A Ph.D. Synopsis

Of the thesis entitled

Impurity Profiling And Quantification Of Selected Anti-HIV Drugs in Their Dosage Forms By Chromatography Technique

Submitted for the award of the degree of

DOCTOR OF PHILOSOPHY

in

Pharmacy

to

GUJARAT TECHNOLOGICAL UNIVERSITY (GTU) Ahmedabad

By:

Nirav Rajendrakumar Soni

(Enrollment No. 189999901009)

Under The Supervision Of:

Dr. Pragnesh Patani (M. Pharm, Ph.D.)

Principal, Khyati College of Pharmacy, Ahmedabad, Gujarat-380058

GUJARAT TECHNOLOGICAL UNIVERSITY

AHMEDABAD

JUNE-2025

CONTENTS

1 Abstract

1.1 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 0.05 M 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.

1.2 Etravirine (ETR)

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. 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.

2 Introduction

2.1 Basic information about impurities profiling

Impurities are substances such as starting materials, intermediates, or by-products that coexist with the parent drug or are formed through side reactions. Interest in the presence of

impurities in active pharmaceutical ingredients (APIs) has grown significantly. Today, regulatory agencies not only require information on the purity profile but also mandate detailed impurity profiling.

An impurity is defined as any constituent present in an excipient, drug substance (Active Pharmaceutical Ingredient -API), or drug product (finished dosage form) that is not the active drug substance, excipient, or part of the intended formulation. This broad definition includes a wide range of chemical entities that may arise during synthesis, formulation, packaging, or storage.

Notably, this definition is inclusive of degradation products, which are considered a major class of impurities.

According to the International Council for Harmonization (ICH) guidelines (www.ich.org, 2012), a Degradation Product (DP) is defined as**:**

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

Such degradation products may form due to environmental factors such as heat, light, moisture, pH, oxidation, or interactions with excipients and packaging materials, and are critical to evaluate for drug safety and efficacy.

Impurity profiling refers to the process of detecting, identifying, characterizing, and quantifying both known and unknown impurities in new drug substances. This includes organic and inorganic impurities as well as residual solvents. Regulatory bodies like the ICH, USFDA, UKMHRA, and CDSCO (India) have developed specific guidelines to ensure the proper management and control of impurities. Impurities are classified into different categories based on their source, chemical nature, and biological safety. The presence of these unwanted substances can impact the safety, efficacy, and overall quality of pharmaceutical products. This review highlights the sources and classification of impurities and outlines the various analytical techniques used for their identification and quantification. Common terms related to impurities include residual solvents, by-products, transformation products, decomposition products, reaction products, and related substances.

The regulatory guidelines for impurities in API monitoring and control can have varying interpretations. Therefore, it is important to use simple and consistent terminology when discussing impurity-related topics.

The U.S. Food and Drug Administration (USFDA) follows guidelines developed by the International Conference on Harmonisation (ICH). These ICH guidelines for impurities were jointly created by regulatory agencies from the European Union (EU), Japan, and the United States to ensure consistency in data requirements submitted to different authorities.

This guidance helps sponsors of new drug applications (NDAs) and abbreviated new drug applications (ANDAs) prepare the necessary impurity-related documentation. It also assists FDA reviewers and researchers in applying and interpreting the regulatory requirements consistently.

The following are various regulatory requirements of ICH Guideline

1. Stability Testing of New Drug Substances and Products.

2. Impurities in New Drug Substances.

3. Impurities in New Drugs.

4. Recommendations Impurities: Residual Solvent.

5. USFDA Guidelines for Impurities in NDAs in New Medicinal Substances.

6. ANDA Impurities in New Drug Substances.

7. Australian Prescription Drug Regulatory Guide, Treatment Australia (TGA)

2.2 Introduction of Anti-HIV Drugs

HIV-the Human Immunodeficiency Virus is the retrovirus that causes AIDS. HIV belongs to the retrovirus subfamily lentivirus. HIV attaches to cells with CD4 receptors (T_4 cells and macrophages). AIDS remains a serious threat because of the expense and inaccessibility of antiretroviral agents in the developing countries in which the disease is most prevalent. In addition, the effectiveness of antiretroviral drugs has been diminished by the emergence of multidrug- resistant virus,[1,5,7]

3 Brief description on the state of the art of the research topic

Impurity profiling in pharmaceuticals is essential for ensuring drug safety and quality. Regulatory agencies like ICH, USFDA, and CDSCO require detailed impurity analysis in APIs and formulations. Techniques such as HPLC are widely used for detecting and quantifying impurities. Validated methods for drugs of Atazanavir sulphate (ATZ) and

Etravirine (ETR) demonstrate high accuracy and compliance with international guidelines. This is particularly crucial in anti-HIV therapy, where drug resistance and patient safety are major concerns**.**

4 Literature Review

An extensive literature survey was conducted to explore existing analytical methods related to anti-HIV drugs. Numerous studies have focused on the development and validation of stability-indicating methods, chemical synthesis, and characterization techniques, particularly for drugs such as Darunavir, Ritonavir, Atazanavir, and Efavirenz. These methods primarily employed chromatographic techniques including HPLC, RP-HPLC, UPLC, and UPLC-MS/MS. A few reports have also documented simultaneous estimation and validation approaches for selected antiretroviral (ARV) combinations. Despite the clinical significance of these agents, a significant research gap remains in the area of impurity profiling and quantification, especially for Atazanavir sulphate (ATZ) and Etravirine (ETR) in their pharmaceutical dosage forms. Most published methods are limited to assay, stability studies, or process-related impurities in APIs and biological matrices, lacking in comprehensive impurity characterization from finished products.[26-56]

Only one reported study discusses forced degradation of Etravirine, identifying seven impurities, two of which align with those listed in the USP Draft 2023. No official monograph for ETR currently exists in Indian Pharmacopoeia (IP), British Pharmacopoeia (BP), or European Pharmacopoeia (EP). The draft method in USP (2023) utilizes RP-HPLC with gradient elution, UV detection at 310 nm, and a 10 mM ammonium formate buffer, but fails to comprehensively address all known and unknown impurities.[75]

Furthermore, existing literature on RP-HPLC and UPLC methods for ARV drug combinations such as Atazanavir sulphate + Cobicistat[55]**,** Atazanavir sulphate + Ritonavir[29] is generally limited to bioanalytical quantification or in-process impurity profiling, rather than complete impurity separation from formulated products.

Hence, there is a need for a simple, sensitive, and well-validated RP-HPLC method capable of simultaneous detection and quantification of both known and unknown impurities of ATZ and ETR in dosage forms individually, which is critical for ensuring safety, efficacy, and regulatory compliance**.**

5 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 Atazanavir sulphate (ATZ) and Etravirine (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.

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.

6 Aims and Objectives

6.1 Aims

An extensive literature review indicates that only a limited number of chromatographic methods have been reported for the determination of ATZ and ETR both in combination with other antiretroviral agents in biological matrices. Additionally, only two analytical methods address 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 fulfil regulatory and pharmaceutical quality requirements.

6.2 Objectives

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

1. **To develop a simple, robust, and reproducible Reverse Phase High-Performance Liquid Chromatographic (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 ETR and ATZ formulations, including degradation products and structurally related substances.

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

3. **To validate the developed RP-HPLC method** as per International Council for Harmonisation (ICH) guidelines (Q2(R1)), ensuring parameters such as specificity, linearity, accuracy, precision, robustness, detection limit (LOD), and quantification limit (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.

This analytical approach provides a critical tool for pharmaceutical quality control, especially for impurity profiling in anti-HIV drug formulations.

7 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 impurities at trace levels, meet regulatory requirements, and ensure the safety, efficacy, and quality of the drug product. It

also addresses the need to analyse structurally similar impurities commonly present in single-molecule drugs like ATZ and ETR**.**

8 Original contribution by the thesis

This thesis outlines the development and validation of a novel, robust reverse-phase high-performance liquid chromatography (RP-HPLC) method designed for the simultaneous separation and quantification of both known and unknown impurities in Atazanavir sulphate (ATZ) and Etravirine (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 centres 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.

9 Methodology of research, results

TABLE 1: - Test & Method of Analysis

Order of Injections

Sr. No	Solution Name	No. of Injections
1.	**Diluents as blank**	Confirm neat blank
2.	**SST Solution**	1
3.	**Diluent as blank**	Confirm neat blank
4.	**Reference solution**	6
5.	**Sample solution preparation-1**	1
6.	**Sample solution preparation-2**	1

7.	**SST solution**	1
8.	**Diluent as blank**	Confirm neat blank
9.	**Reference solution**	1

10 Selected drug and its impurities

10.1 Atazanavir sulphate (ATZ)

10.1.1 Selection of impurities

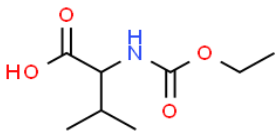
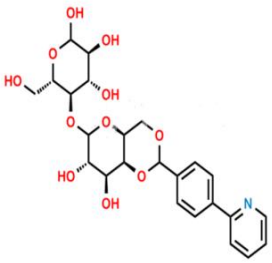
Potential impurities of Atazanavir sulphate (ATZ) were not separated from main analytes in the reported methods and it is the most common in their dosage forms. It has produces genotoxicity.[74]

Hence, no liquid chromatographic methods (HPLC and UPLC) were reported for the determination of ATZ 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 ATZ and its impurities in the fixed dose products.

Finally, it was selected two impurities in this project Impurity 1 (Atazanavir related compound A) and Impurity 2 (Atazanavir Impurity 5) respectively.

TABLE 2: - Selected impurities of ATZ

Sr. No	Parameter	Atazanavir related compound A	Atazanavir impurity-5
1.	**CAS no.**	162537-11-3	NA
2.	**Molecular structure**		
3.	**Molecular formula**	$C_8H_{15}NO_4$	$C_{24}H_{29}NO_{11}$

4.	**Molecular weight**	189.21	507.5
5.	**CAT no.**	SZ-A009003	SZ-A009D01
6.	**Category**	USP standards	USP standards

10.1.2 Experimental works

Development and optimization of the RP-HPLC method

Method development and optimization has been carried out in a systematic approach by considering various aspects which will play major role in the separation. Different factors such as buffer pH, column chemistry, organic solvent and other chromatographic parameters were chosen as summarized below.

Selection of buffer strength and pH

Optimum buffer strength shall be maintained for attaining reproducible separation between the impurities. Since phosphate buffer is having wide range of pKa values, 0.05M concentration potassium dihydrogen orthophosphate (KH_2PO_4) buffer was selected for initial study. Considering the presence of "amine" functional groups in both the drug components, initial trials were taken by adjusting the buffer pH to 4.0 ± 0.05. However, better separation of all the desired peaks with good resolution was achieved at pH 4.0.

Hence, the buffer pH was fixed at 4.0 to ensure improved column performance at this acidic pH. To attain baseline resolution of impurities and for eluting late eluting non-polar impurities, methanol was used as organic solvent and found satisfactory separation with reduced retention times. Also, sharp peaks with good responses were observed. Hence, Methanol and 0.05 M Potassium dihydrogen phosphate buffer (pH-4.0) was used as organic solvent.

Preparation of Mobile Phase

After considering the varying combinations of various mobile phases, Buffer: Methanol used by gradient RP-HPLC method. [Buffer (0.05 M KH_2PO_4, pH 4.0) Take 6.8 gm KH_2PO_4 in to a 1000 ml beaker, add 800 ml water and dissolve, adjust pH 4.0 with 0.1 N NaOH, make

up Volume 1000 ml with water] was finalized as it was showing good peak shapes and a significant amount of resolution.

Evaluation of stationary phase

Selection of stationary phase (column) plays critical role in the separation of impurities along with both the drug components. Since impurities of ATZ and the main drug is having different polarities, column used in the method shall separate all these compounds with satisfactory resolution. Available RP-HPLC column chemistries such as High Strength Silica (HSS) and Hypersil Base Deactivated Silica (BDS) column were tried for this purpose. Among these, hypersil BDS C18 (250 x 4.6 mm, 5µm) column shows optimum separation between all the desired peaks. Hypersil BDS C18 column contains trifunctional ligand bonded C18 ligand chemistry which produce superior low pH stability and ultra-low column bleed. This low pH stability is combined with the high pH stability of the 5 µm BDS particle to deliver the widest usable pH range. This new chemistry also utilizes new, proprietary end capping process which produce outstanding peak shape for bases. Hence, this column was considered for the entire study.

Optimization of gradient program

Since impurities present in ATZ having wide range of polarities, it is necessary to adopt gradient elution mode instead of isocratic elution mode. Trials were taken by changing the composition of buffer and methanol at fixed flow rate of 1 ml/min. Among different trials performed, gradient program was finalized in which, all the required components were well separated.

TABLE 3:- Gradient program

Time(min)	0-7	7-20	20-25
Flow rate (ml/min)	1	1	1
% Buffer	60	15	60
% Methanol	40	85	40

Evaluation of flow rate and column oven temperature

For optimum retention times 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**.**

Selection of detector wavelength

Impurities of ATZ show spectral absorption maxima at ~225 nm. Hence, wavelength of ~225 nm has been chosen for quantification of impurities due to satisfactory sensitivity and optimum responses.

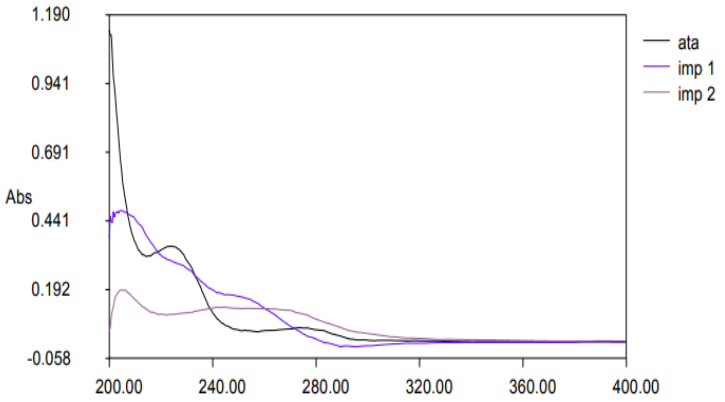


FIGURE 1: - ATZ and impurities spectral absorption

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.

TABLE 4:- Optimized chromatographic conditions for proposed method

Parameter	Method
Types of chromatography	Reverse phase (RP)
Mode of operation	Gradient
Stationary phase (Column)	Hypersil BDS C18 column (250mm X 4.6mm i.d.,5µ)
Mobile phase	Methanol: 0.05M potassium phosphate buffer (pH 4.0)
Flow rate (mL/min)	1.0
Run time (Minutes)	15
Column temperature (^{0}C) :	40
Volume of injection loop (µL)	20
Detection wavelength (nm)	210

Diluent	Methanol
Detector	PDA

Preparation of stock solution

Weigh and powdered 20 capsules. Disperse the content of capsules containing about 20 mg ATZ with 60 ml of the methanol in the 100 ml volumetric flask. Sonicate for 15 minutes and make up volume with methanol. Filter this solution with Whatman filter paper no-1. (ATZ-200 µg/ml)

Preparation of standard working solution

From the stock solution pipette out 1ml into 10 ml volumetric flask and makeup the final volume with mobile phase (ATZ-20 µg/ml).

Preparation of mobile phase

Mobile Phase A: 0.05 M Potassium dihydrogen phosphate buffer (pH-4.0). Take 6.8 gm potassium dihydrogen orthophosphate (KH_2PO_4) into a 1000 ml beaker. Add 800 ml water and dissolve with water. Adjust pH 4.0 with 1% orthophosphoric acid. Make up volume with 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)

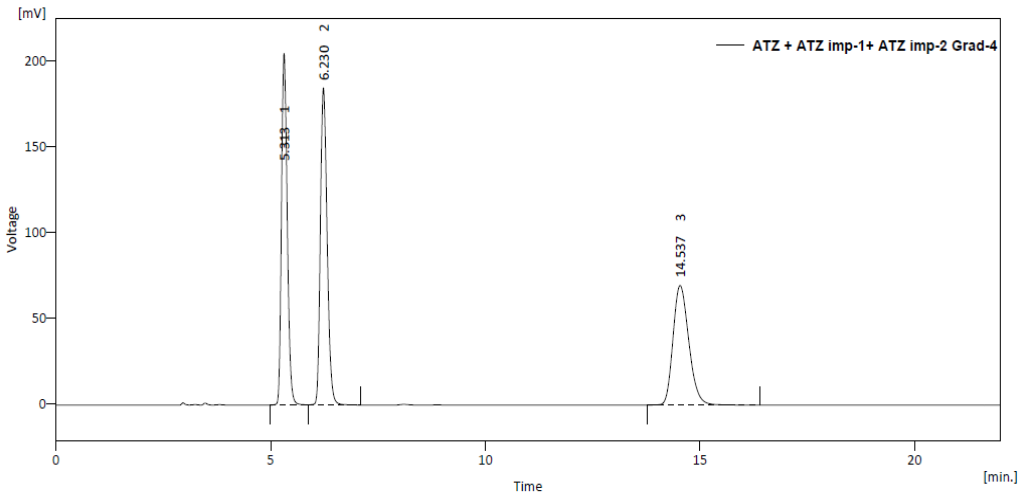


FIGURE 2: - ATZ sample chromatogram

Analysis of commercial formulation

The proposed method was applied for the determination of ATZ impurities estimation in marketed capsules 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**.**

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 5: - Analysis of ATZ formulation

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

10.1.3 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 0.05 M 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.**

10.2 Etravirine (ETR)

10.2.1 Selection of Impurities

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 liquid chromatographic methods (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 two impurities in this project impurities 1 (**3- amino bromo)** and 2 (**Des amino impurity).**

TABLE 6:- Selected impurities of ETR

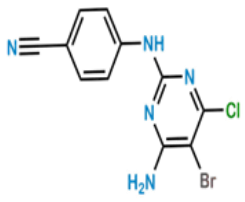
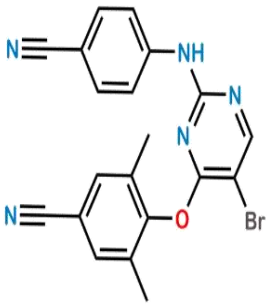
Sr.No	Parameter	Impurity-1	Impurity-2
1.	**Marketed Name**	**3- amino bromo**	**Des amino impurity**
2.	**CAS no**	1398507-09-9	269055-04-1
3.	**Molecular structure**		
4.	**Molecular Formula**	$C_{11}H_7BrC_1N_5$	$C_{20}H_{14}BrN_5O$
5.	**Molecular weight**	324.6	420.26

TABLE 7 :- Optimized chromatographic conditions for proposed method

Parameter	Method
Types of chromatography	Reverse phase (RP)
Mode of operation	Gradient
Stationary phase (Column)	X select 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 (^{0}C):	35
Volume of injection loop (µl)	10
Detection wavelength (nm)	310
Detector	PDA
Sample concentration	0.7 mg/ml
Retention Time (Rt)	Etravirine Peak between 15.2-15.6 min

10.2.2 Experimental Work

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.

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

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.

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.

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

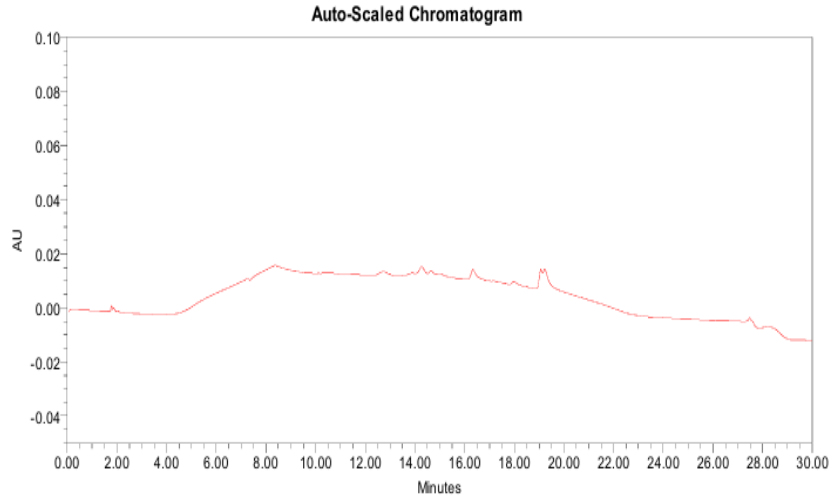
Mobile phase B: Methanol: Acetonitrile: Water (90:05:05 v/v/v)

TABLE 8 :- Gradient programme

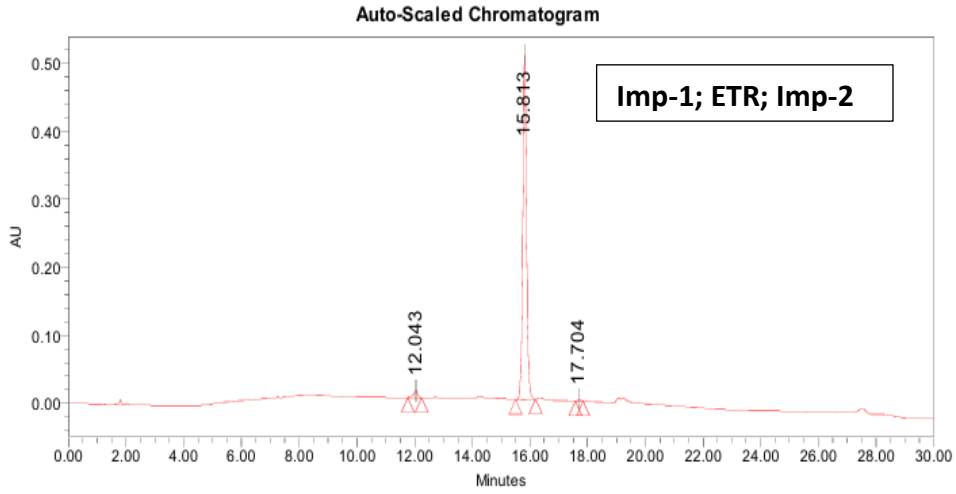
Time (in mins)	Mobile Phase-A (%)	Mobile Phase- B (%)
0	30	70
10	30	70
22	5	95
35	5	95
35.5	30	70
42	30	70

10.2.3 Final analytical method

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. 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 impurity-2 was found to be 15.813, 12.043 and 17.704 respectively minutes.



(FIGURE 3: - Representative chromatogram of blank solution)



(FIGURE 4: - Representative chromatogram of ETR and its impurities)

11 Achievements with respect to objectives

11.1 Successful Development of RP-HPLC Method

A novel and efficient RP-HPLC method was successfully developed for the simultaneous separation of Atazanavir sulphate (ATZ) and Etravirine (ETR), along with their impurities, in marketed formulations.

11.2 Identification and Quantification of Impurities

The method allowed for accurate identification of specified (known) impurities and quantification of unspecified (unknown) impurities at trace levels, in compliance with ICH Q3A/B guidelines for impurity limits in pharmaceutical substances and products.

Identification and Profiling of Impurities

Each drug was found to contain only one major impurity. Complete impurity profiling was achieved using a combination of advanced techniques including **Mass Spectroscopy (MS)**, **Nuclear Magnetic Resonance (NMR)**, and **Fourier Transform Infrared Spectroscopy (FT-IR)**, enabling accurate structural elucidation of these impurities.

11.3 Validation as per ICH Guidelines

The developed method was validated according to ICH Q2(R1) guidelines and met all critical parameters including accuracy, precision, linearity, limit of detection (LOD), limit of quantitation (LOQ), specificity, and robustness.

11.4 High Sensitivity and Selectivity

The method displayed excellent sensitivity with LOD and LOQ values sufficient to detect even the lowest impurity concentrations, ensuring a high level of safety and efficacy of the drug products.

11.5 Short Runtime and Operational Efficiency

The optimized method offered a reduced analysis time without compromising on resolution and sensitivity, making it suitable for routine quality control in pharmaceutical manufacturing. The developed method offered a significantly reduced runtime, enabling faster analysis without compromising on quality, thereby enhancing operational efficiency in laboratory workflows.

11.6 Applicability to Marketed Formulations

The method was successfully applied to analyse commercial formulations of Atazanavir sulphate (ATZ) and Etravirine (ETR) individually confirming its practical utility in real-world pharmaceutical analysis.

12 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.

13 Paper publication

[1]. Impurities Profiling and Quantification of Atazanavir sulphate (ATZ) and its Impurities in Their Dosage Forms by Gradient RP-HPLC Method. (2022). *Der Pharma Chemica*, 14(10), pp. 1–13. doi:10.4712/0975-413X.14.10.1-13

[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

14 Achievements

1. Recently selected in Harvard University (**The Harvard Project for Asian and International Relations- HPAIR)-2025 conference**, USA, MA sponsored by **Harvard university.**
2. Meritorious academic background.
3. Qualified **GPAT Exam**, Got stipend **12.400 INR** per month up to two years during post-graduation program from AICTE.
4. Qualified National Institute of Pharmaceutical Education and Research **(NIPER)** entrance test.

5. I had done **PGDIPR** (Post Graduate Diploma Intellectual Property Rights) and **PGDRD** (Post Graduate Diploma Rural Development) course
6. Certificate of merit received from **"Dr. P D. Shethi Award"** in M. Pharm (QA)-Research paper
7. 7 books author and published articles in reputed national and international journal like WoS and Scopus.
8. Editorial member and reviewer in various national and international journals.
9. Selected in **"TOP-50 Smart education projects in India"** for pharmaceutical research by **Skoch Order-of-Merit.**

15 References

1. U.S. Department of Health and Human Services, Replication cycle, AIDS info. [Online]. Available: https://hivinfo.nih.gov/understanding-hiv/fact-sheets/hiv-life-cycle [Accessed: 9 April 2025].
2. NIAID, HIV Life Cycle. [Online]. Available: http://www.niaid.nih.gov/daids/dtpdp/virpage1.htm [Accessed: 5 Oct 2004].
3. Jones A. (2019) Types of anti-retroviral medication, NAM AIDS Map, HIV & AIDS Sharing Knowledge. [Online]. Available: http://www.aidsmap.com/about-hiv/types anti-retroviral-medications [Accessed: 14 April 2024].
4. Talla V. (2014) Anti-retroviral drugs. PowerPoint presentation, Slideshare.net. [Online]. Available: https://www.slideshare.net/DrVijayBhushanam/vj-antiretroviral [Accessed: 15 January 2014].
5. Soni N.R. (2014) Modern technology of pharmaceutical analysis, Pharmamedix India Publication Pvt. Ltd., pp. 90-151.
6. Soni N.R. (2014) Modern technology of pharmaceutical analysis, Pharmamedix India Publication Pvt. Ltd., pp. 314-320.
7. Soni N.R. (2013) Illustrated description of modern analytical technique (MAT), Lambert Academic Publishing House (LAP), Germany Pvt. Ltd., pp. 18-107.
8. Soni N.R. (2013) Illustrated description of modern analytical technique (MAT), Lambert Academic Publishing House (LAP), Germany Pvt. Ltd., pp. 344-363.
9. Benish M., Bartal I., Goldfarb Y., Levi B., Avraham R., Raz A., Eliyahu S.B. (2008) J. Clin. Oncol., 15, pp. 2042-2052.

10. Gudrun F. (2001) Pharmaceutical regulatory guidelines. Drug Inf. J., 35, pp. 865–874.

11. Tzanavaras P.D., Verdoukas A., Balloma T. (2006) Spectrophotometric determination of pharmaceutical substances. J. Pharm. Biomed. Anal., 41, pp. 437–441.

12. Food and Drug Administration. (1997) Guidance for Industry: Dissolution Testing of Immediate Release Solid Oral Dosage Forms. US Department of Health and Human Services/Food and Drug Administration/Center for Drug Evaluation and Research, Rockville, MD.

13. Siewert M., Dressman J., Brown C.K., Shah V.P. (2003) Dissolution testing of pharmaceutical products. AAPS PharmSciTech, 4, Article 7.

14. Kasture A.V., Mahadik K.R., Wododkar S.G., More H.N. (2002). A Textbook of Pharmaceutical Analysis, 17th ed., Nirali Prakashan, Pune, pp. 48–57.

15. Shethi P.D. (1996) HPLC: Quantitative Analysis of Pharmaceutical Formulations. CBS Publishers & Distributors, New Delhi, pp. 3–46.

16. Chatwal G.R. (2002) Instrumental Method of Chemical Analysis, Part-1, 5th ed., Himalaya Publishing House, pp. 2624–2631.

17. Robinson J.W., Skelly Frame E.M., Frame G.M. (2005) Undergraduate Instrumental Analysis, 6th ed., Marcel Dekker, pp. 806.

18. Ahuja S., Scypinski S. (2001) Handbook of Modern Pharmaceutical Analysis. Academic Press, pp. 356–367.

19. Yord L.L., Snyder R., Kirkland J.J., Glajch J.L. (1997) Practical HPLC Method Development, 2nd ed., John Wiley & Sons, New York.

20. NIAID. HIV Life Cycle. [Online]. Available: http://www.niaid.nih.gov/daids/dtpdp/virpage1.htm [Accessed: Oct 2004].

21. Baltimore D. (1971) Expression of animal virus genomes. Bacteriol. Rev., 35(3), pp. 235–241. doi:10.1128/MMBR.35.3.235-241.1971

22. De Clercq E. (2002) New anti-HIV agents and targets. Med. Res. Rev., 22(6), pp. 531–565.

23. El Kouni M.H. (2002) Trends in the design of nucleoside analogues as anti-HIV drugs. Curr. Pharm. Des., 8(8), pp. 581–593.

24. Block J.H., Beale J.M. (2004) Antiviral Agents. In: Wilson and Gisvold's Textbook of Organic Medicinal and Pharmaceutical Chemistry, 11th ed. Maryland: Lippincott Williams & Wilkins, pp. 379, 943.

25. De Clercq E., Vandamme A.-M. (2004) Combination Therapy of AIDS. Germany: Birkhäuser Verlag.

26. Indian Pharmacopoeia (2018) Monograph of Atazanavir sulphate, Volume I, pp. 1279–1280. Available: https://ipc.gov.in/images/Atazanavir_Sulphate.pdf [Accessed: 7 Feb 2024].

27. Chitturi S.R. et al. (2011) Gradient RP-HPLC method for the determination of potential impurities in Atazanavir sulphate. J. Pharm. Biomed. Anal., 55(1), pp. 31–47. doi: 10.1016/j.jpba.2011.01.002

28. Chitturi S.R. et al. (2011) Gradient RP-HPLC method for impurities in Atazanavir sulphate. J. Pharm. Biomed. Anal., 55(1), pp. 31–47.

29. Mantripragada M.K.V.V.N. et al. (2015) Simultaneous determination of impurities of Atazanavir and Ritonavir. J. Chromatogr. Sci., 56(3), pp. 270–284. doi:10.1093/chromsci/bmx110

30. Killi G.D. et al. (2014) A novel validated UPLC method for Lopinavir and Ritonavir. Braz. J. Pharm. Sci., 50. doi:10.1590/s1984-82502014000200009

31. Hamarapurkar P.D., Parate A.N. (2013) HPLC for emtricitabine and degradation products. J. Chromatogr. Sci., 51(5), pp. 419–424. doi:10.1093/chromsci/bms157

32. Rao R.N. et al. (2013) RP-HPLC and impurity analysis of Darunavir. J. Pharm. Biomed. Anal., 75, pp. 186–191. doi: 10.1016/j.jpba.2012.10.022

33. Devrukhakar P.S. et al. (2017) LC-MS/MS of Zidovudine degradation. J. Pharm. Anal., 7(4), pp. 231–236. doi:10.1016/j.jpha.2017.01.006

34. Zhang L.K. et al. (2016) Impurity analysis of Doravirine by UHPLC-MS. J. Mass Spectrom., 51(10), pp. 959–968. doi:10.1002/jms.3807

35. Chitturi S.R. et al. (2008) Lopinavir impurity profiling by HPLC. J. Pharm. Biomed. Anal., 48(5), pp. 1430–1440. doi: 10.1016/j.jpba.2008.09.015

36. He J. et al. (2015) Identification of impurities in Tenofovir. Pharmazie, 70(5), pp. 283–288. doi:10.1691/ph.2015.4132

37. Mondhe C.C., Dabhade P.S. (2017) RP-HPLC for Atazanavir assay. World J. Pharm. Pharm. Sci., 6(8), pp. 1317–1334.

38. Srinivasu K. et al. (2011) RP-HPLC of Atazanavir dosage forms. E-Journal of Chemistry, 8(1), pp. 453–456.

39. Naazneen S., Sridevi A. (2017) RP-HPLC of Atazanavir and Cobicistat. IOSR-JPBS, 12(5), pp. 61–68.

40. Lakshmana Rao A., Raghu Ram M.S. (2012) RP-HPLC of Raltegravir. Int. J. Res. Pharm. Chem., pp. 217–220.

41. Annapurna M.M. et al. (2018) RP-HPLC of Raltegravir. Int. J. Green Pharm., 12(1), p. S180.

42. Estan-Cerezo G. et al. (2017) UV-HPLC of Darunavir and Raltegravir. Rev. Esp. Quimioter., 30(3), pp. 195–200.

43. Gupta A. et al. (2015) LC-MS/MS for Raltegravir in plasma. J. Pharm. Anal., 5(2), pp. 101–109. doi: 10.1016/j.jpha.2014.10.002

44. Vels University. Method development for Raltegravir and Rilpivirine. [Online]. Available: https://shodhganga.inflibnet.ac.in/bitstream/10603/54508/14/15_chapter6.pdf

45. Bhavyasri K. et al. (2015) Raltegravir degradation by LC-MS/MS. J. Pharm. Sci. Res., 7(9), pp. 685–689.

46. Notari S. et al. Simultaneous HPLC-UV of Maraviroc and Raltegravir. IUBMB Life, 61(4), pp. 470–475.

47. Singh V.D., Daharwal S.J. (2017) RP-HPLC for Lamivudine and Raltegravir. Eurasian J. Anal. Chem., 12(3), pp. 179–195. doi:10.12973/ejac.2017.00162a

48. Lakshmi T., Annapurna A., Gupta K.R. (2015) RP-HPLC of Raltegravir in blood plasma. Int. J. Pharma Bio Sci., 6(1), pp. 113–120.

49. Chaves J. et al. (2013) Stability of Darunavir Ethanolate by HPLC. Chromatogr. Res. Int., Article ID: 834173. doi:10.1155/2013/834173

50. Rami Reddy B.V. et al. (2013) HPLC for Darunavir Ethanolate. J. Chromatogr. Sci., 51(5), pp. 471–476. doi:10.1093/chromsci/bms165

51. Bhaskar Reddy V. et al. (2016) UPLC-MS/MS of Darunavir impurities. J. Pharm. Biomed. Anal., 128, pp. 141–148. doi: 10.1016/j.jpba.2016.05.026

52. Venugopal N. et al. (2014) UPLC-MS/MS of phenol impurities in Ritonavir. J. Pharm. Biomed. Anal., 90, pp. 127–133. doi: 10.1016/j.jpba.2013.11.02

53. Ahamed A. et al. (2012) UPLC method for Etravirine. Int. J. Pharm. Pharm. Sci., 4(1), pp. 255–261.

54. Mulla S. et al. (2014) Impurity in Efavirenz. Int. J. Pharm. Pharm. Sci., 6(1), pp. 426–431.

55. Purnima B.V. et al. (2016) RP-HPLC of Cobicistat and Atazanavir. Eur. J. Biomed. Pharm. Sci., 3(5), pp. 450–461.

56. Vora P.K. et al. (2016) RP-HPLC for Cobicistat and Atazanavir. Eur. J. Biomed. Pharm. Sci., 5(4), pp. 111–132.

57. Sirisha V.S. et al. (2014) RP-HPLC for Darunavir. Int. J. Res. Pharm., 5(1), pp. 13–16.

58. Wikipedia. Atazanavir sulphate. [Online]. Available: https://www.wikidata.org/wiki/Q27114238

59. PubChem. [Online]. Available: https://pubchem.ncbi.nlm.nih.gov

60. Drug Bank. [Online]. Available: https://www.drugbank.ca/drugs/DB01072 [Accessed: 26 Dec 2019].

61. WIPO IPC. [Online]. Available: http://www.wipo.int/classifications/ipc/

62. Drugs.com. Atazanavir sulphate. [Online]. Available: https://www.drugs.com/monograph/atazanavir-sulfate.html [Accessed: 22 May 2019].

63. Piliero P.J. (2002) Atazanavir: a novel HIV-1 protease inhibitor. Expert Opin. Investig. Drugs, 11, pp. 1295–1301.

64. AK Scientific. [Online]. Available: https://aksci.com/item_detail.php?cat=N349

65. Singh J. et al. Bisulfate salt of HIV protease inhibitor. Patent.

66. Xu Z. et al. (2002) Synthesis of BMS-232632. Org. Process Res. Dev., 6(3), pp. 323–328.

67. Kim S. et al. (2005) Synthesis and forms of Atazanavir bisulfate. WO 2005108349 A2.

68. Piliero P.J. (2002) Atazanavir: a novel HIV-1 protease inhibitor. Expert Opin. Investig. Drugs, 11(9), pp. 1295–1301.

69. Karlgren M. et al. (2012) OATP inhibitors and drug interactions. J. Med. Chem., 55(10), pp. 4740–4763. doi:10.1021/jm300212s

70. BOC Sciences. Atazanavir and impurities. [Online]. Available: https://www.bocsci.com/im-atazanavir-and-impurities-list-366.html [Accessed: 17-21 August 2025].

71. Syn Zeal. Atazanavir. [Online]. Available: https://www.synzeal.com/rac-atazanavir-impurity-9-5

72. Sigma-Aldrich. IR spectrum table. [Online]. Available: https://www.sigmaaldrich.com/technical-documents/articles/biology/ir-spectrum-table.html [Accessed: 17-21 August 2025].

73. Indian Pharmacopoeia (2018) FTIR of Atazanavir sulphate, Volume I, pp. 419.

74. Bhavani K.G. et al. (2017) Genotoxic impurity in Atazanavir. J. Pharm. Biomed. Anal., 132(5), pp. 156–158. doi: 10.1016/j.jpba.2016.09.025

75. United States Pharmacopeia – USP-NF Online. [Online]. Available: https://online.uspnf.com/ [Accessed: 2023].

76. Ahamed A., Krishnamurthy G., Bhojya Naik H.S., Ramesha S. (2012) Development and validation of stability-indicating ultra-performance liquid chromatographic method for Etravirine. *Int. J. Pharm. Pharm. Sci.*, 4(1), pp. 255–261. ISSN: 0975-1491.

77. Raman S.G. (2021) Analytical method development and validation of Etravirine tablets by RP-HPLC. *Int. J. Res. Pharm. Nano Sci.*, 10(3), pp. 174–178.

78. Abhilash R., Punugoti R., Rao V., Jupally V. (2013) HPTLC method development and validation for determination of Etravirine in bulk and tablet dosage form. *Int. J. Pharm. Biol. Sci.*, 3, pp. 515–522.

79. Runja C., Pigili R.K. (2013) Development and validation of a new RP-HPLC method for estimation of Etravirine in bulk and pharmaceutical dosage form. *Semantic Scholar*.

80. Damle M. et al. (2017) Determination of Etravirine in human plasma by HPLC. *Indo Am. J. Pharm. Sci.*, 4(06).

81. Ghosh S., A. S., Bvv R. (2015) Analytical method development and validation of Etravirine in its bulk dosage form by using RP-HPLC method as per ICH guidelines. *Asian J. Pharm. Clin. Res.*, 8(2), pp. 147–150

82. Barath M., Chandan R.S., Maruthi R., Paramakrishnan N. (2021) Analytical method development and validation of Etravirine by RP-UFLC. *Asian J. Res. Chem.*, 14(7), pp. 3537–3542.

83. Satyanarayana L., Naidu S.V., Narasimha Rao M., Priyadarshini D. (2011) The estimation of Etravirine in tablet dosage form by RP-HPLC. *Asian J. Res. Chem.*, 4(10).

84. Quaranta S., Woloch C., Paccou A., Giocanti M., Solas C., Lacarelle B. (2009) Validation of an electrospray ionization LC-MS/MS method for quantitative analysis of Raltegravir, Etravirine, and 9 other antiretroviral agents in human plasma samples. *Ther. Drug Monit.*, 31(6), pp. 695–702. doi: 10.1097/FTD.0b013e3181c05adf.

85. Drug Bank. Etravirine. [Online]. Available: https://go.drugbank.com/drugs/DB06414 [Accessed: 25 Aug 2023].

86. Drugs.com. Etravirine Monograph. [Online]. Available: https://www.drugs.com/monograph/etravirine.html [Accessed: 08 May 2014].

87. FDA Approval History – Drugs.com. Etravirine (Intelence). [Online]. Available: http://www.etravirine/Intelenceetravirine.html [Accessed: 08 May 2014].

88. Synthink Chemicals. Etravirine Impurities. [Online]. Available: https://synthinkchemicals.com/product-category/impurities/etravirine-impurities/ [Accessed: 2023].

89. John J., Robinson K., John M., Caballero J.L., Ma J., Liang D., Abobo C.V. (2012) Simultaneous LC-MS/MS determination of racemic warfarin and Etravirine in rat plasma and its application in pharmacokinetic studies.

90. Quantification of darunavir and etravirine in human peripheral blood mononuclear cells using high performance liquid chromatography, ClinBiochem (2015) http://dx.doi.org/10.1016/j.clinbiochem.2015.12.011

91. Quaranta S., Woloch C., Paccou A., Giocanti M., Solas C., Lacarelle B. (2009) Validation of an electrospray ionization LC-MS/MS method for quantitative analysis of Raltegravir, Etravirine, and 9 other antiretroviral agents in human plasma samples. Ther. Drug Monit., 31(6), pp. 695–702. doi: 10.1097/FTD.0b013e3181c05adf. PMID: 19865000.

92. Ramesh K., Chandra Shekar B., Khadgapathi P. (2015) Formulation and evaluation of poorly soluble Etravirine by spray drying method. Int. J. Pharm. Pharm. Sci., 7(4), pp. 98–103.

93. Bhattacharyya S., Adhikari H., Regmi D., Hosuru R.R.V. (2022) Study on solubility enhancement of Etravirine by crystal engineering method. Indian J. Pharm. Sci., 84(3), pp. 575–585.

94. U.S. Food and Drug Administration. NDA Approval Document for Etravirine (Intelence). [Online]. Available: https://www.accessdata.fda.gov/drugsatfda_docs/nda/2008/022187TOC.cfm [Accessed: 17 Mar 2008].