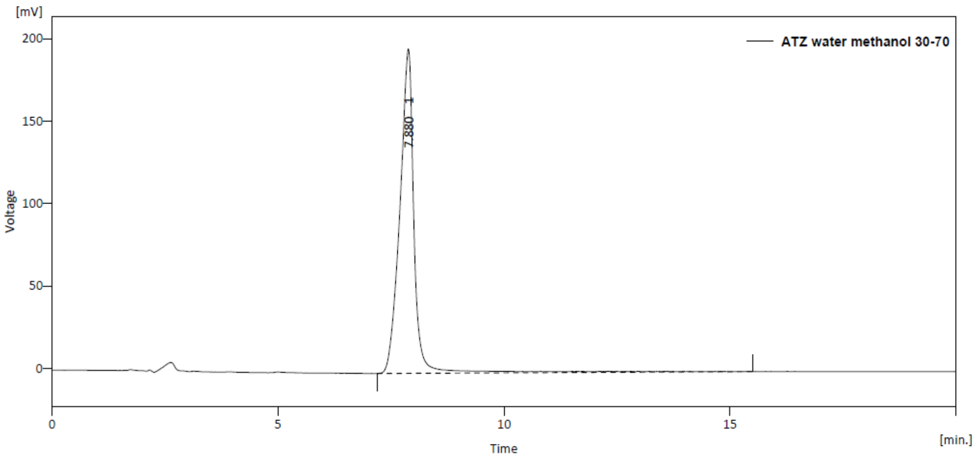


FIGURE 20 Trial 1 - Chromatogram of ATZ in Water: Methanol (30:70 v/v)

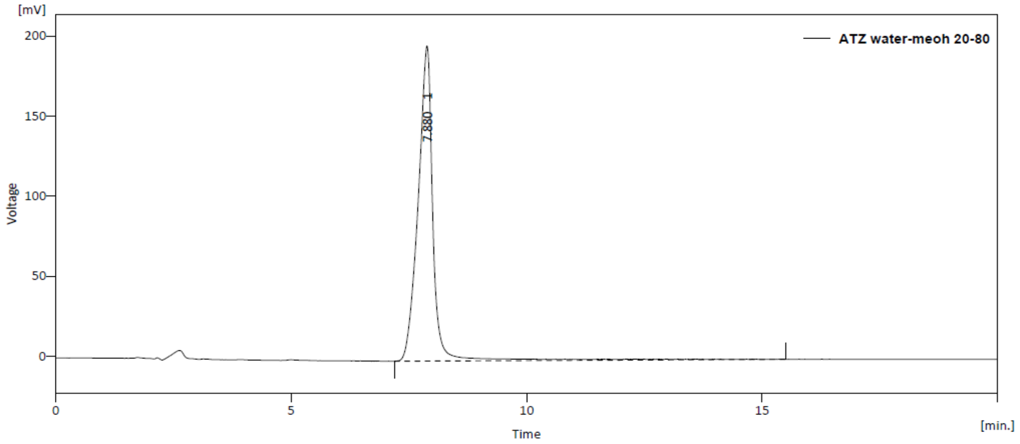


FIGURE 21 Trial 2 - Chromatogram of ATZ in Water: Methanol (20:80 v/v)

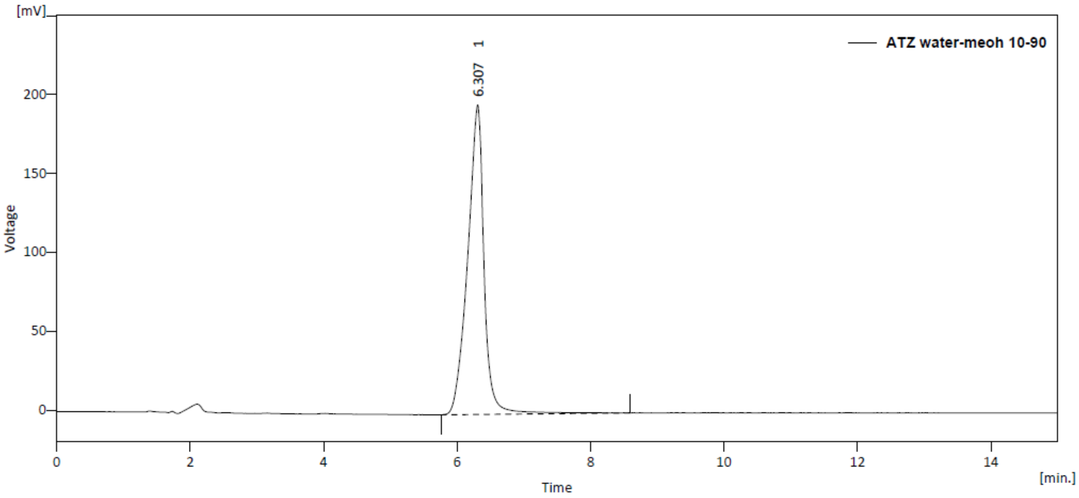


FIGURE 22 Trial 3 - Chromatogram of ATZ in Water: Methanol (10:90 v/v)

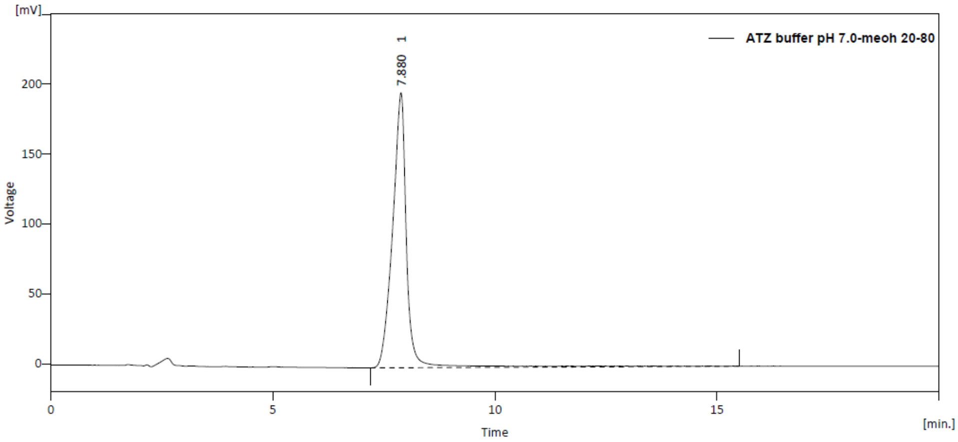


FIGURE 23 Trial 4 - Chromatogram of ATZ in Buffer (pH 7.0) : Methanol (20:80 v/v)

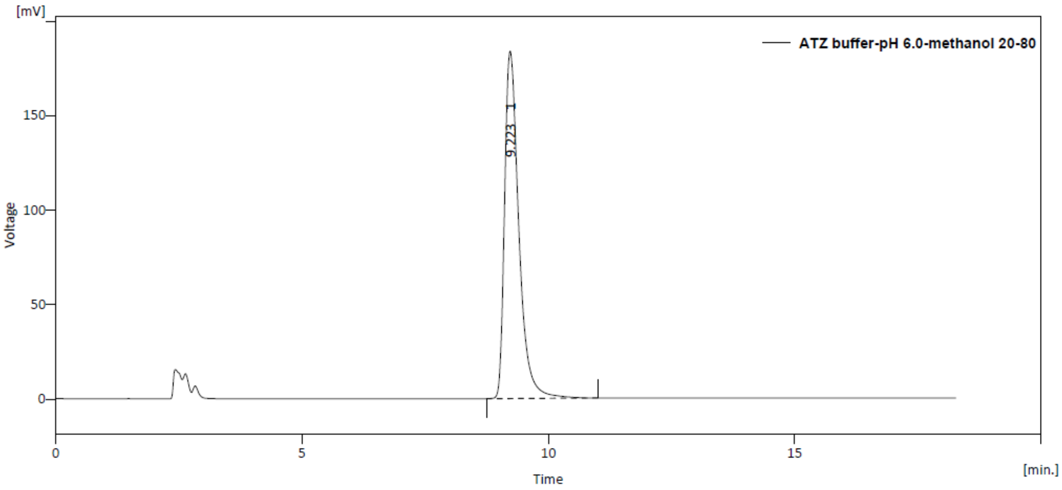


FIGURE 24 Trial 5 - Chromatogram of ATZ in Buffer (pH 6.0) : Methanol (20:80 v/v)

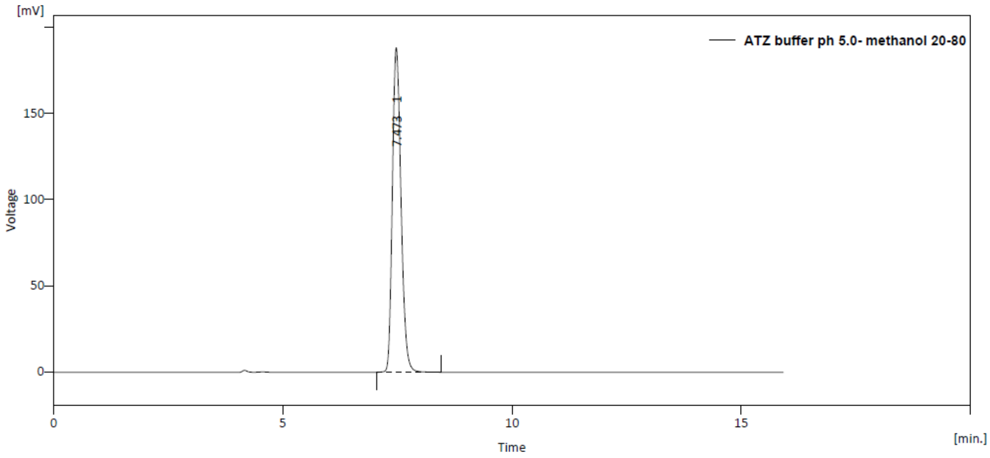


FIGURE 25 Trial 6-Chromatogram of ATZ in Buffer (pH 5.0) : Methanol (20:80 v/v)

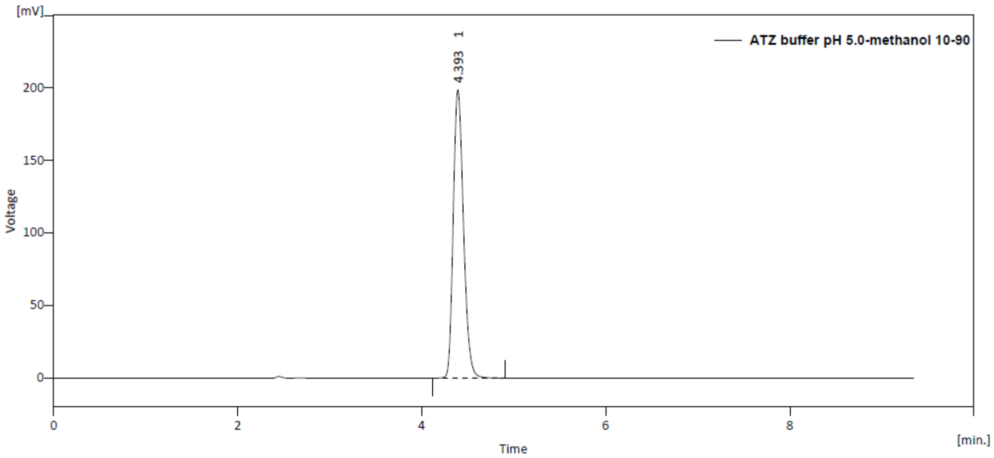


FIGURE 26 Trial 7 -Chromatogram of AZT in Buffer (pH 5.0) : Methanol (10:90 v/v)

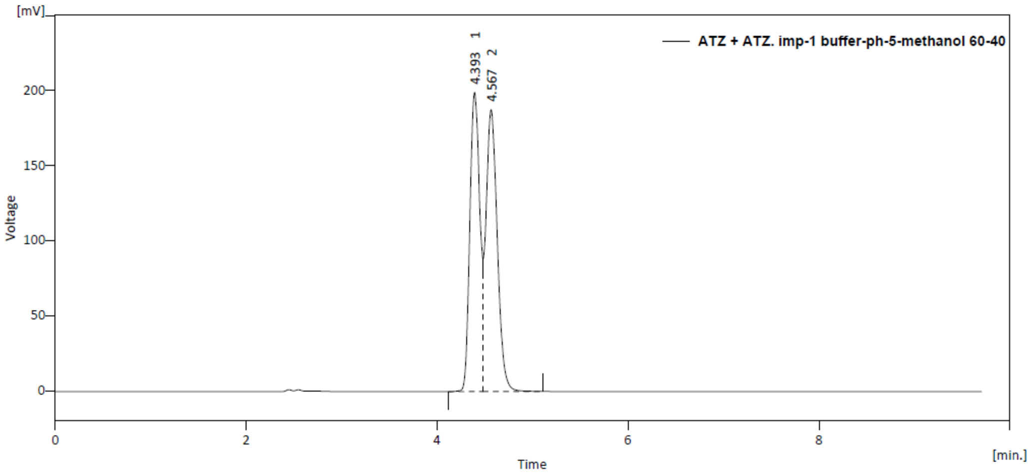


FIGURE 27 Trial 8 - Chromatogram of ATZ +Impurity 1 in Buffer (pH 5.0) : Methanol (60:40 v/v)

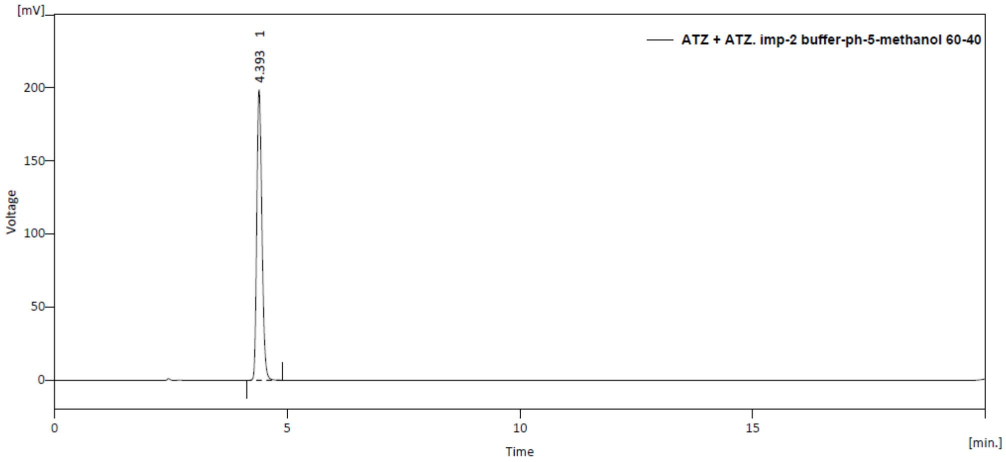


FIGURE 28 Trial 9 - Chromatogram of ATZ + Impurity 2 in Buffer (pH 5.0): Methanol (60:40 v/v)

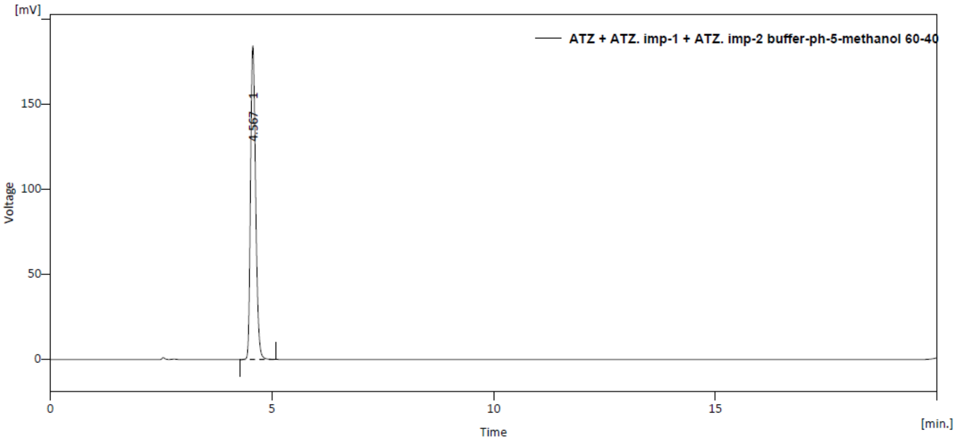


FIGURE 29 Trial 10 - Chromatogram of ATZ Impurity 1+ Impurity 2 in Buffer (pH 5.0): Methanol (60:40 v/v)

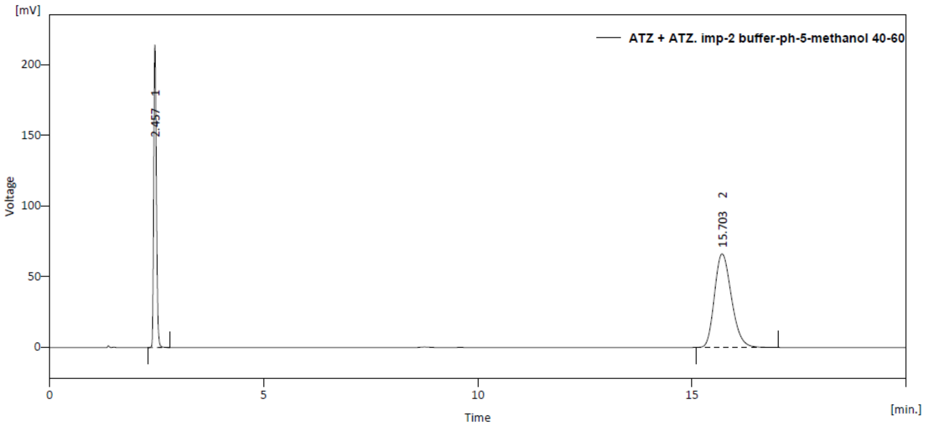


FIGURE 30 Trial 11 - Chromatogram of ATZ + Impurity 2 in Buffer (pH 5.0) : Methanol (40:60v/v)

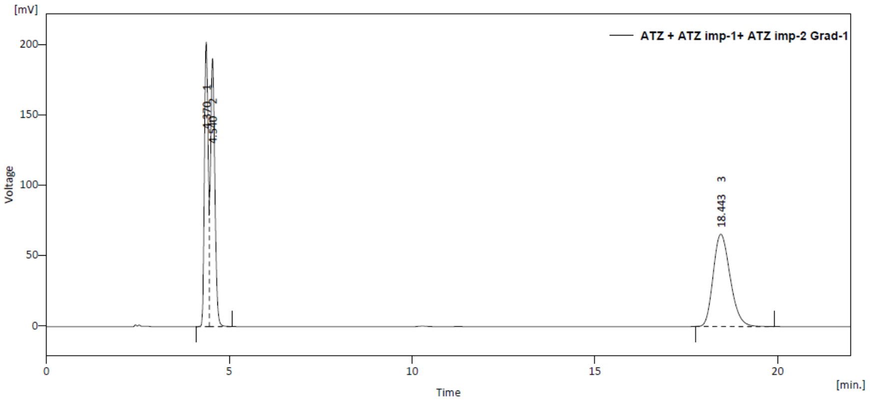


FIGURE 31 Trial 12 - Chromatogram of ATZ + Impurity 1+ Impurity 2 in Gradient 1 technique

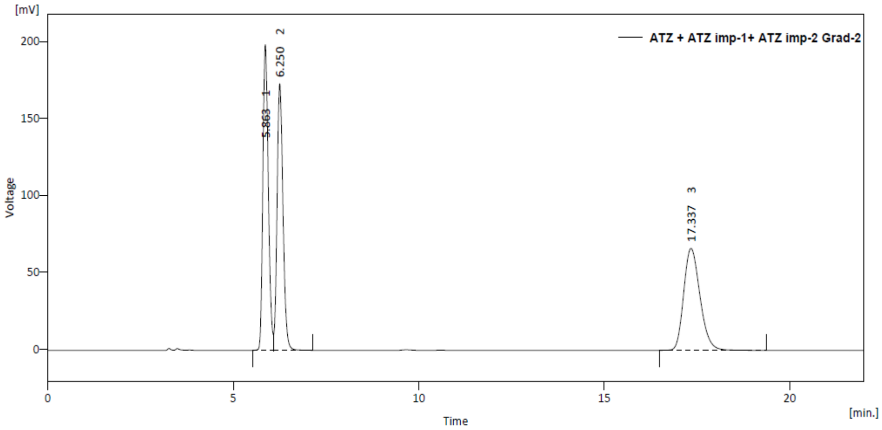


FIGURE 32 Trial 13 - Chromatogram of ATZ Impurity 1+ Impurity 2 in Gradient 2 technique

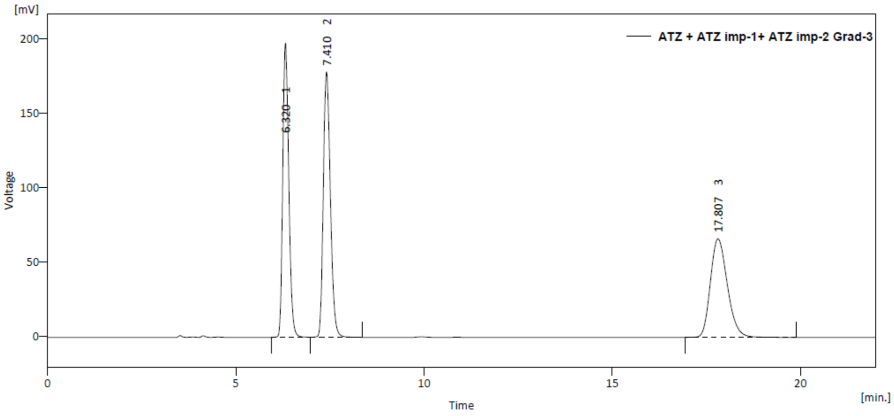


FIGURE 33 Trial 14 - Chromatogram of ATZ + Impurity 1+ Impurity 2 in Gradient 3 technique

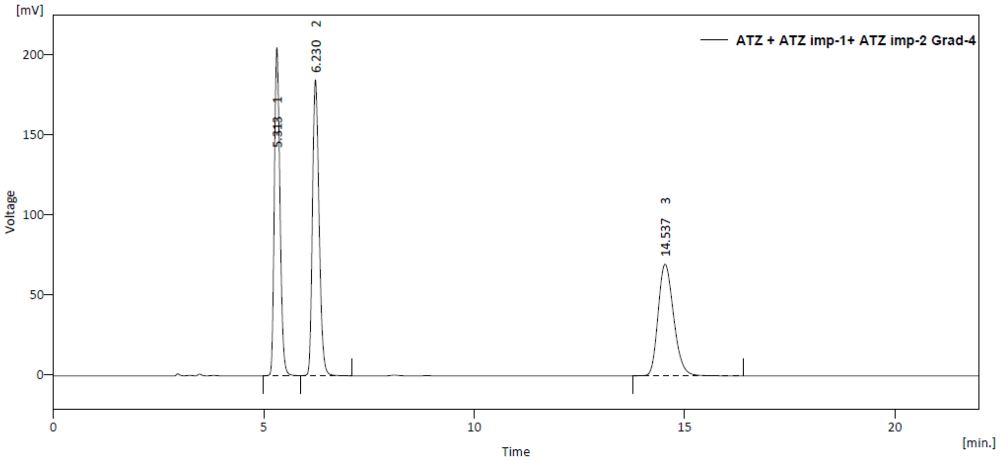


FIGURE 34 Trial 15 - Chromatogram of ATZ + Impurity 1+ Impurity 2 in Gradient 4 technique

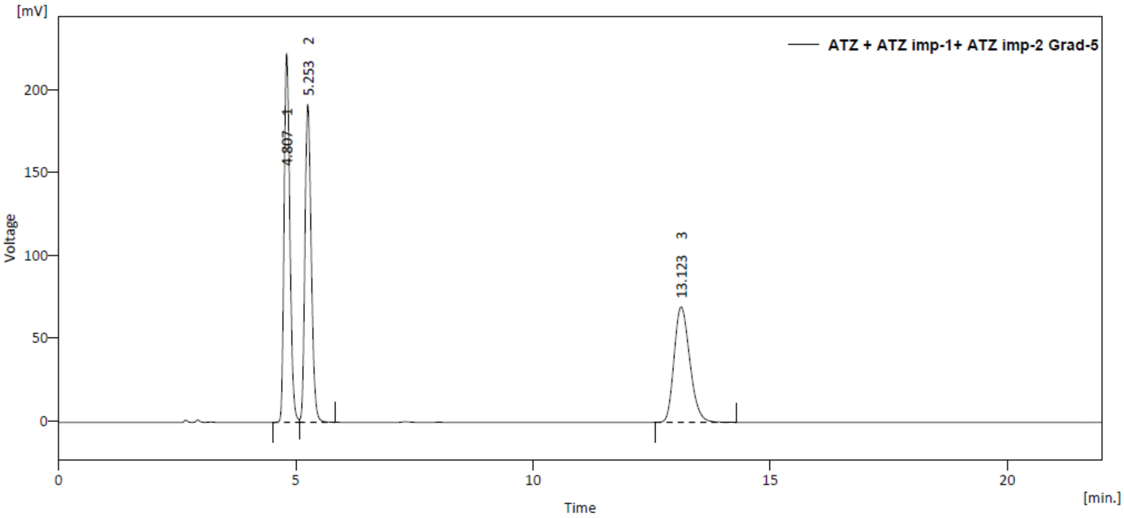


FIGURE 35 Trial 16 - Chromatogram of ATZ + Impurity 1+ Impurity 2 in Gradient 5 technique

4.21 Final Analytical Method: Analytical method was developed using HPLC Shimadzu [with power stream] gradient chromatographic technique. Data were passed through the spinchrom software. Separation was achieved on hypersil BDS C18 (250 x 4.6 mm, 5 µm) column using mobile phase composition of 0.05M potassium phosphate buffer: methanol (70:30 v/v), (15:85 v/v), (60:40 v/v), pH adjusted to 4 with 1 % OPA (OPA). Make up volume with water. Flow rate was maintained at 1 ml/min with 210 nm UV detection. The Rt obtained for ATZ was at 5.3 min. with injection volume 20 µl and the detection was made at 210 nm.

4.22 Method validation: The method was validated for its linearity range, accuracy, precision, sensitivity, and specificity. Method validation is carried out as per ICH guidelines.

4.22.1 Linearity: The linearity method was checked by preparing different types of solution of ATZ from 50% to 100%.20 µL of each solution was injected in to the HPLC system and the peak area of the chromatogram obtained was noted. Then, a linear regression equation was derived by plotting the graph between the sample dissolved and recovered by the method. From the observation and calculation are given in **Table 26**. The peak area vs. concentration data was analyzed with least squares linear regression. The slope and y-intercept of the calibration curve was reported.

TABLE 26 Linearity of ATZ, Impurities 1 and Impurities 2

		ATZ		**Impurities 1**		**Impurities 2**	
Sr. No	**Level**	**Conc. (µg/ml)**	**Response (Area)**	**Conc. (µg/ml)**	**Response (Area)**	**Conc. (µg/ml)**	**Response (Area)**
1.	**LOQ**	**0.5**	31.087	0.5	55.993	**0.2**	34.86
2.	**Linearity-1**	**2.5**	101.133	2.5	153.158	**2.5**	130.737
3.	**Linearity-2**	**3.75**	156.255	3.75	222.875	**3.75**	190.671
4.	**Linearity-3**	**5**	207.854	5	302.874	**5**	260.029
5.	**Linearity-4**	**6.25**	255.678	6.25	378.95	**6.25**	325.376
6.	**Linearity-5**	**7.5**	311.047	7.5	452.428	**7.5**	388.485
7.	**LOQ**	**0.5**	31.087	0.5	55.993	**0.2**	34.86
Slope		**41.540**		**60.369**		**150.434**	

Correlation Coefficient	**0.9997**	**0.9974**	**0.9998**

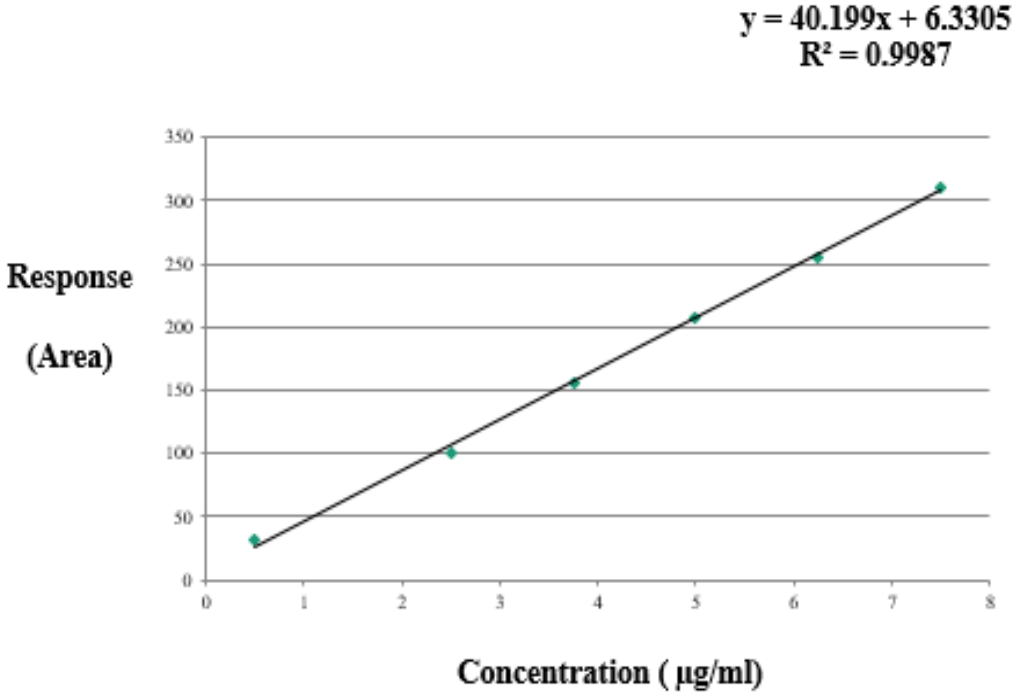


FIGURE 36 Linearity of ATZ

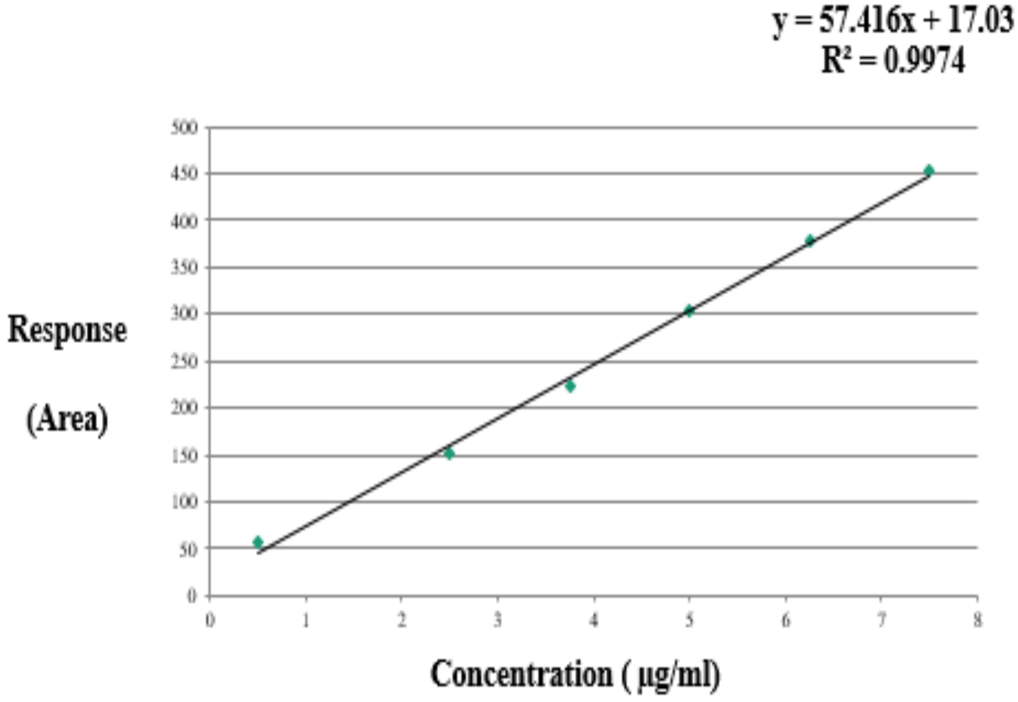


FIGURE 37 Linearity of ATZ impurity-A

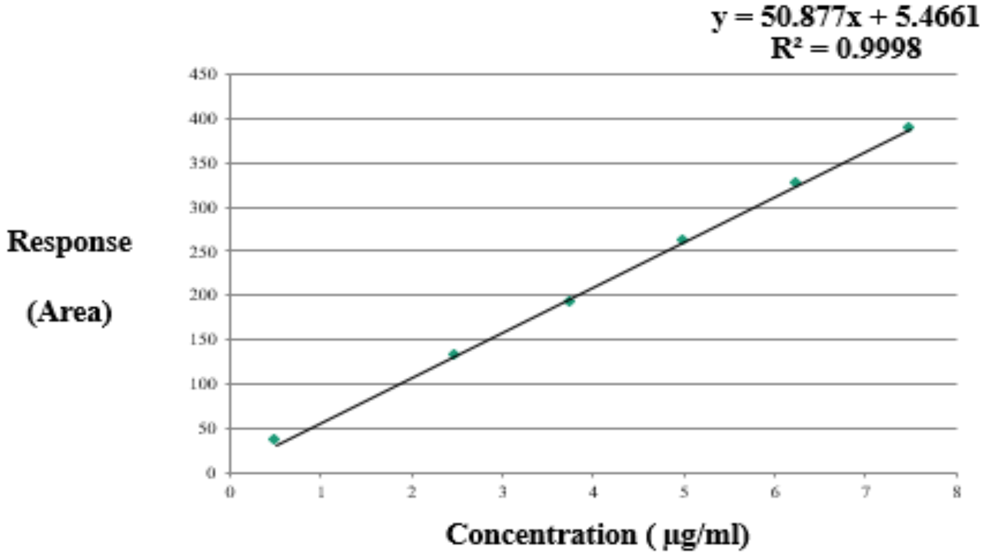


FIGURE 38 Linearity of ATZ impurity-5

4.22.2 Accuracy: - Accuracy Sample of ATZ was spiked with known impurities at five different levels: LOQ, 80%, 100% and 120% of the specification limit in triplicate (total 12 determinations) and then proceed with sample preparation as described under Methodology.

The Acceptance Criteria of Mean Recovery should be in the range of 90.0% to 110.0% for LOQ, 80%, 100% and 120% levels. Recovery studies were carried out in triplicate and the percentage recovery and standard deviation of the percentage recovery was calculated. The Mean Recovery for known Impurities is within limits. Therefore, the HPLC Method for the determination of ATZ, impurity A and Impurity 5 are accurate**.** The data are presented in the table below **Table 27** and **Table 28** for impurity A and impurity 5 respectively.

4.22.3 Precision: - The intra-day precision of the assay method was evaluated by carrying out 9 independent assays of a test sample of ATZ and its impurities at three levels (LOQ, 100% and 150%) against the qualified reference standard. The %RSD of three obtained assay values at three different concentration levels was calculated. The interday precision study was performed on three different days i.e. day 1, day 2 and day 3 at three different concentration levels (LOQ, 100% and 150%, n=3). The % RSD of three obtained assay values on three different days was calculated. The data are presented in the table below **Table 29** for intraday precision **(Ruggedness)** and **Table 30** for interday precision

4.22.4 Robustness: - The robustness of an analytical procedure refers to its ability to remain unaffected by small and deliberate variations in method parameters and provides an indication of its reliability for routine analysis. The robustness of the method was evaluated by performing the assay of ATZ and its impurities A and 5 both under different analytical conditions deliberately changing from the original condition. Slight changes in mobile phase composition, flow rate, and pH affects the chromatographic response such as Rt and peak area as given in **Table.** The % RSD obtained for peak area was 0.64-2.68 indicating that the proposed method is robust. The low % RSD values of intra-day and inter-day of ATZ, impurity A and impurity 5 for ATZ and its both impurities reveal that the proposed method is precise.

4.22.5 Repeatability: -Repeatability is demonstrated by calculating %RSD for replicate injections at the same concentration. **% RSD** is within the **acceptable limit (NMT 5.0%)** for all samples. Indicates **analytical method precision** is satisfactory for ATZ and its impurities at 5 µg/ml. **% RSD** is widely used in **chromatographic method validation** to ensure the consistency and reliability of detector response**. Values below 2% are considered excellent for API.**

4.23 Analysis of commercial formulations (Capsules):

The proposed method was applied for the determination of ATZ impurities estimation in marketed capsules (Atavir-300 mg and Atazor-300 mg) results of its impurities RSD < 5.0 % The results indicate that the method is selective for the assay of ATZ without interference from the excipients used in these capsules.

4.23.1 Stock and Sample preparation

Preparation of Stock solution of ATZ: - Weighed 50 mg of ATZ and diluted in 100 ml volumetric flasks to obtain 500 ppm solution.

Preparation of Mix Standard solution of ATZ: - Taken 1 ml of the above stock solution of ATZ and diluted up to the 10 ml volumetric flask to obtain 50 ppm solution.

Preparation of sample solution: -Weighed 50 mg of ATZ and diluted in 100 ml volumetric flasks to obtain 500 ppm solution. Again Take 1 ml of this prepared solution diluted up to the 10 ml volumetric flask to obtain 50 ppm solution.

TABLE 27 Recovery result of Impurity A

Recovery Level	Area of recovery spiked with Test	Area of imp in Test	Net area of std	Area of std	Amount added (µg/ml)	Amount recovered (µg/ml)	% Recovery	Mean ± SD	%RSD
LOQ	114.4	38.97	75.46	150.8	2.5	2.502	100.1	100.098 ± 1.788	**1.786**
LOQ	115.8	38.965	76.834	150.81	2.5	2.547	101.9		
LOQ	113.1	38.97	74.14	150.8	2.5	2.458	98.32		
80%	158.8	38.97	119.8	150.8	4	3.972	99.31	99.585 ± 1.196	**1.201**
80%	160.69	38.965	121.73	150.81	4	4.036	100.89		
80%	157.9	38.97	118.9	150.8	4	3.942	98.55		
100%	189.3	38.97	150.3	150.8	5	4.984	99.67	99.371 ± 1.175	**1.183**
100%	190.3	38.97	151.4	150.8	5	5.018	100.4		
100%	186.9	38.97	147.9	150.8	5	4.904	98.07		
120%	218.7	38.97	179.7	150.8	6	5.958	99.3	100.114 ± 0.713	**0.712**
120%	220.65	38.965	181.69	150.81	6	6.024	100.4		
120%	221.1	38.97	182.1	150.8	6	6.039	100.6		

TABLE 28 Recovery result of Impurity 5

Recovery Level	**Area of recovery spiked with Test**	**Area of imp in Test**	**Net area of std**	**Area of std**	**Amount added(µg/ml)**	**amount recovered (µg /ml)**	**% Recovery**	**Mean ± SD**	**% RSD**
LOQ	114.43	38.965	75.463	150.81	2.5	2.502	100.08	100.098 ± 1.788	**1.786**
LOQ	115.8	38.965	76.834	150.81	2.5	2.547	101.9		
LOQ	113.1	38.965	74.138	150.81	2.5	2.458	98.32		
80%	158.78	38.965	119.81	150.81	4	3.972	99.309	99.585 ± 1.196	**1.201**
80%	160.69	38.965	121.73	150.81	4	4.036	100.89		
80%	157.86	38.965	118.9	150.81	4	3.942	98.55		
100%	189.28	38.965	150.32	150.81	5	4.984	99.673	99.371 ± 1.175	**1.183**
100%	190.33	38.965	151.36	150.81	5	5.018	100.37		
100%	186.87	38.965	147.91	150.81	5	4.904	98.074		
120%	218.67	38.965	179.71	150.81	6	5.958	99.303	100.114 ± 0.713	**0.712**
120%	220.65	38.965	181.69	150.81	6	6.024	100.4		
120%	221.1	38.965	182.13	150.81	6	6.039	100.64		

TABLE 29 Intraday Precision (Ruggedness) of ATZ

	Sr.no	**1**	**2**	**3**
	Level	**LOQ**	**100**	**150**
ATZ	**Conc. (µg/ml)**	**0.5**	**5**	**7.5**
	Mean ± SD	31.855 ± 1.364	215.779 ± 5.527	318.499 ± 10.657
	%RSD	4.282	2.561	3.346
Impurity A	**Conc. (µg/ml)**	**0.5**	**5**	**7.5**
	Mean ± SD	63.004 ± 2.469	311.464 ± 11.659	456.746 ± 12.732
	%RSD	3.919	3.743	2.787
Impurity 5	**Conc. (µg/ml)**	**0.2**	**5**	**7.5**
	Mean ± SD	48.351 ± 1.363	262.182 ± 5.889	390.022 ± 5.550
	%RSD	2.818	2.246	1.423

***Mean of three replicates**

TABLE 30 Interday Precision of ATZ

	Sr.no	**1**	**2**	**3**
ATZ	**Conc. (µg/ml)**	**0.5**	**5**	**7.5**
	Mean ± SD	36.263 ± 1.338	219.824 ± 7.470	320.181 ± 9.034
	%RSD	3.689	3.398	2.822
Impurity A	**Conc. (µg/ml)**	**0.5**	**5**	**7.5**
	Mean ± SD	67.287 ± 2.741	310.250 ± 13.095	448.087 ± 5.452
	%RSD	4.073	4.221	1.217
Impurity 5	**Conc. (µg/ml)**	**0.2**	**5**	**7.5**
	Mean ± SD	51.527 ± 1.712	263.964 ±7.814	386.250 ± 4.975
	%RSD	3.323	2.96	1.288

***Mean of three replicates**

TABLE 31 Robustness of ATZ and its impurities

			ATZ		Impurity A		Impurity 5	
Sr.No	**Parameter**	**Condition**	**Mean ±SD**	**%RSD**	**Mean ±SD**	**%RSD**	**Mean ±SD**	**%RSD**
1	**Flow rate +0.2 ml**	**1.2**	201.3923 ± 4.656829	**2.312317**	291.4833 ± 3.422955	**1.174323**	251.55467 ± 4.142761	**1.6468633**
2	**Flow rate -0.2 ml**	**0.8**	219.704 ± 4.659113	**2.120632**	319.11866 ± 2.755327	**0.863418**	269.3927 ± 3.963373	**1.471225**
3	**Mobile phase +2%**	**62:38 17:83 62 :38**	201.4623 ± 2.17482	**1.079517**	296.288 ±7.948018	**2.6825312**	251.767 ± 3.575102	**1.4200044**
4	**Mobile phase -2%**	**58:42 13:87 58:42**	221.637 ± 1.698928	**0.766536**	319.6743 ± 3.453901	**1.080444**	271.61 ± 1.739094	**0.640291**
5	**pH +0.2 unit**	**4.2**	209.229 ± 3.920163	**1.873623**	299.0187 ± 4.539861	**1.518253**	259.4377 ±1.677521	**0.646599**
6	**pH -0.2 unit**	**3.8**	206.2097± 5.16774	**2.506061**	302.065 ± 2.809774	**0.930188**	259.2493 ±4.159856	**1.604577**

TABLE 32 Repeatability of ATZ and its impurities

ATZ		Impurity A		Impurity 5	
At 100%	**5 µg/ml**	**At 100%**	**5 µg/ml**	**At 100%**	**5 µg/ml**
Std	Area	**Std**	Area	**Std**	Area
1	205.206	**1**	296.875	**1**	256.403
2	208.988	**2**	304.97	**2**	263.618
3	207.37	**3**	311.882	**3**	270.28
4	214.41	**4**	303.515	**4**	261.543
5	210.381	**5**	321.496	**5**	277.167
6	203.358	**6**	326.784	**6**	263.966
Avg.	**208.286**	**Avg.**	**310.92**	**Avg.**	**265.496**
SD	**3.924**	**SD**	**11.419**	**SD**	**7.256**
% RSD	**1.884**	**% RSD**	**3.673**	**% RSD**	**2.733**
Limit: % RSD for area NMT 5.0%		**Limit: % RSD for area NMT 5.0%**		**Limit: % RSD for area NMT 5.0%**	

TABLE 33 Analysis of commercial formulation of ATZ

Formulation	Impurities A		Impurities 5		Total impurities	
Brand name	**Mean ± SD**	**%RSD**	**Mean ± SD**	**%RSD**	**Mean ± SD**	**%RSD**
1. Atavir 300 (Cipla)	0.254 ± 0.003	1.19	0.195 ± 0.005	2.453	0.449 ± 0.007	1.561
2. ATAZOR-300 (Emcure)	0.256 ± 0.006	2.473	0.199 ± 0.002	1.189	0.455 ± 0.008	1.704

TABLE 34 Estimation of ATZ

Label claim	**Mean±SD**
1. Atavir 300 (Cipla)	99.73 ± 1.66
2. ATAZOR-300 (Emcure)	100.57 ± 1.39

CHAPTER-5

IMPURITY PROFILING OF ETRAVIRINE (ETR)

5.0 Introduction

ETR (ETR) is a non-nucleoside reverse transcriptase inhibitor (NNRTIs) used to treat HIV-1, approved by the FDA for its efficacy against the virus[1]. It is chemically classified as a 4-[[6-amino-5-bromo-2[(4-cyanophenyl)-amino] pyrimidinyl] oxy]-3,5-dimethylbenzonitrile and belongs to BCS Class IV due to its low solubility and permeability.

Literature survey [3-20] reveals that analytical methods for ETR are limited, with only three HPLC methods available and it being listed in the USP Draft-2023[4]. The brand of this molecule mainly **intelence**.

Existing literature shows a lack of comprehensive methods for analysing ETR and its impurities, with only one study addressing force degradation impurities and detecting seven, two of which are noted in the USP draft.

Controlling organic impurities in pharmaceutical products is essential for ensuring drug purity, safety, and efficacy. An accurate, sensitive, and robust SIAM is crucial for monitoring drug stability and reducing production losses. Key parameters in developing such a method include the detection wavelength for impurities, LOD, and the drug's label claim in the finished product.

There is a need for a method to identify both known and unknown impurities, particularly those causing toxicity. The aim of the study is to develop a simple and accurate RP-HPLC method for routine ETR analysis

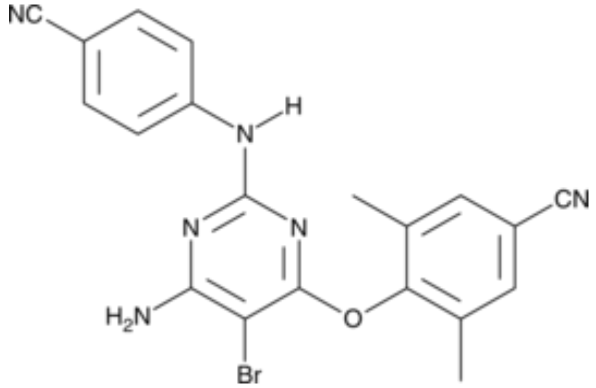


FIGURE 39 Structure of ETR (ETR) [4]

5.1 Selection of Impurities [30-57,74]

- Potential impurities of ETR were not separated from main analytes in the reported methods and it is the most common in their dosage forms. It has produces genotoxicity.[74]
- Hence, no RP-HPLC and UPLC were reported for the determination of ETR impurities in their fixed dosage forms.
- RP-HPLC system enables improved sensitivity, selectivity, rapid analysis, environment friendly due to lower solvent consumption, RP-HPLC equipment was chosen for the determination of ETR and its processed impurities in the fixed dose products.

Finally, it was selected four impurities in this project impurities 1 and 2

TABLE 35 ETR impurity Profile Summary

Name	**Impurity-1**	**Impurity-2**
Marketed Name	**3- amino bromo**	**Des amino impurity**
IUPAC name	4-((4-Amino-5-bromo-6-chloropyrimidin-2-yl)amino)benzonitrile	4-((5-Bromo-2-((4-cyanophenyl)amino)pyrimidin-4-yl)oxy)-3,5-dimethylbenzonitrile
CAS no	1398507-09-9	269055-04-1

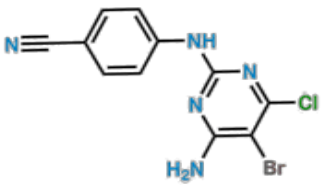
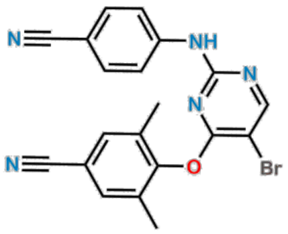
Molecular structure		
Molecular Formula	$C_{11}H_7BrC_lN_5$	$C_{20}H_{14}BrN_5O$
Molecular weight	324.6	420.26

TABLE 36 Materials and Instrumentals Specification

Sr. No.	Name	Description
1.	**HPLC**	**Shimadzu [with power stream]** **Column: -** C18 Hypersil BDS (250mm × 4.6mm × 5µm) **Pump: -** LC-20 AT **Syringe: -** Rheodyne injector valve with 20.0 µl loop **Detector: -** PDA **Software: -** Spinchrom
2.	**pH Meter**	Systronics PH361
3.	**Digital Balance**	Mettler Toledo ML 204
4.	**Glass wares**	Borosil
5.	**Ultrasonicator**	Toshcon
6.	**Melting Point Apparatus**	Supertek

TABLE 37 List of Reagents and Materials

Sr. No.	Name	Description
1.	**Water**	HPLC Grade
2.	**Methanol**	HPLC Grade

TABLE 38 Detail of Drug Procurement

ETR (ETR) standards procured from Hetero pharma and impurities were obtained from Acun pharm life science, Ahmedabad.

Sr. No.	Name	Source
1.	**ETR (ETR)**	HETERO pharma, Hydrabad
2.	**Impurity -1 and 2**	Acun Pharm Life science, Ahmedabad

5.2 Identification of Drugs:

5.2.1 Identification by Melting Point

Taken the ETR in capillary and place into the supertek melting point apparatus. Melting point observed and compared with the reference

TABLE 39 Melting Point comparison

Sr. No.	Drug	Reported Melting Point [60]	Observed Melting Point
1.	**ETR (ETR)**	262-265°C	250-263°C

5.2.2 Identification by Solubility

The sample of ETR was taken in test tubes and observed for solubility in various solvents like water, methanol, ACN, 0.1 N HCl and 0.1 N NaOH.

TABLE 40 Solubility Determination

Solvent	Solubility	Criteria (Parts of solvent per 1 part solute) as per IP/USP
Water	Slightly soluble	100-1000
0.1 N NaOH	Slightly soluble	100-1000
0.1 N HCl	Insoluble	> 10,000
Methanol	Soluble	10-30

ACN	Soluble	10-30

5.3 Identification of impurities

5.3.1 Mass Spectroscopy: The MS was performed on the SCIEX Triple Quad 5500+ mass spectrometer instrument with the ESI method in a positive ion mode with turbo ion spray as an interface. Different parameters like IS (Ion Source) =55,00 V, DP (Declustering Potential) = 60 V, and EP (Entrance Potential) = 10 V, were set along with the pressure of 50 and 60 psi of air and nitrogen as nebulizer and curtain gas to acquired mass spectra. **The obtained MS spectrum was recorded and compared (M+) peak with molecular weight.**

1. Mass-to-Charge Ratio (m/z):

- The **x-axis** represents the mass-to-charge ratio (m/z).
- **Significant peaks:**
    - **Base peak: - m/z 271.22**: The **base peak**, indicating the most abundant ion detected. This corresponds to the primary impurity in the sample.
    - **Molecular Ion Peak: m/z 324.01**: A secondary ion, which might be a fragment or a related impurity.

2. Intensity:

- The **y-axis** indicates ion intensity.
- The peak at **m/z 271.22** has the highest intensity, confirming its dominance in the ion profile.

3. Molecular Structure:

- The chemical structure linked with the base peak (m/z 271.22) contains:
    - **Aromatic ring with a cyano group (–C≡N):** Indicates potential synthetic origin or a degradation product.
    - **Halogen atoms (Chlorine and Bromine):** These might increase the molecular weight and provide characteristic fragmentation patterns.

  - **Amine group (–NH2):** Suggests it might interact with the active pharmaceutical ingredient during synthesis or degradation.

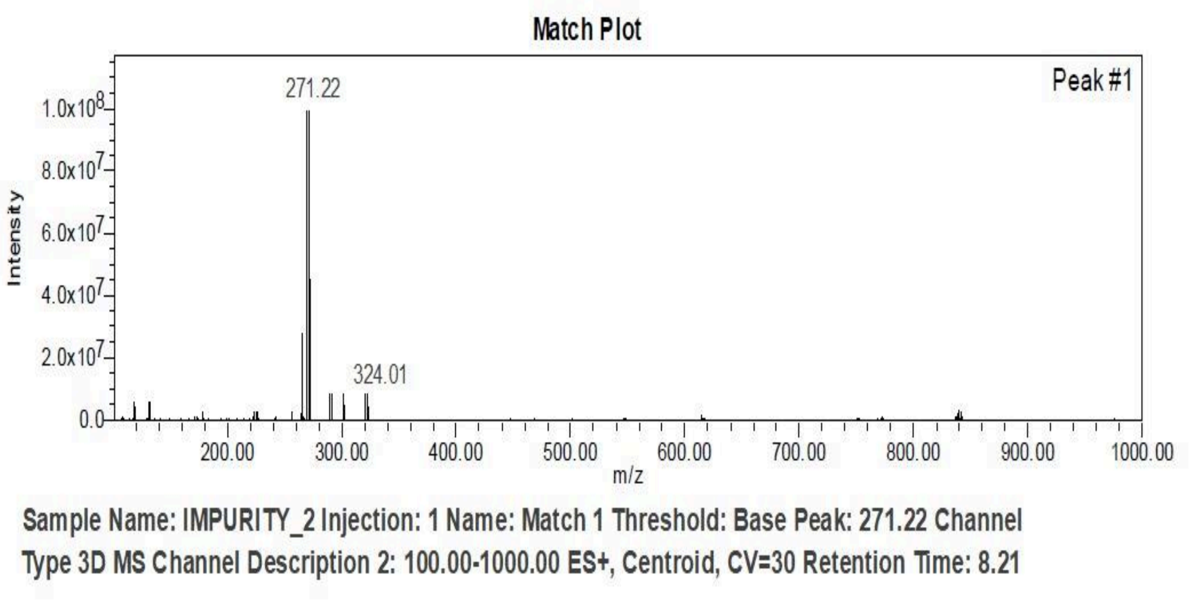
4. Interpretation:

- The **base peak at m/z 271.22** is most likely the primary impurity associated with the drug compound. Its structure indicates synthetic complexity due to the presence of halogens and aromatic functionalities.
- The **secondary peak at m/z 324.01** might correspond to a higher molecular weight impurity or a fragment resulting from ionization.

5. Applications:

- This type of analysis is crucial for impurity profiling and quantification, ensuring drug safety and efficacy, especially in anti-HIV medications like Atazanavir.
- Such analysis is critical in impurity profiling for pharmaceuticals to meet regulatory guidelines (like ICH Q3A/B for impurities).

The identified structure aids in understanding the origin of the impurity, whether from degradation, side reactions, or residual solvents



Sample Name: IMPURITY_2 Injection: 1 Name: Match 1 Threshold: Base Peak: 271.22 Channel Type 3D MS Channel Description 2: 100.00-1000.00 ES+, Centroid, CV=30 Retention Time: 8.21

FIGURE 40 Mass spectrum of ETR Impurity A

5.3.2 NMR Spectroscopy

The 1H and 13C NMR spectra of impurities were carried out using NMR spectroscopy. Chemical shifts from both experiments were reported on the δ scale in ppm with respect to tetramethyl silane (TMS).

5.3.3 ETR Impurity-A H1 NMR

The Below **figure 41** represents the **NMR spectrum** of **Impurity-2**, identified as **4-((4-amino-5-bromo-6-chloropyrimidin-2-yl) amino) benzonitrile**, commonly called as 3-amino bromo impurity, dissolved in DMSO (dimethyl sulfoxide) -d6. Here's a detailed explanation:

1. Acquisition Parameters:

- **Solvent:** DMSO-d6 is used, known for its high solubility and suppression of exchangeable proton signals.
- **Instrument:** AvanceNeo Ascend 400 MHz NMR spectrometer.
- **NS (Number of Scans):** 16, sufficient for a good signal-to-noise ratio.
- **Line Broadening (LB):** 0.30 Hz ensures sharp peak resolution.

2. Interpretation:

- **Aromatic Region (7.2–7.7 ppm):**
    - Peaks in this region confirm the presence of aromatic protons on the benzonitrile and pyrimidine rings.
    - Splitting patterns provide insights into substitution patterns and proton-proton coupling.
- **3.4–3.5 ppm (Amino Protons):**
    - The singlet nature suggests isolated protons without coupling.
    - These are attributed to -NH or -NH2 groups present in the structure.

3. Molecular Structure:

- The structure shows:

  - **Pyrimidine ring** with substituents:
    - **Amino group (-NH_2)**
    - **Bromine (Br)** and **Chlorine (Cl)** atoms.
  - **Benzonitrile group (-C≡N):** A characteristic functional group contributing to the downfield region of the spectrum.

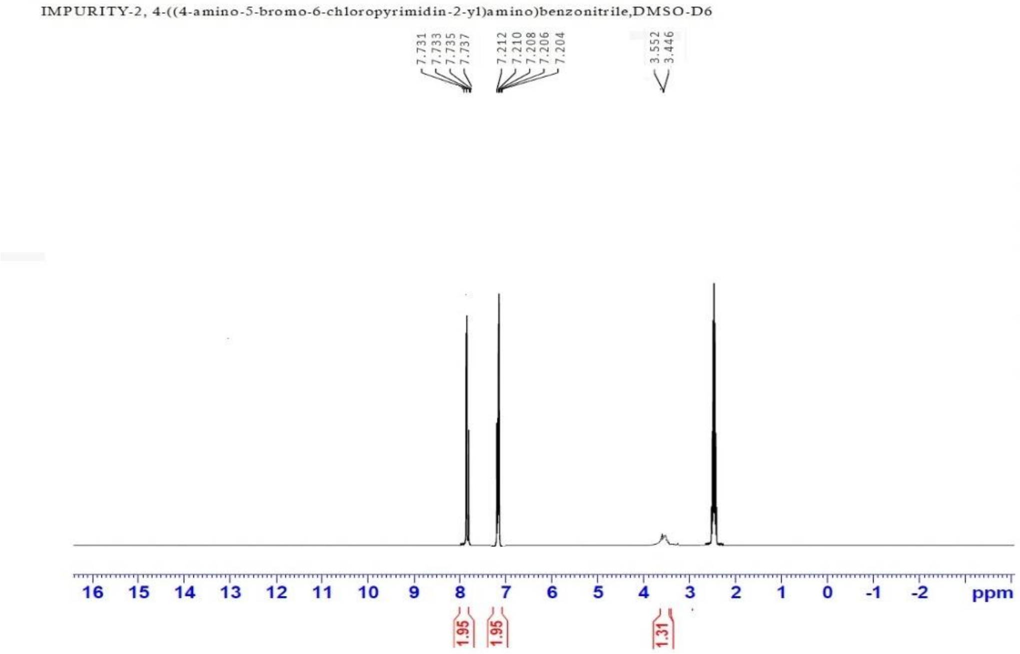
4. NMR Spectrum Features:

- **X-Axis (ppm):**
  - The spectrum spans from ~16 ppm to 0 ppm, with key chemical shift regions identified.
  - Peaks:
    - **7.2–7.7 ppm (Aromatic region):**
      - These correspond to protons on the aromatic rings (benzene and pyrimidine).
      - Splitting indicates coupling between adjacent protons.
    - **3.4–3.5 ppm:** Likely corresponds to exchangeable protons such as those on the amine (-NH or -NH2) group due to their chemical environment in DMSO.
- **Y-Axis:**
  - The intensity shows the relative abundance of specific proton environments.

5. Applications:

- **Impurity Identification:** The NMR confirms the structure of Impurity-2, essential for regulatory compliance and quality control in pharmaceuticals.
- **Quantitative Analysis:** Integration of peak areas can help quantify the impurity in a sample.

- **Regulatory Standards:** Identifying and characterizing impurities like this one ensures adherence to guidelines like ICH Q3A/B.



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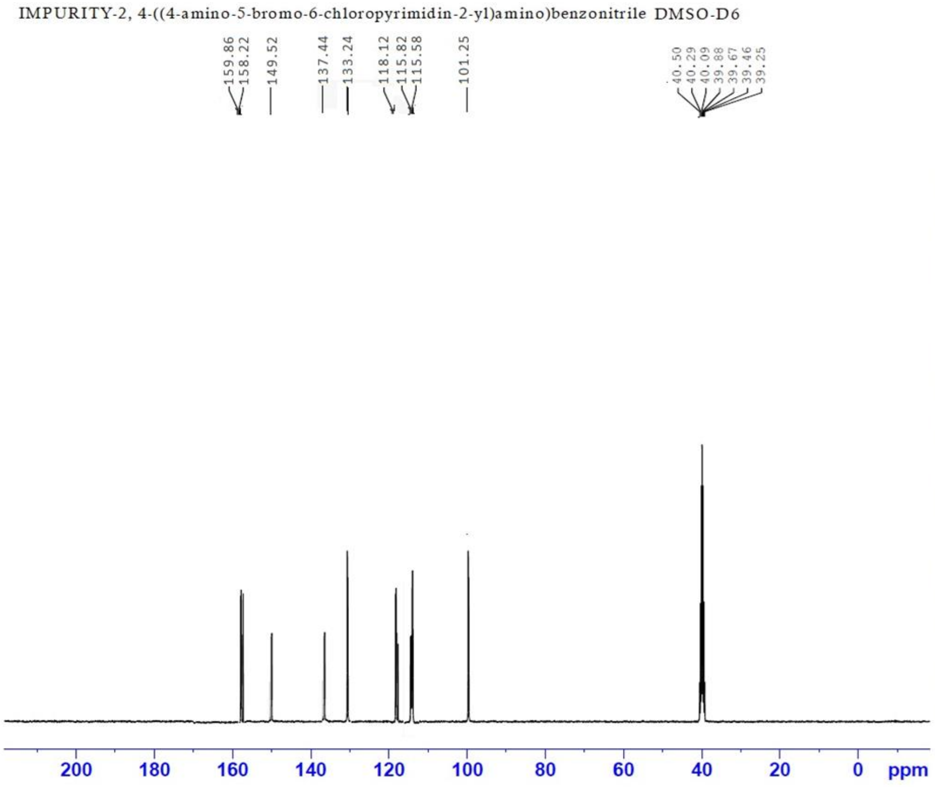
EXPNO                      1
PROCNO                     1

F2 - Acquisition Parameters
Date_               20230817
Time                 14.26 h
INSTRUM   AvanceNeo  Ascend400MHz
PROBHD    Z163739_0198 (
PULPROG                 zg30
TD                     65536
SOLVENT                 DMSO
NS                        16
DS                         0
SWH                 8196.722 Hz
FIDRES              0.250144 Hz
AQ                 3.9976959 sec
RG                   11.2736
DW                    61.000 usec
DE                     13.89 usec
TE                     295.4 K
D1                1.00000000 sec
TD0                        1
SFO1             400.3024719 MHz
NUC1                      1H
P0                      2.67 usec
P1                      8.00 usec
PLW1             24.20299911 W

F2 - Processing parameters
SI                     65536
SF               400.3000000 MHz
WDW                       EM
SSB                        0
LB                      0.30 Hz
GB                         0
PC                      1.00
```

FIGURE 41 ETR Impurity-A H1 NMR



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Current Data Parameters
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EXPNO                      2
PROCNO                     1

F2 - Acquisition Parameters
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PROBHD    Z163739_0198 (
PULPROG               zgpg30
TD                     65536
SOLVENT                 DMSO
NS                      1024
DS                         0
SWH                23809.523 Hz
FIDRES              0.726609 Hz
AQ                 1.3762560 sec
RG                       101
DW                    21.000 usec
DE                      6.50 usec
TE                     296.1 K
D1                2.00000000 sec
D11               0.03000000 sec
TD0                        1
SFO1             100.6655806 MHz
NUC1                     13C
P0                      2.67 usec
P1                      8.00 usec
PLW1             95.69300079 W
SFO2             400.3016012 MHz
NUC2                      1H
CPDPRG[2             waltz65
PCPD2                  90.00 usec
PLW2             24.20299911 W
PLW12             0.19123000 W
PLW13             0.09618800 W

F2 - Processing parameters
SI                     32768
SF               100.6555151 MHz
WDW                       EM
SSB                        0
LB                      3.00 Hz
GB                         0
PC                      1.40
```

FIGURE 42 ETR Impurity-A C13 NMR

5.3.4 FT-IR Spectroscopy: The FT-IR spectrum indicates the presence of: Amine groups (-NH, -NH2), Nitrile group (-C≡N), Aromatic rings and associated C-H bonds, Halogens (e.g., Cl or Br). This analysis is crucial for:

1. **Impurity Profiling:** Identifying functional groups in pharmaceutical impurities.
2. **Structure Confirmation:** Verifying the structure of synthesized compounds.
3. **Regulatory Compliance:** Ensuring the compound aligns with chemical standards.

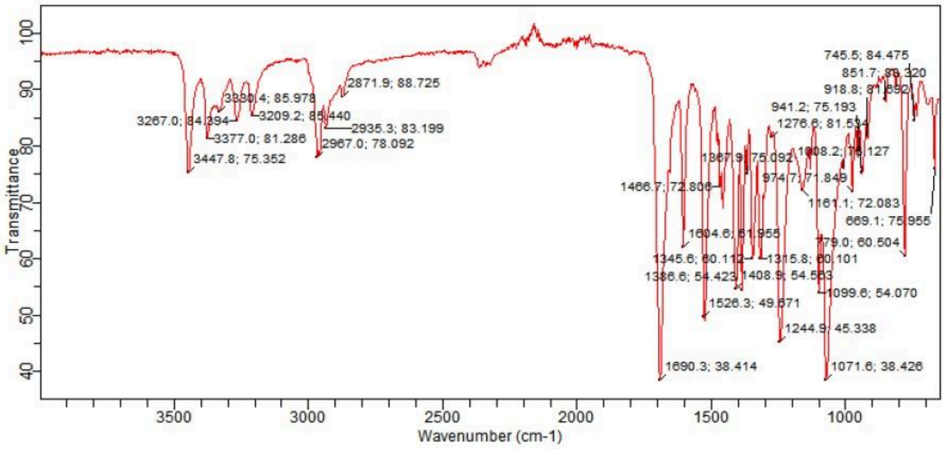

FIGURE 43 FT-IR of Etravirine Impurity-A

TABLE 41 FTIR of Impurity A

Assigned group	Observed frequency (cm^{-1})	Reference Range (cm^{-1})
-C≡N Stretch	2200	**2200-2260**
N–H / NH_2 stretch	3447	**3200-3500**
C=N or aromatic C=C	1690	**1650-1750**
CH_2/CH_3 bending	1456	**1350-1500**
C–N or C–Cl/C–Br	1244	**1000-1300**

C–N, C–Cl / C–Br	941.2	**900-1100**
Aromatic ring bending (Substituted benzene ring)	745.5	**690-900**
N–H / NH_2 stretch	3377	**3200-3500**

5.4 Experimental works

5.4.1 Preparation of stock solution

Weigh accurately about 7 mg of ETR standard into a 10 ml volumetric flask, add about 4-5 ml of diluent and sonicate to dissolve. Make up to the mark diluent and mix well. Dilute 100 µl (0.1 ml) of the above solution into a 100 ml volumetric flask. Make up to the mark with diluent and mix well.

5.4.2 Preparation of impurity-1 stock solution

Weigh accurately about 7 mg of impurity-1 into a 10 ml volumetric flask, add about 4-5 ml of diluent and sonicate to dissolve and make up to the mark with diluent and mix well

5.4.3 Preparation of impurity-2 stock solution

Weigh accurately about 7 mg of impurity-2 into a 10 ml volumetric flask, add about 4-5 ml of diluent, and sonicate to dissolve and make up to the mark with diluent and mix well.

5.4.4 LOD solution preparation

Transferred 2.3 µl impurity -1 stock solution, 1.4 µl impurity -2 stock solution and 1.0 µl of standard stock solution into a 100 ml volumetric flask containing 50 ml of diluent, mixed well and diluted to volume with diluent and mixed well.

5.4.5 Preparation of mobile phase

Buffer: First filter 1000 ml of Milli-Q- water through 0.45 µm nylon membrane filter paper and transfer accurately 1.0 ml of perchloric acid (70%). Mix well and sonicate to degas.

Mobile Phase A: Buffer

Mobile phase B: Methanol: Acetonitrile: Water (90:05:05 v/v/v)

TABLE 42 Gradient Programme

Time (in mins)	0	10	22	35	35.5	42
Mobile Phase-A (%)	30	30	5	5	30	30
Mobile Phase- B (%)	70	70	95	95	70	70

Diluent:

Mobile phase is used as diluents.

TABLE 43 Optimised chromatographic conditions

Parameter	Method
Types of chromatography	Reverse phase (RP)
Mode of operation	Gradient
Stationary phase (Column)	Xselect HSS T3,150 ×4.6 mm,3.5 µm
Diluent	Methanol: Acetonitrile (50:50 v/v)
Flow rate (mL/min)	1.0
Run time (Minutes)	30
Column temperature (^{O}C) :	35
Volume of injection loop (µl)	10
Detection wavelength (nm)	310
Detector	PDA
Sample concentration	0.7 mg/ml
Retention Time (Rt)	ETR Peak between 15.2-15.6 min

TABLE 44 Order of injections (Test Method of analysis)

Sr. No	Solution Name	No. of injections
1	Diluent as blank	**Confirm neat blank**
2	SST solution	**1**
3	Diluent as blank	**Confirm neat blank**

4	Reference solution	**6**
5	Sample solution preparation-01	**1**
6	Sample solution preparation-02	**1**
7	SST solution	**1**
8	Diluent as blank	**Confirm neat blank**
9	Reference solution	**Confirm neat blank**

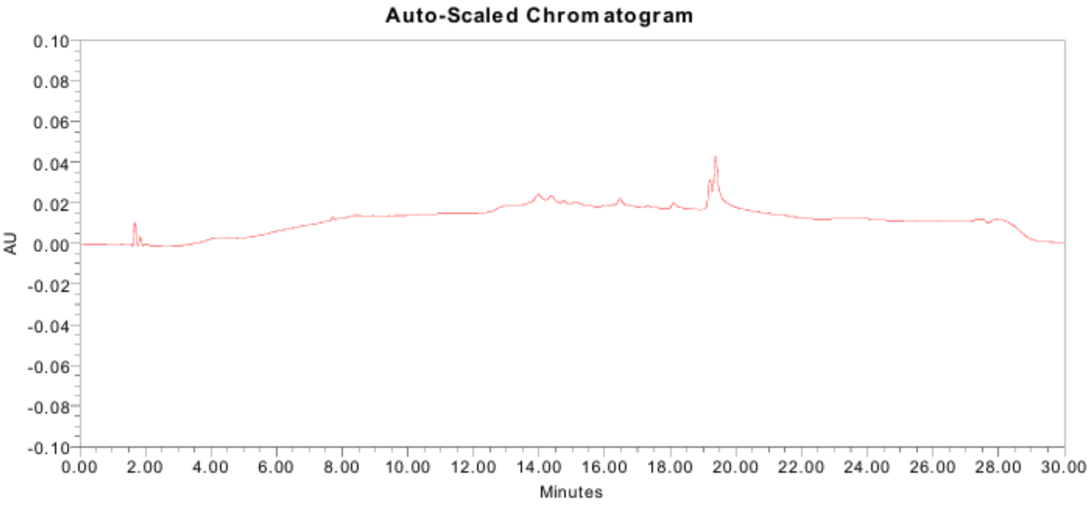

FIGURE 44 Representative chromatogram of blank solution

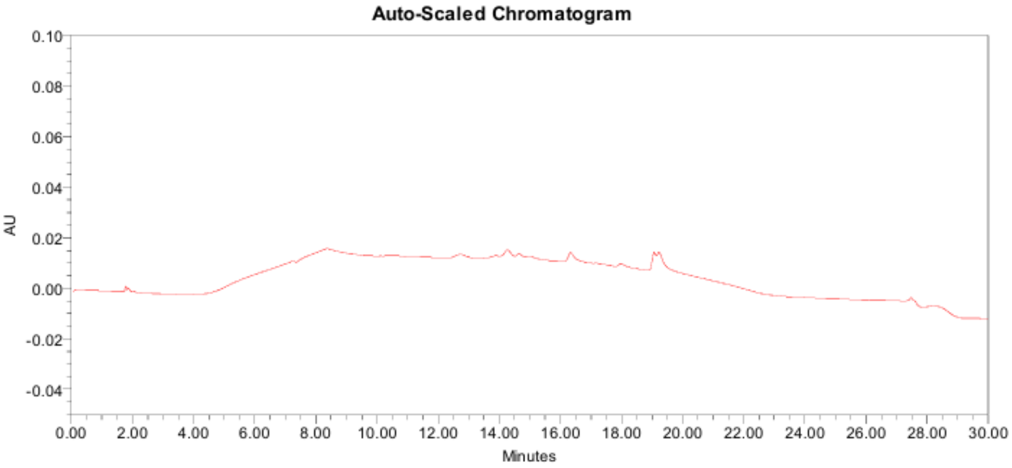

FIGURE 45 Representative chromatogram of placebo solution

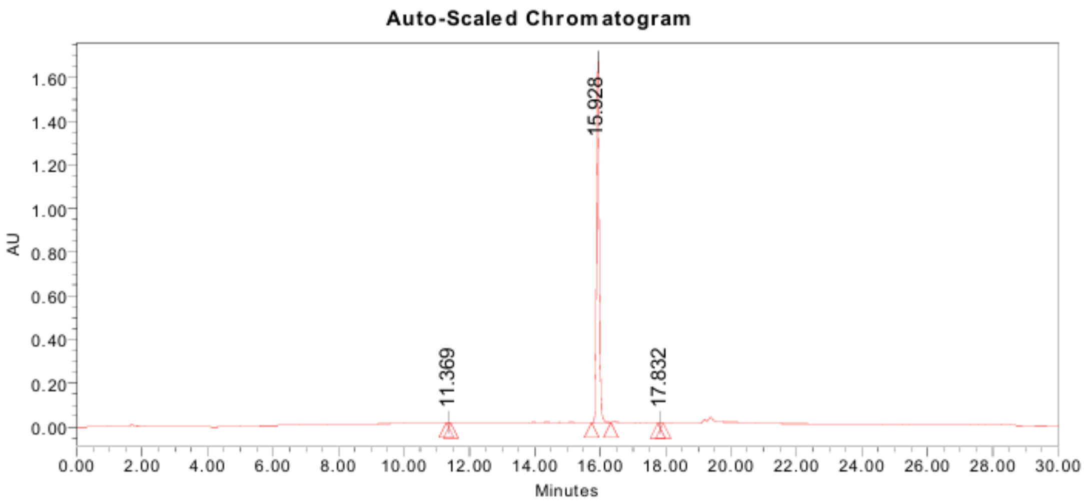

FIGURE 46 Representative chromatogram of system suitability solution

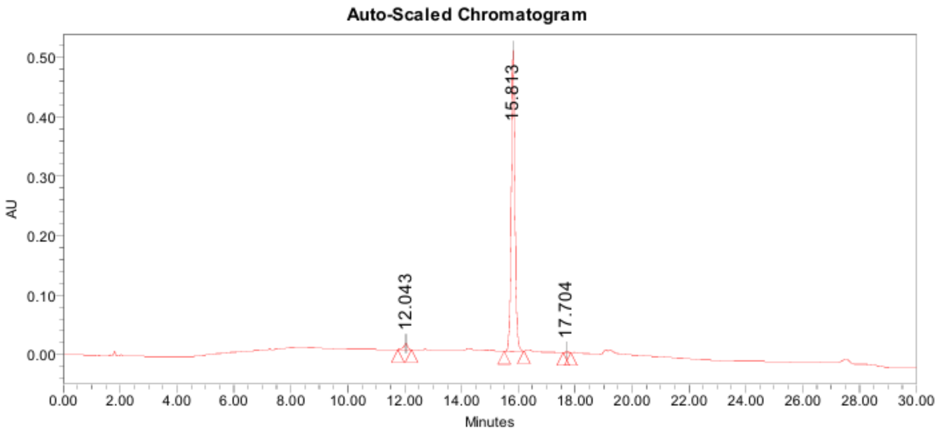

FIGURE 47 Representative chromatogram of sample solution

TABLE 45 Method Development Trials

Trial	**Mobile Phase**	**Flow rate (ml/min)**	**Ratio (v/v)**	**Remarks**	**Figure no.**
1.	**Methanol and water**	1	50:50	Chromatogram not suitable for SST Criteria.	**48**
2.	**ACN and Water**	1	50:50	Merged Peak as per not SST.	**49**

3.	ACN	1	-	Single peak observed at 10.296 minutes.	**50**
4.	**Buffer: ACN**	1	50:50	Peaks are merged with each other's and Retention time of ETR was 11.217 minutes.	**51**
5.	**Buffer: ACN**	1	30:70	Peaks are merged with each other's as per not SST criteria. And retention time of ETR was 11.209 minutes.	**52**
6.	**Buffer: ACN**	1	60:40	Retention time (Rt) reduced and tailing and ETR peak was at 5.532 minutes.	**53**

Trial 7. Chromatogram of Mobile phase A (%Buffer) and Mobile Phase B (% ACN: Water) by gradient technique

Time	**Mobile phase A (%) (Buffer)**	**Mobile Phase B (%) ACN: Water**	**Flow rate (ml/min)**	**Peak**	**Retention time (Rt)**	**Area**	**Area (%)**
0	**30**	**70**	**1**	Peak-1	6.133	61649	0.42
5				Peak-2	6.320	23993	0.17
7	**5**	**95**		Peak-3	7.707	38075	0.26
13				Peak-4	8.147	20415	0.14
15				Peak-5	8.467	87150	0.60
22	**40**	**60**		Peak-6	11.520	11721483	80.66
25				Peak-7	13.960	114163	0.79
28				Peak-8	16.400	14750	0.10
35	**30**	**70**		Peak-9	20.000	70091	0.48

35.5				Peak-10	20.093	382463	2.63
42				Peak-11	34.507	1998383	13.75
				Total		14532615	100.00

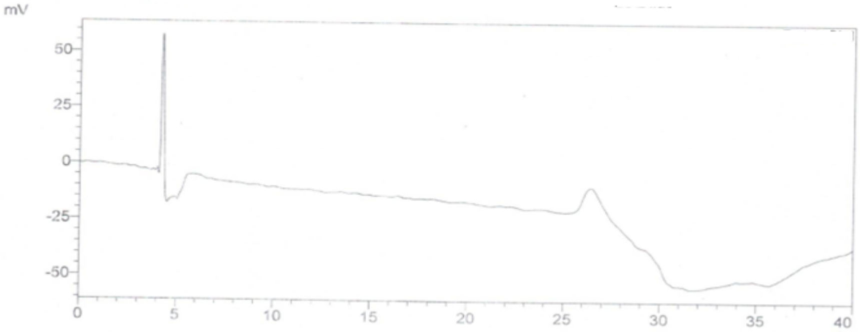

FIGURE 48 Trial 1:- Chromatogram of Diluent Methanol and water (50:50 v/v)

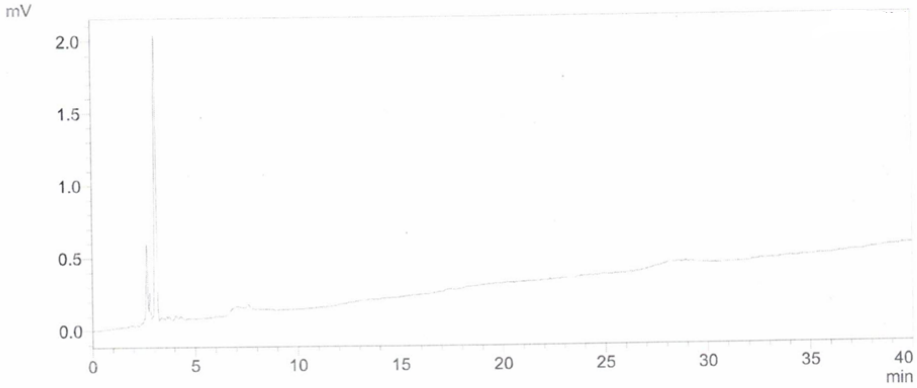

FIGURE 49 Trail 2:- Chromatogram of Diluent ACN and water (50:50 v/v)

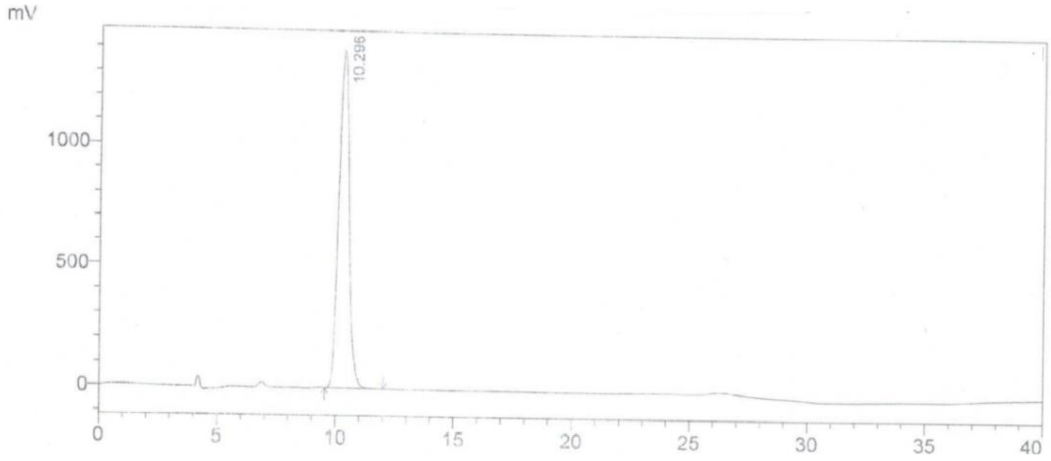

FIGURE 50 Trial 3:- Chromatogram of ACN

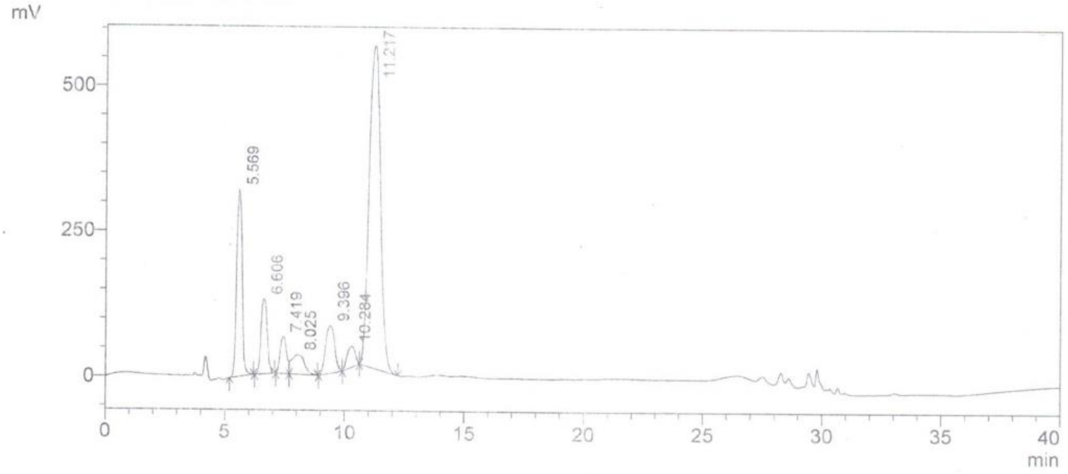

FIGURE 51 Trial 4: - Chromatogram of Water: ACN (50 :50 v/v)

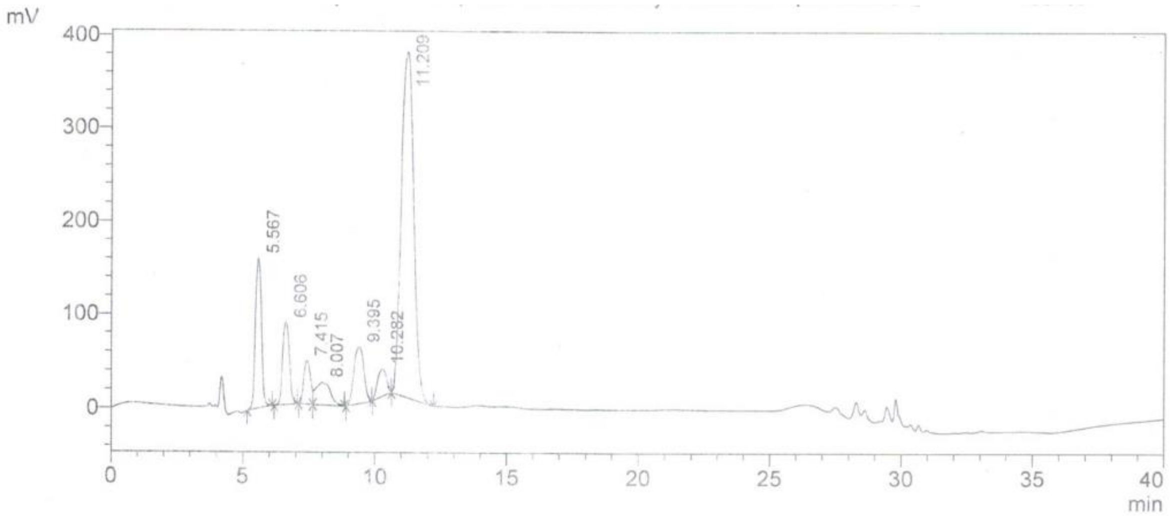

FIGURE 52 Trial 5: - Buffer: ACN (30 :70 v/v)

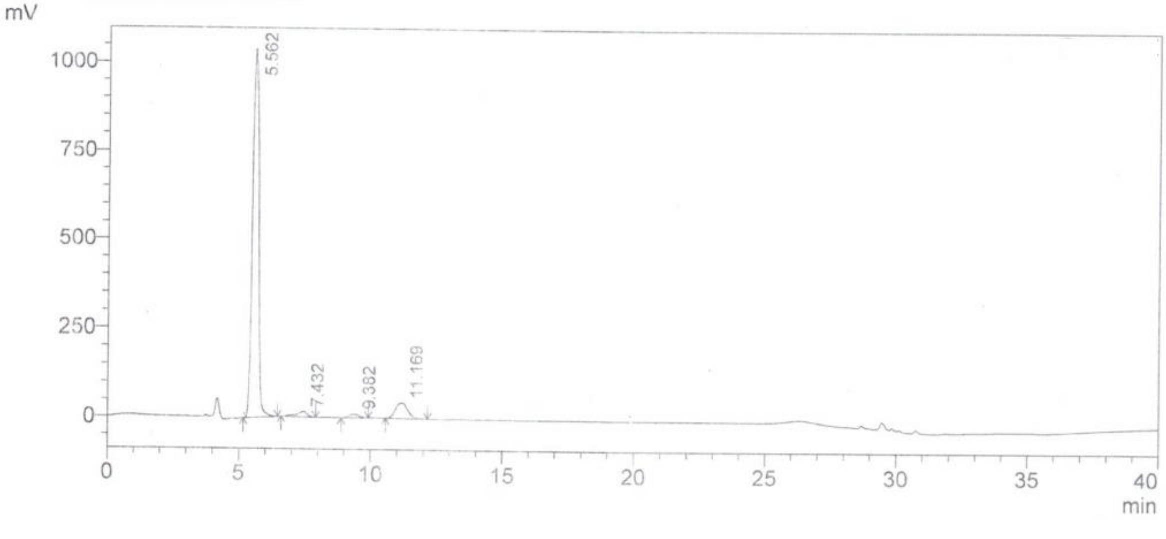

FIGURE 53 Trial 6: - Buffer: ACN (60 :40 v/v)

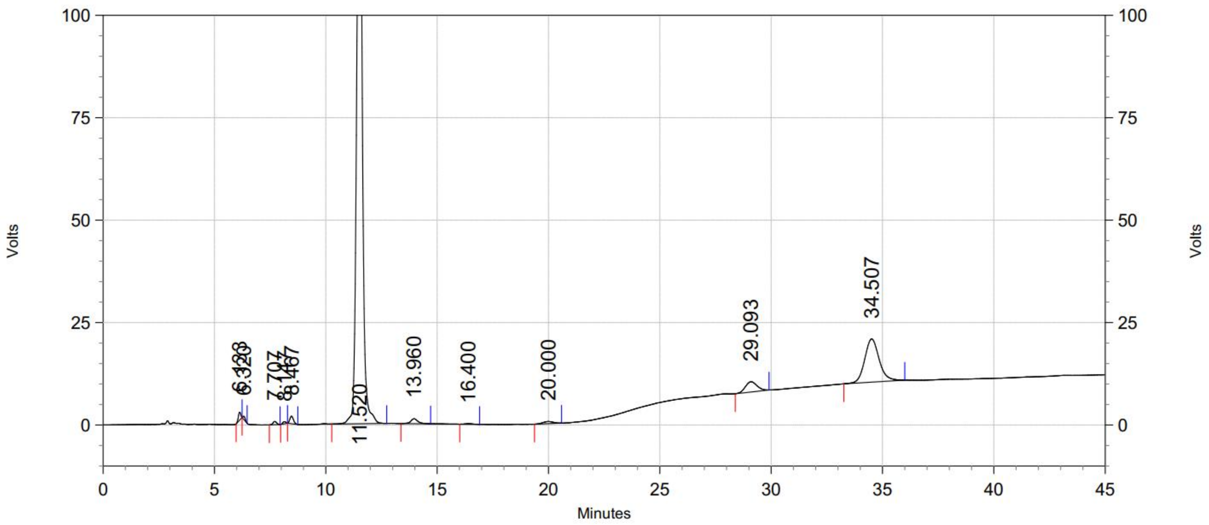

FIGURE 54 Trial 7: - Chromatogram of Mobile phase A (%Buffer) and Mobile Phase B (% ACN: Water) by gradient technique

5.5 Method validation

The HPLC method developed for the analysis of ETR was validated in line with the ICH Q2 (R1) guidelines. The validation process encompassed key analytical parameters such as system suitability, specificity (including selectivity and forced degradation), sensitivity (limit of detection and quantification), linearity, precision, accuracy, filter compatibility, solution stability, and robustness. The method was tailored for the evaluation of ETR in its bulk form, with particular emphasis on the detection and quantification of impurities 1 and 2.

5.5.1 System suitability test (SST)

SST were conducted to ensure the reliability of both the initial and final injections, and the results were evaluated against predefined acceptance criteria. To support the development of a robust analytical method, key system suitability parameters such as resolution, capacity factor, signal-to-noise ratio, and theoretical plates-were established. Given the extended run time and potential concerns regarding solution stability, a similarity factor was calculated by injecting two separate standard solutions (Standard 1 and Standard 2) to confirm consistency throughout the analysis.

a) Resolution between any two known impurities and main peak **should not be less than 1.5**

b) Number of theoretical plates for ETR (ETR) Peak: - **Not less than 3000**

c) Tailing factor for ETR (ETR) Peak: - **NMT 2.0**

5.5.2 Reference solution preparation /Standard preparation

Accurately weigh approximately 7 mg of ETR standard and transfer it into a 10 ml volumetric flask. Add 4–5 ml of diluent and sonicate to ensure complete dissolution. Dilute to volume with the same diluent and mix thoroughly. From this stock solution, pipette 100 µl (0.1 ml) into a 100 ml volumetric flask, dilute to the mark with diluent, and mix well to obtain the final working solution.

5.5.3 Linearity and Range (Calibration curve)

The linearity of the analytical method was established by injecting a series of solutions prepared in the concentration range of 10–60 µg/ml, corresponding to 25% to 200% of the target test concentration. A minimum of six different concentration levels were analyzed to demonstrate a linear response over the specified range.

5.5.4 Level solutions

To determine accuracy, three concentrations of the analyte-50%, 100%, and 150% were prepared and chromatograms recorded. Six preparations at the LOQ and three each at 50%, 100%, and 150% levels were injected into the system. The recovery percentages for each impurity and the principal analyte peak were calculated. The recovery limits were set at 80%-120% for LOQ and 85%-115% for other levels. Acceptance criteria included meeting system suitability standards, ensuring that the RSD for impurity peak areas and the ETR peak area did not exceed 5.0%, and that the regression coefficient for each impurity was at least 0.99 with a Y-intercept within ± 5.0% [6,7] The data are reported in **Table 50 and Figure 55 & 56.**

5.5.5 Determination of Limit of Detection (LOD) and Limit of quantification (LOQ)

The limit of detection and limit of quantification of the impurities were determined by calculating the signal-to-noise ratio (S/N, i.e., 3.3 for LOD and 10 for LOQ) using the following equations designated by International Conference on Harmonization (ICH).[6]

$$\mathbf{LOD = 3.3 \times \sigma/S}$$

$$\mathbf{LOQ = 10 \times \sigma/S}$$

Where σ = standard deviation

5.5.6 Precision

The precision was demonstration under three categories.

5.5.6.1 System precision

The precision was demonstration under three categories. System Precision Single individual preparation of ETR drug substance was prepared with target concentration of about 0.1 mg/ml for system precision. The data are reported in **Table 48.**

5.5.6.2 Method Precision

The precision was demonstrated under three categories. For system precision, the standard solution was prepared and six replicate injections 10 µl of above preparation into the chromatographic system and the area was measured for all six injections in HPLC. The % RSD for the area of six replicate injections was found to be within the specified limits and for ruggedness six individual preparations of ETR drug substance with impurities were prepared and analysed by different analysis systems, columns and in different days under similar conditions at different time.

5.5.6.3 Intermediate precision (Ruggedness)

Demonstration of ruggedness was conducted by different analysts, systems, columns and in different days under similar conditions at different times. Six individual preparations of ETR drug substance spiked with impurities were prepared and each was analysed.

5.5.7 Specificity

The specificity of the method was demonstrated by interference check by injecting the diluent blank, ETR and impurities solutions to determine whether any peaks in the diluent and impurities solutions are co-eluting with ETR peaks.

5.5.8 Forced degradation study

A forced degradation study was conducted to assess the selectivity and stability-indicating nature of the method. Samples and placebos were subjected to various stress conditions: acid degradation with 0.5 N HCl at 60°C for 2 hours, alkali degradation with 1 N NaOH at room temperature for 5 hours, thermal degradation at 80°C for 2 days, oxidative stress with 0.3% H_2O_2 for 12 hours, and photolytic degradation by exposing the sample to 1.2 million lux hours at 200 Wh/sq. These stressed samples were then analysed using RP-HPLC.

5.5.9 LOD and LOQ determination

Series of known impurity solutions were prepared over a range starting from 1 to 50% of the working concentration and injected in triplicates. The linearity graph was plotted for average area at each level against the concentration in ppm. The correlation coefficient, slope and intercept were determined for each known impurity.

5.5.10 Precision at LOQ Level

Six solutions were prepared by spiking the ETR working standard along with all the known impurities at LOQ level in the placebo. The % RSD of peak area and % results were calculated.

5.5.11 Solution stability

The stability of the solutions was tested over 48 hours by injecting 10 µl of the standard solution into the chromatographic system at 0, 24, and 48 hours. The peak areas were recorded, and the RSD of these peak areas was calculated. The acceptance criteria required that the system suitability pass under variable conditions and that the % RSD for the peak area of ETR not exceed 2.0%. The data are reported in **Table 53 and 54.**

5.5.12 Filter validation study

A filter validation study was performed to ensure that the molecule and its impurities did not interact with or get adsorbed by the filters used during sample and standard preparation. This is important because such interactions can lead to inconsistent results. The study involved filtering the sample solution through 0.45 µm porosity Nylon (MDI, India) and PVDF (Merck Millipore, Germany) filters, discarding the initial 2 ml, 3 ml, and 5 ml of filtrate to optimize the filtration process for accurate results.

5.5.13 Robustness

As part of the robustness, deliberate change in the temperature and flow rate variation was made to evaluate the impact on the method. This study was conducted by intentionally altering the critical method parameters like flow rate, change pH, change in column temperature, and change in gradient composition. The diluent, standard solution, sample solution and impurity spiked sample solution were injected to check the robustness of analytical method. The data are reported in **Table 51.**

5.6 Results and discussion:

The developed method was validated as per ICH guide line Q2B (R1).

Impurities stock solution preparation: - Weigh accurately each impurity about 7 mg (Impurity-1 and Impurity-2) into a 50 ml volumetric flask, add about 25-30 ml of diluent and sonicate to dissolve and make up to the mark with diluent and mix well.

Accuracy: The developed method was found capable to recover the contents accurately when spiked at 50 % to 150 % of the working concentration. The obtained results in % as well as µg/ml are summarized in **Tables 46 and 47.**

Precision: The % similarity factor for standard 1 and standard 2 was found between 95 and 105% during the complete validation study.

System precision: Single individual preparation of ETR drug substance was prepared with target concentration of about 0.1 mg/ml for system precision. The data are reported in **Table 48.**

Method precision: The % RSD of known impurities, single maximum and total impurities was found to be less than 15% from six sample preparations during the method and intermediate precision study.

Intermediate precision (Ruggedness):

The acceptance criteria for system, method and intermediate precision were that the system suitability criteria should be meet and the % RSD for the peak area of ETR in reference solution from the six replicate injections **should NMT be 5.0 %. The % Relative standard deviation for the peak area of each impurity should NTM 5.0** and for **ETR should NMT 2.0 %.** The data are reported in **Table 49.**

Specificity

Selectivity:

No interference was observed at the retention time of known impurities and principle analyte peaks from blank and placebo solution. All the peaks were properly resolved from each other.

LOD and LOQ determination:

The LOD and LOQ values of ETR impurities-1 and 2 were found to be 0.001, 0.002, 0.001 µg/ml and 0.005, 0.005, 0.005 respectively**.** The data are reported in **Table 52.**

TABLE 46 Study results in % recovered of ETR and its impurities

Analytes Name	Recovery at LOQ	Recovery at 50%	Recovery at 100 %	Recovery at 150 %
ETR	90.8	98.0	99.4	98.9
Impurity-1	97.5	98.4	102.6	97.9
Impurity-2	97.1	99.6	107.8	103.9

TABLE 47 Recovery study results in µg/ml of ETR and its impurities

Analytes Name	Recovery in µg/ml at LOQ		Recovery in µg/ml at 50%		Recovery in µg/ml at 100 %		Recovery in µg/ml at 150 %	
	Added	**Recovered**	**Added**	**Recovered**	**Added**	**Recovered**	**Added**	**Recovered**
ETR	0.215	0.195	1.076	1.054	2.147	2.138	3.324	3.190
Impurity-1	0.223	0.217	1.108	1.088	2.210	2.265	3.315	3.245
Impurity-2	0.225	0.209	1.074	1.073	2.147	2.313	3.218	3.332

TABLE 48 System precision of ETR

No. of injections	Area (mV*s)
1	25698.52
2	25391.26
3	25397.9
4	25024.67
5	25461.67
Average	**25394.804**
SD	**241.731**
% RSD	**0.952**

TABLE 49 Method precision and Intermediate precision study of ETR and its impurities

	Method precision			**Intermediate precision (Ruggedness)**		
No. of injections	**Area (mV*s)**			**Area (mV*s)**		
	ETR	**Impurity-1**	**Impurity-2**	**ETR**	**Impurity-1**	**Impurity-2**
1	25718.42	25.17	23.49	25325.23	22.48	21.47
2	25790.36	25.48	23.44	25212.41	22.27	22.3
3	25229.31	25.28	24.34	25118.21	22.9	21.5
4	25942.03	25.41	23.99	25675.62	22.05	21.56
5	25295.36	25.53	23.86	25271.76	22.41	21.78
6	25973.37	25.78	23.83	25696.03	22.53	22.15
Average	**25658.142**	**25.44**	**23.16**	**25383.210**	**22.44**	**21.793**
SD	**321.429**	**0.212**	**23.770**	**244.352**	**0.284**	**0.355**
%RSD	**1.253**	**0.833**	**0.416**	**0.963**	**1.265**	**1.628**

TABLE 50 Linearity of Impurity-1 and Impurity-2

Analyte name		**Impurity-1**					**Impurity-2**		
% Level or Concentration (µg/ml)	**Conc. (µg/ml)**	**Average**	**Calculated value**	**Residual value**	**% Level or**	**Conc. (µg/ml)**	**Average**	**Calculated value**	**Residual value**
LOQ	4.76	1745	1513	232	**LOQ (3.62)**	3.62	2692	2634	58
25	7.5	7063	6897	166	**25**	7.5	7362	7252	110
50	15.0	13944	13547	398	**50**	15.0	13227	12956	271
80	24.0	20878	21526	-648	**80**	24.0	19757	19801	-45
90	27.0	24088	24186	-98	**90**	27.0	21878	22083	-205
100	30.0	26682	26846	-164	**100**	30.0	24344	24365	-21
110	33.0	29034	29506	-472	**110**	33.0	26298	26646	-348
120	36.0	32172	32166	7	**120**	36.0	28872	28928	-56
150	45.0	40406	40145	261	**150**	45.0	35667	35773	-106
200	60.0	53764	53445	320	**200**	60.0	47524	47181	342
Slope		**267.04**	**Sum of Residual values**	**-1.0**	**Slope**		**228.43**	**Sum of Residual values**	**0.0**
Y-intercept		**113.48**			**Y-intercept**		**1514.5**		
Correlation coefficient		**0.9993**			**Correlation coefficient**		**0.9997**		

Acceptance Criteria: - 1) Correlation coefficient should not be less than 0.99 and 2) Sum of residual should be around 0.0

TABLE 51 Robustness for ETR, impuritiy-1 and impurity-2 standard

Analytes Name	Chromatographic condition	Retention time	Theoretical plate count	Tailing factor
ETR	**Actual condition**	15.81	2128	0.99
Impurity-1		12.04	2176	0.99
Impurity-2		17.70	2102	0.93
ETR	**Flow rate 1.3 ml /min**	16.92	2203	0.96
Impurity-1		12.07	2089	0.93
Impurity-2		17.72	2166	0.91
ETR	**Flow rate 1.7 ml/min**	15.67	2183	0.99
Impurity-1		11.54	2208	0.98
Impurity-2		17.52	2186	0.98
ETR	**Column temperature 30 ^{0}C**	15.32	2087	0.93
Impurity-1		12.11	2100	1.00
Impurity-2		16.98	2109	0.99
ETR	**Column temperature 40 ^{0}C**	15.11	2066	1.01
Impurity-1		11.89	2045	1.01
Impurity-2		17.02	2184	0.99
ETR	**pH 3.8**	15.73	2302	1.00
Impurity-1		12.31	2777	0.97
Impurity-2		17.63	2097	0.97
ETR	**pH 4.2**	14.78	2775	1.13
Impurity-1		12.37	2759	0.99
Impurity-2		16.86	2118	0.92

TABLE 52 LOQ and LOD S/N ratio results

		LOQ		LOD	
Sr. No	**Analyte Name**	**S/N Ratio**	**% level of impurity w.r.t to sample concentration**	**S/N Ratio**	**% level of impurity w.r.t to sample concentration**
1.	**ETR**	9.5	0.005	2.3	0.001
2.	**Impurity-1**	10.0	0.005	2.9	0.002
3.	**Impurity-2**	10.2	0.005	2.5	0.001
Acceptance criteria	The signal to noise ratio for each individual ETR and impurity-1, impurity-2 should be in between 9.5 to 10.2			The signal to noise ratio for each individual ETR and impurity-1, impurity-2 should be in between 2.0 -3.0	

TABLE 53 Average % of impurities for mobile phase stability “0” hour to “48” hours.

Analyte name	**0 hour**	**18 hours**	**24 hours**	**48 hours**	**Limit in %**
Impurity-1	ND	ND	ND	ND	NMT 0.15
Impurity-2	0.03	0.03	0.03	0.03	NMT 0.15
% highest individual unspecified impurity	0.01	0.01	0.01	0.01	NMT 0.10
% of total impurities	0.04	0.04	0.04	0.04	NMT 1.0
Acceptance criteria	The difference for the % of each known and unknown individual impurities from the “0” hour to “48” hour for sample solution injections and fresh sample solution injections should not be more than ± 0.04 and for the total impurities should not be more than ±0.1.				

TABLE 54 Average % of impurities for sample solution stability “0” hour to “48” hours.

Analyte name	0 hour	18 hours	24 hours	48 hours	Limit in %
Impurity-1	ND	ND	ND	ND	NMT 0.15
Impurity-2	0.03	0.03	0.03	0.03	NMT 0.15
% highest individual unspecified impurity	0.01	0.01	0.01	0.01	NMT 0.10
% of total impurities	0.04	0.04	0.04	0.04	NMT 1.0
Acceptance criteria	The difference for the % of each known and unknown individual impurities from the “0” hour to “48” hour for sample solution injections and fresh sample solution injections should not be more than ± 0.04 and for the total impurities should not be more than ±0.1.				

TABLE 55 Force degradation studies of ETR standard and sample

Sr. No	**Stress condition and time**	**% Std. degradation**	**% Drug recovered**	**Std. mean peak area**	**% Sample degradation**	**% Drug recovered**	**Sample mean peak area**
1.	**Acid hydrolysis 0.5 N HCl at 60 ^{0}C for 2 hours**	14.64	85.35	**21872.42**	16.44	83.56	**21412.621**
2.	**Alkali degradation with 1 N NaOH at RT for 5 hours**	15.60	84.39	**21625.27**	15.29	84.7	**21705.639**
3.	**Thermal degradation at 80^{o}C for 2 days**	15.14	84.86	**21745.86**	15.268	84.73	**21712.45**
4.	**Oxidative stress with 0.3 % hydrogen peroxide for 12 hours**	18.55	81.45	**20872.55**	19.32	80.67	**20674.265**
5.	**Photolytic degradation (48 hours)**	11.71	88.29	**22625.52**	12.51	87.48	**22418.56**

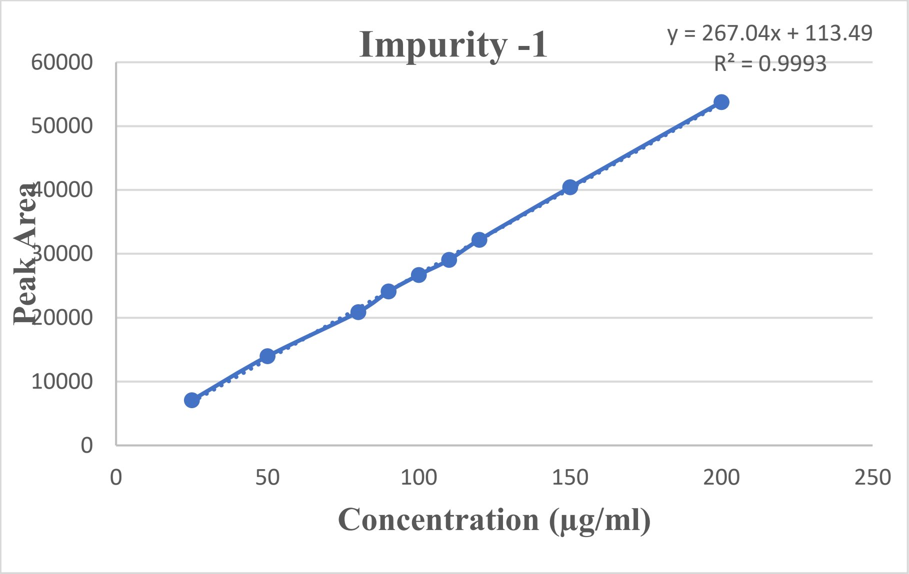

FIGURE 55 Linearity of Impurity-1

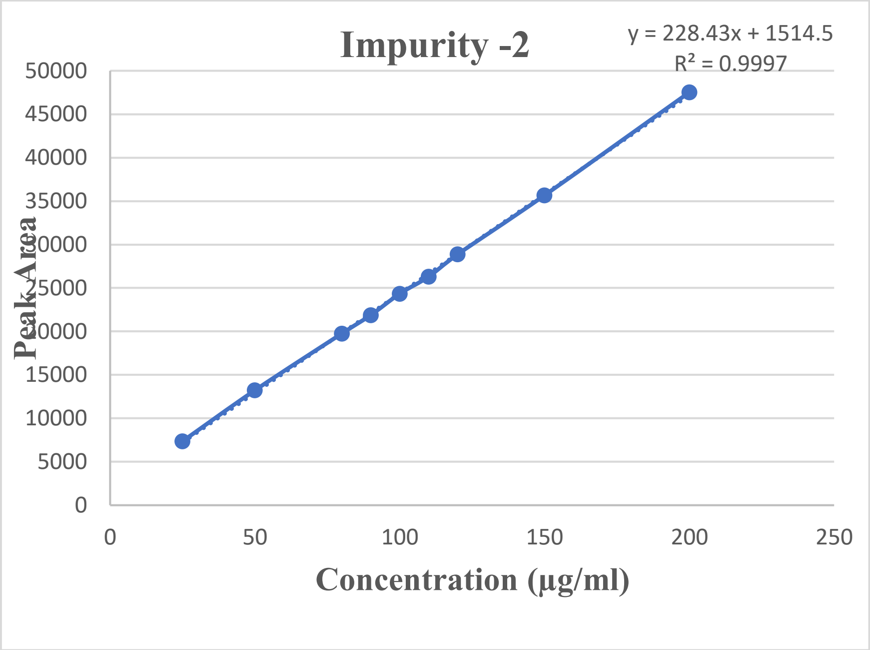

FIGURE 56 Linearity of Impurity-2

CHAPTER-6

CONCLUSION

- The present research successfully achieved the development and validation of a novel, robust, and reliable Reverse Phase High-Performance Liquid Chromatographic (RP-HPLC) method for the simultaneous separation, identification, and quantification of impurities in Atazanavir sulphate (ATZ) and Etravirine (ETR).
- The method complied with ICH Q2 (R1) guidelines and demonstrated excellent sensitivity, specificity, and resolution, making it suitable for routine pharmaceutical quality control. **Comprehensive impurity profiling revealed the presence of only one major impurity in each drug**. These impurities were structurally characterized using advanced analytical techniques including **Mass Spectroscopy (MS), Nuclear Magnetic Resonance (NMR), and Fourier Transform Infrared Spectroscopy (FT-IR)**, **thus confirming their identity and chemical nature**.
- The developed method not only ensures the safety, efficacy, and regulatory compliance of anti-HIV drug formulations but also fills a significant gap in existing literature, where limited work has been done on impurity profiling for these specific APIs.
- **Overall, this study contributes a valuable analytical tool for impurity analysis and enhances the quality control standards of pharmaceutical formulations containing ATZ and ETR.**

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LIST OF PUBLICATIONS

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[2]. Impurities profiling, method development and validation of Etravirine (ETR) in dosage forms by chromatography as per ICH guidelines. (2024) *Afr. J. Biomed. Res.*, 27(4s), pp. 98–107. doi:10.53555/AJBR.v27i4S.3262

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Impurities Profiling and Quantification of Atazanavir Sulphate (ATZ) and its Impurities in their Dosage Forms by Gradient RP-HPLC Method

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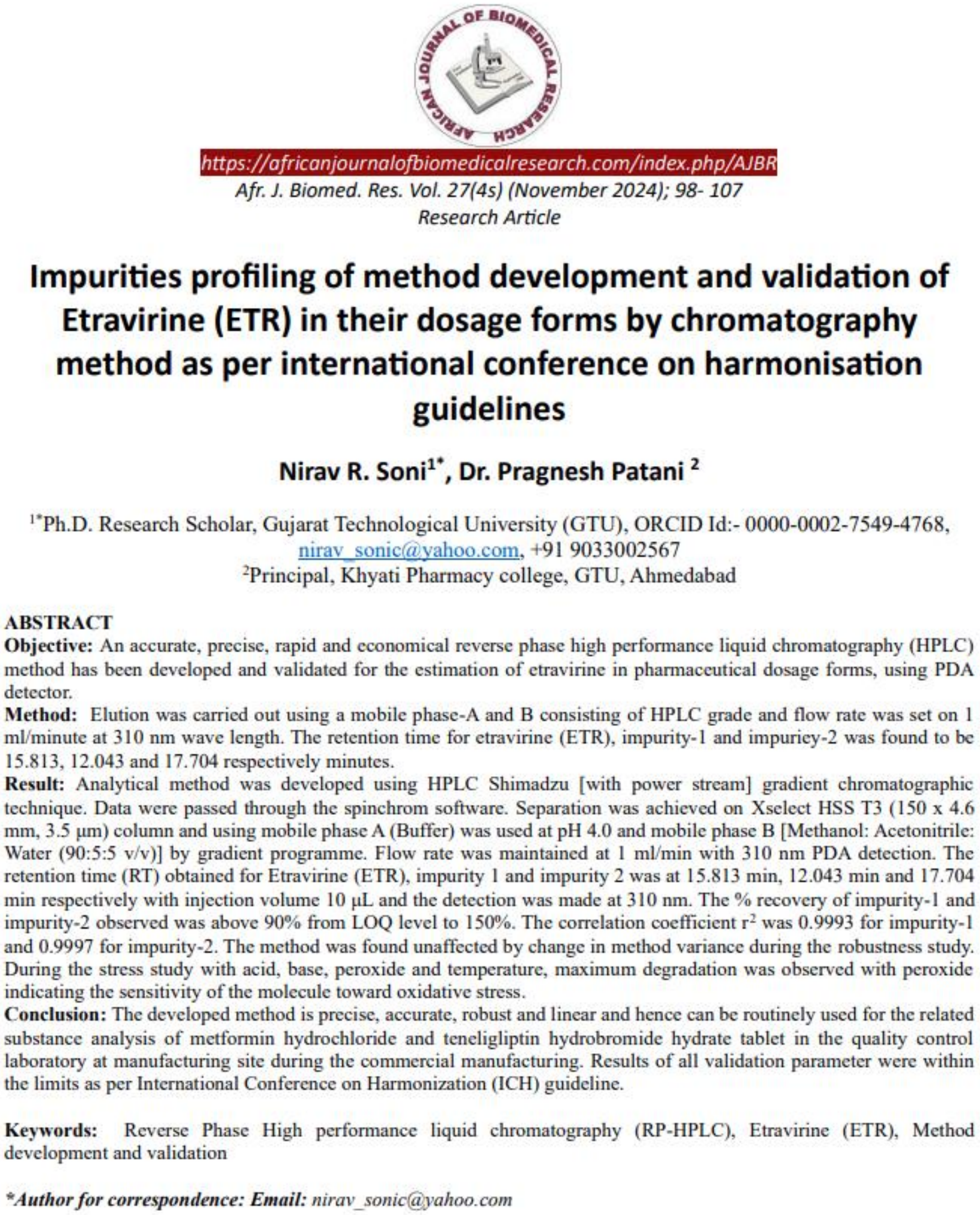
ABSTRACT

Analytical method was developed using HPLC Shimadzu (with power stream) gradient chromatographic technique. Data were passed through the spinchrom software. Separation was achieved on hypersil based deactivated silica C18 (250 mm × 4.6 mm, 5 μm) column using mobile phase composition of 0.05 M Potassium phosphate buffer: Methanol (60 v/v:40 v/v), (15 v/v:85 v/v), (60 v/v:40 v/v), adjusted to pH 4 with 1% orthophosphoric acid. Makeup volume with water. The flow rate of mobile phase was maintained at 1 ml/min with wavelength 225 nm UV detection. The Retention Time (RT) found for Atazanavir sulphate (ATZ), impurity A and impurity 5 was at 5.3 min, 6.23 min and 14.53 min respectively with an injection volume of 20 ml and the detection was made at 225 nm. Validation of the method was successfully established by performing various validation parameters such as accuracy, precision, specificity, linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), ruggedness, robustness, according to ICH guidelines.

Keywords: Atazanavir sulphate; Impurity-A and impurity-5; RP-HPLC; Gradient elution; Force degradation study; Atazor capsules (300 mg); Atavir (300 mg)

INTRODUCTION

Chemically, Atazanavir sulphate(ATZ)1 is a methyl N-[(2S)-1-[2-[(2S,3S)-2-hydroxy-3-[[(2S)-2-(methoxycarbonylamino)-3,3-dimethylbutanoyl]amino]-4-phenylbutyl]-2-[(4-pyridin-2-ylphenyl)methyl]hydrazinyl]-3,3dimethyl-1-oxobutan-2-yl] carbamate. ATZ is an oral antiretroviral drug that specifically belongs to the protease inhibitors class used in the treatment of HIV/AIDS. Literature survey reveals few chromatographic methods for the determination of ATZ in combination with other anti-retroviral drugs in bodyfluids, one assay with quantification of impurity method in active pharmaceutical excipients and one assay in dosage form 10. The present paper aims at reporting precise, accurate, selective, sensitive, robust and rugged validated RP-HPLC method for the estimation of ATZ and its known impurity A and impurities in the marketed dosage form (Figures 1-3) [1].



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Research Article

Impurities profiling of method development and validation of Etravirine (ETR) in their dosage forms by chromatography method as per international conference on harmonisation guidelines

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ABSTRACT

Objective: An accurate, precise, rapid and economical reverse phase high performance liquid chromatography (HPLC) method has been developed and validated for the estimation of etravirine in pharmaceutical dosage forms, using PDA detector.

Method: Elution was carried out using a mobile phase-A and B consisting of HPLC grade and flow rate was set on 1 ml/minute at 310 nm wave length. The retention time for etravirine (ETR), impurity-1 and impuriey-2 was found to be 15.813, 12.043 and 17.704 respectively minutes.

Result: Analytical method was developed using HPLC Shimadzu [with power stream] gradient chromatographic technique. Data were passed through the spinchrom software. Separation was achieved on Xselect HSS T3 (150 x 4.6 mm, 3.5 µm) column and using mobile phase A (Buffer) was used at pH 4.0 and mobile phase B [Methanol: Acetonitrile: Water (90:5:5 v/v)] by gradient programme. Flow rate was maintained at 1 ml/min with 310 nm PDA detection. The retention time (RT) obtained for Etravirine (ETR), impurity 1 and impurity 2 was at 15.813 min, 12.043 min and 17.704 min respectively with injection volume 10 µL and the detection was made at 310 nm. The % recovery of impurity-1 and impurity-2 observed was above 90% from LOQ level to 150%. The correlation coefficient r^2 was 0.9993 for impurity-1 and 0.9997 for impurity-2. The method was found unaffected by change in method variance during the robustness study. During the stress study with acid, base, peroxide and temperature, maximum degradation was observed with peroxide indicating the sensitivity of the molecule toward oxidative stress.

Conclusion: The developed method is precise, accurate, robust and linear and hence can be routinely used for the related substance analysis of metformin hydrochloride and teneligliptin hydrobromide hydrate tablet in the quality control laboratory at manufacturing site during the commercial manufacturing. Results of all validation parameter were within the limits as per International Conference on Harmonization (ICH) guideline.

Keywords: Reverse Phase High performance liquid chromatography (RP-HPLC), Etravirine (ETR), Method development and validation

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