METHOD DVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF ATAZANAVIR SULPHATE AND RITONAVIR IN BULK AND TABLET DOSAGE FORM BY RP-HPLC

A Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY
Chennai-600032

In partial fulfillment of the requirements for the award of degree of

MASTER OF PHARMACY
IN
PHARMACEUTICAL ANALYSIS

Submitted by

Register No. 261230553

Under the Guidance of

Dr. S. ANANDA THANGADURAI, M.Pharm., Ph.D.,

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

SWAMY VIVEKANANDHA COLLEGE OF PHARMACY

ELAYAMPALAYAM, TIRUCHENGODE - 637 205

TAMILNADU, INDIA.

APRIL-2014

Dr. N. N. Rajendran, M. Pharm., Ph. D.,

Principal,

SwamyVivekanandha College of Pharmacy,

Tiruchengode-637 205, Namakkal (DT), Tamil Nadu.

Ph.: +91-4288-234417

CERTIFICATE

This is to certify that the dissertation entitled **"METHOD DVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF ATAZANAVIR SULPHATE AND RITONAVIR IN BULK AND TABLET DOSAGE FORM BY RP-HPLC"** submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, is a bonafide project work of **SARAVANA KUMAR.S (Reg. No: 261230553)** in the Department of Pharmaceutical Analysis, Swamy Vivekanandha College of Pharmacy, Tiruchengode, for the partial fulfillment of award of the degree in Master of Pharmacy under the guidance of **Dr. S. ANANDA THANGADURAI, M.Pharm., Ph.D.,** Swamy Vivekanandha College of Pharmacy, Tiruchengode.

Date:

Place: Elayampalayam   **[Dr. N. N. RAJENDRAN]**

Dr. S. AnandaThangadurai, M. Pharm., Ph. D.,

Professor & Head,

Department of Pharmaceutical Analysis,

SwamyVivekanandha College of Pharmacy,

Tiruchengode-637 205, Namakkal (DT), Tamil Nadu.

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Date:

Place: Elayampalayam   **[Dr. S. ANANDA THANGADURAI]**

EVALUATION CERTIFICATE

This is to certify that the dissertation entitled **"METHOD DVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF ATAZANAVIR SULPHATE AND RITONAVIR IN BULK AND TABLET DOSAGE FORM BY RP-HPLC"** submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, is a bonafide project work of **SARAVANAKUMAR. S (Reg.No: 261230553),** in the Department of Pharmaceutical Analysis, SwamyVivekanandha College of Pharmacy, Tiruchengode, for the partial fulfillment of award of the degree in Master of Pharmacy under the guidance of **Dr. S. ANANDA THANGADURAI, M.Pharm., Ph.D.,** Swamy Vivekanandha College of Pharmacy, Tiruchengode.

Internal Examiner   **External Examiner**

Examination Center: Swamy Vivekanandha College of Pharmacy.

Date:

Affectionately dedicated

To

My beloved parents, staff & friends

ACKNOWLEDGEMENT

ACKNOWLEDGEMENT

Behind every successful venture, it is always said that there is an invisible force, which shapes the things in the right way and directions in which they should be. I found it moral duty to bow to that divine power and thank him for imbibing in me the strength required for the successful completion of the thesis work.

Ability and ambition are not enough for success many an able person fails to achieve anything worth while because he has not been properly guided. Success of any project depends solely on support, guidance and encouragement received from the guide and well-wishers.

It is with a sense of pride and pleasure that, I humbly look back to acknowledge, those who have been a source of encouragement in my entire endeavor.

Many Thanks to **God** for it are he who began this work in me and carried it throughout completion.

The completion of this dissertation is not only fulfillment of my dreams but also the dreams of my Parents, who have taken lots of pain for me in completion of my higher studies.

I take this privilege and pleasure to acknowledge the contributions of many individuals who have been inspirational and supportive throughout my work undertaken and endowed me with the most precious knowledge to see success in my endeavor.

We submit our sincere thanks to our beloved chairman and secretary "VidyaRatna, Rashtriya Rattan, Hind Ratna" **Prof. Dr. M. Karunanithi, B.Pharm., M.S., Ph.D., D. Litt.,** for providing all the facilities to carry out this thesis work.

It gives me great pleasure to record my deep sense of gratitude and indebtedness to **Dr. S. AnandaThangadurai, M.Pharm., Ph.D.,** Prof & Head., Department of Pharmaceutical Analysis, whose guidance, support, critical evaluations and professional eminency have inspired me a lot to put optimum efforts towards the completion of my thesis work.

It is a great pleasure to utilize this unique opportunity to express my deep sense of gratitude to **Dr. N.N.Rajendran, M.Pharm., Ph.D.,** Principal, Swamy Vivekanandha College of Pharmacy, for his encouragement and advice in completion of the work.

I also take this opportunity to express my deep sense of gratitude and offer my most sincere and humble regards to **Mr. C. Jothimanivannan, M.Pharm., (Ph.D.,)** Prof, & Head., Department of Pharmaceutical Chemistry, for his continuous encouragement and support in completion of my course and dissertation successfully.

CONTENTS

Chapter 1

INTRODUCTION

1. INTRODUCTION

Analytical chemistry may be defined as the science and art of determining the composition of material in terms of elements or compounds contained in it[1]. Analytical chemistry is divided into two branches quantitative and qualitative. A qualitative method is the information about the identity of atomic or molecular species or functional groups in the sample. A quantitative method provides numerical information as to the relative amount of one or more of these components.

For analysis of these drugs different analytical methods are routinely being used. These analytical methods are classified as chemical and instrumental. The chemical methods include Gravimetric and Titrimetric. These methods are simple but less precise and more time consuming so now days these methods are not suggested for the routine analysis. The instrumental methods include electrochemical methods - Electro gravimetric method, Potentiometry, Colorimetry, Conductometry, Polarography and Optical methods - Absorption and Emission methods. The Absorption methods include Visible Spectrophotometry, Ultraviolet Spectrophotometry, Atomic Absorption Spectrophotometry, Infrared Spectrophotometry, and Emission methods include Emission Spectroscopy, Flame Photometry, Fluorimetry, etc. The other outstanding methods include isotopes, radioactivity, X-ray fluorescence and separation methods as various chromatographic principles viz. HPLC, GC, HPTLC, LC-MS[2].

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC):

HPLC is a fast growing analytical technique for the analysis of drugs. Its simplicity, specificity and wide range of sensitivity make it ideal for the analysis of many drugs in both dosage forms and biological fluids[3].

The rate of distribution of drugs between stationary and mobile phase is controlled by diffusion process. If diffusion is minimized, a faster and effective separation can be achieved. The technique of high performance liquid chromatography is so called because of its improved performance when compared to conventional column chromatography. Advances in column technology, high pressure pumping system and sensitive detectors have transformed liquid column chromatography into high efficient, accurate and highly resolved method of separation[4]. For the present study, the drug Atazanavir Sulphate and Ritonavir was selected for the estimation.

The HPLC method was considered the choice of estimation, since this method is the most powerful of all chromatographic and other separative methods. The HPLC method has enabled analytical chemist to attain great success in solving his analytical problems.

Method Development:

The development of a method of analysis of any compound is usually based on existing literature, using same or quite similar instrumentation. But now days HPLC based method is not similar as compare to existing literature based approaches. The development of new or any improved method should be beneficial in any way than the existing method. Method development usually requires selecting the method requirement and deciding the instrumentation to utilize for what purpose[5].

Method development is done for, new drug products and already existing products. The various parameters that include to be optimized during method development are

- ➢ Mode of separation.
- ➢ Selection of stationary phase.
- ➢ Selection of mobile phase.
- ➢ Selection of detector

Method validation:

Validation of an analytical method is the process by which it is established, by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical applications.

Validation is defined by different agencies. According to Food and Drug administration (FDA), validation is establishing documentation evidence, which provides a high degree of assurance that specific process will consistently produce a product meeting its predetermined specification and quality attributes.

According to World Health Organization (WHO), validation is an action of providing that any procedure, process, equipment, material, activity, or system actually leads to the expected results[6].

Validation parameter includes accuracy, precision, system suitability, linearity, ruggedness, robustness, limit of detection and limit of quantitation.

The principal purpose of analytical method validation is to ensure that test methods, which are used for assessing compliance of pharmaceutical products with established specifications, will give accurate, reliable and reproducible results.

The number of drugs and drug formulations introduced into the market has an alarming rate. These drugs or formulations may be either in the new entities in the market or partial structural modification of the existing drugs or novel dosage forms or multi component dosage forms.

Applications:

a. Chemistry and biochemistry research analyzing complex mixtures
b. Developing processes for synthesizing chemical compounds
c. Purifying chemical compounds
d. Quality control to ensure the purity of raw materials, to control and improve process yields, to quantify assays of final products or to evaluate product stability and monitor degradation.
e. Isolating natural products or predicting physical properties.

The HPLC method was considered the choice of estimation, since this method is the most powerful of all chromatographic and other separative methods.   The HPLC method has enabled analytical chemist to attain great success in solving his analytical problems.
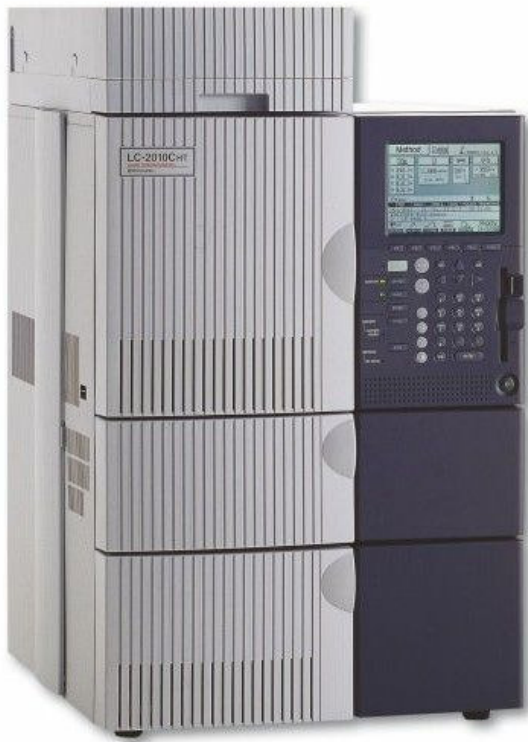
Atazanavir sulphate is an antiretroviral drug of the protease inhibitor class, it is used to treat infection of human immunodeficiency virus Used in combination with other antiretroviral agents for the treatment of HIV-1 infection, as well as post exposure prophylaxis of HIV infection in individuals who have had occupational or no occupational exposure to potentially infectious body fluids of a person known to be infected with HIV when that exposure represents a substantial risk for HIV transmission. Ritonavir an HIV protease inhibitor that works by interfering with the reproductive cycle of HIV is an antiretroviral drug of the protease inhibitor class, it is used to treat infection of human immunodeficiency virus.
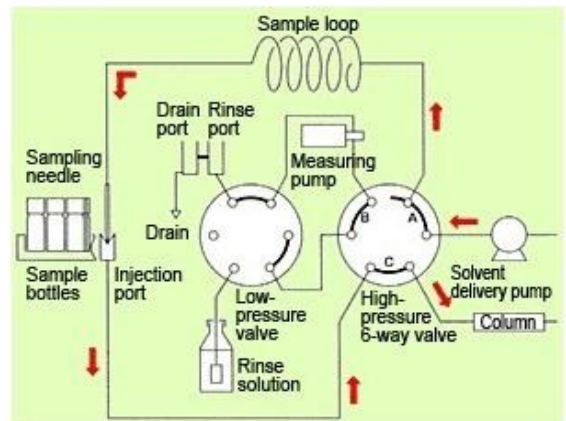
Chapter 2

LITERATURE REVIEW

2. LITERATURE REVIEW

Normal-phase HPLC (NP-HPLC), or adsorption chromatography, this method separates analytes based on their affinity for a polar stationary surface such as silica, hence it is based on analyte ability to engage in polar interactions (such as hydrogen-bonding or dipole-dipole type of interactions) with the sorbent surface. To select an optimum mobile phase it is better to start with pure hydro carbon mobile phase such as heptanes. In normal phase mode, separations of oil soluble vitamins, essential oils, can be done using binary mobile phase[7].

Solvent Pumping system

Shimadzu   LC 2010 HPLC

UV detector

Reverse phase HPLC (RP-HPLC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica which has been surface-modified with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}.With such stationary phases, retention time is longer for molecules which are less polar, while polar molecules elute more readily. Generally the lower the polarity of the mobile phase the higher is its elution strength. Thus water is the weaker solvent. Methanol and acetonitrile are popular solvents because of its low viscosity and are readily available with excellent purity. We have selected Atazanavir sulphate and Ritonavir for the present work and literature cited on the above drugs were listed below.

A sensitive method for the simultaneous estimation of Ritonavir in their dosage form was developed and validated. The method was carried out on a Thermo Hypersil RP C-18 column (150 mm x 4.6 mm, 5 μm) with a mobile phase consisting of Acetonitrile: Potassium dihydrogen Phosphate & di potassium hydrogen Phosphate (45: 55 V/V) at a flow rate of 1 mL min^{-1} and the detection was carried out at 239 nm. The limits of detection (LOD) and quantification (LOQ) were 0.09 and 0.027 μg/ mL respectively. Precision % RSD values were found to be 0.38% and 0.41% respectively. Recovery of Ritonavir was found to be in the range of 99.90-100.50%. The retention time of Ritonavir was 3.11 min respectively. Linearity for Ritonavir were found in the range of 20-120 μg/ mL^{-1} [8].

A reverse phase high performance liquid chromatographic method was developed and validated for the simultaneous estimation of Lopinavir and Ritonavir in Bulk and Tablet dosage form. A Hypersil (ODS) C8 (250 × 4.6mm, 5 μm) id in isocratic mode with mobile phase containing acetonitrile: methanol: 0.01 M potassium dihydrogen orthophosphate buffer at pH 3.0 (30:20:50 V/V/V) was used. The flow rate was 1 mL min^{-1} and effluents were monitored at 254 nm. The retention time of Atazanavir Sulphate and Ritonavir was 4.66 min and 3.44 min respectively. The concentration curves were linear in the concentration range of 80–120 μg/ mL^{-1} for both the drugs. The developed method was validated for specificity, precision, linearity, accuracy, LOD, LOQ, robustness. Recovery of Lopinavir and Ritonavir in formulations was found to be in the range of 98% -102% respectively confirms the non-interferences of the excipients in the formulation[9].

A high-performance liquid chromatographic method for the determination of atazanavir (ATV) in human plasma is developed and validated. The method involves a rapid and simple solid-phase extraction of ATV using Bond-elut C18 3 mL cartridge. The separation of ATV from internal standard and endogenous components is achieved using an isocratic elution on an octyl column and an UV detector set at 260 nm. The method is linear from 20 to 10,000 μg/ mL (mean r^2 = 0.9991, n = 10). The observed intra- and inter-day assay precision ranged from 2.2% to 14.7% at the lower limit of quantitation (LOQ), where as accuracy varies between 1.0% and 14% (at LOQ). Mean drug recovery is 80.5% for ATV and 78.4% for IS[10].

A new, simple, sensitive, precise and accurate High-performance thin-layer chromatographic method for simultaneous determination of Ritonavir and Atazanavir in their combined tablet dosage form has been developed, validated and used for determination of the compounds in commercial pharmaceutical products. Chromatographic separation was achieved on aluminium plates precoated with silica gel 60 F254 as the stationary phase and chloroform: ethyl acetate: acetone   (5:2:3 V/V/V) as a mobile phase. Densitometric measurements of their spots were achieved at 244 nm over the concentration ranges of 800-2800 μg spot-1 and 2400-8400 μg spot-1, with mean recoveries of 98.57 ± 0.35 and 99.16 ± 0.20 for ritonavir and atazanavir respectively. Limit of detection for ritonavir and atazanavir were found to be 300 μg spot^{-1} and 200 μg spot^{-1} respectively. Chromatogram of standard Ritonavir Rf = 0.28 and Atazanavir Rf = 0.58[11].

Chemometrics-assisted spectrophotometry for the determination of two protease inhibitors, lopinavir and ritonavir, in plasma was evaluated. A set of calibration mixtures was designed according to central composite design. The UV spectra obtained from the calibration set were subjected to partial least square regression to construct the prediction models for lopinavir and ritonavir in unknown samples, which were then validated in a randomly selected set of synthetic mixtures of the drugs. An optimum model was obtained in the wavelength ranges of   215–249 nm and 240–279 nm with principal components 8 for both lopinavir and ritonavir respectively. The prediction models were used to analyse the two drugs in plasma and the results were compared with those obtained by high-performance liquid chromatography (HPLC). The PLS^{-1} model and the HPLC method were found to be comparable[12].

A simple, precise, specific and accurate reverse phase HPLC method has been developed for the determination of Atazanavir and Ritonavir in pharmaceutical dosage forms. The chromatographic separation was achieved on Symmetry C8 (4.6 x 100mm, 5 μm) column using a mixture of Buffer: Acetonitrile (45:55 V/V) as the mobile phase at a flow rate 0.9 mL/ min. The retention time of Atazanavir and Ritonavir was 2.9 min and 4.1 min. The analyte was monitored using UV detector at 235 nm. Results of analysis were validated statistically and by recovery studies. The method was validated according to the ICH guidelines with respect to linearity, accuracy, precision and robustness. The proposed method can be successfully used to determine the drug contents of marketed formulation[13].

A simple, precise, accurate, and rapid HPLC method has been developed, and validated for the determination of Atazanavir and Ritonavir simultaneously in combined tablet dosage form. The mobile phase used was a mixture of phosphate buffer pH 4.0 and Acetonitrile (43:57 V/V). The detection of Atazanavir and Ritonavir was carried out by UV detector at 240 nm. The retention time of Atazanavir and Ritonavir were found to be 4.2 min and 5.2 min respectively. Results of the analysis were validated statistically, and by recovery studies. The proposed method can be successfully used to determine the drug contents of marketed formulation[14].

Simple, precise, rapid and accurate methods for simultaneous determination of Atazanavir and Ritonavir in combined tablet dosage form have been developed. Method A is based on ratio spectra derivative and method B uses integrated area under curve, methanol is used as solvent for both the methods. The amplitudes at 280.01 nm and 286.12 nm of the first derivative of ratio spectra were selected to determine ATV and RTV, respectively by ratio derivative method and wavelength ranges of 246.97-252.03 nm and 240.78-244.16 nm were selected to determine ATV and RTV by AUC method in combined formulation. Beer's law is obeyed in the concentration range of 15-75 μg/ mL and 5-25 μg/ mL for Atazanavir and Ritonavir, respectively by both the methods. The % assay in commercial formulation was found to be in the range 98.81-100.8% for ATV and 98.91-101.2% for RTV by the proposed methods. The methods were validated with respect to linearity, precision and accuracy. Recovery was found in the range of 99.20-99.96% for ATV and 99.12-99.97% for RTV by ratio derivative method and 99.08-99.66% for ATV and 99.78-100.6% for RTV by AUC method. The

methods developed are simple, economical, precise and accurate and can be used for routine quality control of analytes in combined tablets[15].

A validated RP HPLC method for the estimation of Atazanavir sulphate in capsule dosage form on Agilent TC C18 (2) 250 x 4.6 mm, 5 μm column using mobile phase composition of water: acetonitrile (20:80 V/V) pH adjusted to 3.0.Flow rate was maintained at 1 ml/ min at an ambient temperature. Quantification was achieved with ultraviolet detection at 255 nm. The retention time obtained for Atazanavir sulphate was at 3.7 min. The detector response was linear in the concentration range of 10 – 80 μg/ mL. This method has been validated and shown to be specific, sensitive, precise, linear, accurate, rugged, robust and fast. Hence, this method can be applied for routine quality control of Atazanavir sulphate in capsule dosage forms as well as in bulk drug[16].

A validated RP HPLC method for the estimation of atazanavir in capsule dosage form on YMC ODS 150 × 4.6 mm, 5 μm column using mobile phase composition of ammonium dihydrogen phosphate buffer (pH 2.5) with acetonitrile (55:45 V/V). Flow rate was maintained at 1.5 mL/ min with 288 nm UV detection. The retention time obtained for atazanavir was at 4.7 min. The detector response was linear in the concentration range of 30 - 600 μg/ mL. This method has been validated and shown to be specific, sensitive, precise, linear, accurate, rugged, robust and fast. Hence, this method can be applied for routine quality control of atazanavir in capsule dosage forms as well as in bulk drug[17].

To simple, sensitive, rapid spectrophotometric methods have been developed for simultaneous estimation of Atazanavir Sulfate (ATV) and Ritonavir (RTV) in tablets. First method involves solving simultaneous equations based on measurement of absorbance at two wavelengths 249.5 nm and 238.5 nm λ max of ATV and RTV, respectively. Second method is based on area under curve (AUC) and the wavelength ranges selected for analysis were 254.5-244.5 nm for Atazanavir Sulfate and 243.5-233.5 nm for Ritonavir. Beer's law was obeyed in the concentration range of 10-50 μg/ mL and 10-50 μg/ mL for ATV and RTV, respectively. The methods were validated as per ICH guidelines. Statistical analysis proved that the methods were accurate, precise, and reproducible for analysis of ATV and RTV in tablets. The wide linearity range, sensitivity, accuracy and simple procedure imply that the proposed technique demonstrated to be appropriate for routine analysis and quality control assay of tablets[18].

Chapter 3

AIM & OBJECTIVES

3. AIM AND OBJECTIVES

The drug analysis plays an important role in the development of drugs, their manufacture and the therapeutic use. Pharmaceutical industries rely upon quantitative chemical analysis to ensure that the raw materials used and the final product obtained meets the required specification. These drugs or formulation may be either in the new entities in the market or partial structural modification of the existing drugs or novel dosage forms or multi component dosage forms. The multi component dosage form proves to be effective due to the combined mode of action on the body. The complexity of dosage forms including the presence of multiple drug entities possesses considerable challenge to the analytical chemist during the development of assay procedure.

The extensive literature survey carried out and revealed that very few methods are reported for the simultaneous estimation of these drugs in other combinations. Hence an attempt was made to develop a specific, precise, accurate, linear, simple, rapid, validated and cost effective HPLC method for the simultaneous estimation of Atazanavir sulphate and Ritonavir in combined dosage forms.

The specific aim of the research was

➢ To develop a method for the simultaneous estimation of Atazanavir sulphate and Ritonavir in bulk and Pharmaceutical dosage form and validate the proposed methods in accordance with ICH guidelines for the intended analytical application.

➢ To validate the developed RP-HPLC method by using various validation parameters such as linearity, accuracy, system suitability, precision, robustness, ruggedness, limit of detection and limit of quantization.
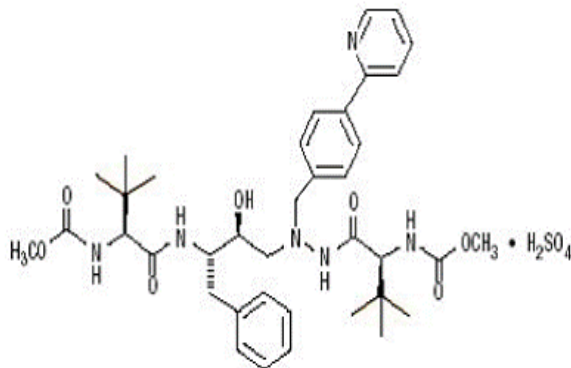
Chapter 4

DRUG PROFILE

4. DRUG PROFILE

Atazanavir sulphate

Structure:

IUPAC Name:(3S,8S,9S,12S)-3,12-Bis(1,1-dimethylethyl)-8hydroxy-4,11-dioxo-9-(phenylmethyl)-6-[[4-(2-pyridinyl)phenyl]methyl]-,5,6,10,13pentaazatetradecanedioic acid dimethyl ester, Sulphate [19,20].

Molecular Formula: $C_{38}H_{52}N_6O_7 \cdot H_2SO_4$

Molecular Weight : 802.93

Category: Atazanavir sulphate is an antiretroviral drug of the protease inhibitor class. Like other antiretrovirals, it is used to treat infection of human immunodeficiency virus (HIV).

Description :A white to pale yellow crystalline powder.

Solubility : It is soluble in methanol,ethanol,isopropanol and practically insoluble in water.

Melting point :195-209°C

pKa value: strongest acidic-11.92, strongest basic-4.42.

Pharmacodynamics:

      Atazanavir sulphate is an azapeptide HIV-1 protease inhibitor (PI) with activity against Human Immunodeficiency Virus Type 1. HIV-1 protease is an enzyme required for the proteolytic cleavage of the viral polyprotein precursors into the individual functional proteins found in infectious HIV-1. Atazanavir sulphate binds to the protease active site and inhibits the activity of the enzyme. This inhibition prevents cleavage of the viral polyproteins resulting in the formation of immature non-infectious viral particles. Protease inhibitors are almost always used in combination with at least two other anti-HIV drugs. Atazanavir sulphate is pharmacologically

related but structurally different from other protease inhibitors and other currently available antiretrovirals.

Mechanism of Action:

Atazanavir sulphate selectively inhibits the virus-specific processing of viral Gag and Gag-Pol polyproteins in HIV-1 infected cells by binding to the active site of HIV-1 protease, thus preventing the formation of mature virions. Atazanavir sulphate is not active against HIV-2.

Pharmacokinetics:

Absorption-Atazanavir is rapidly absorbed with a t_{max} of approximately 2.5 hours. Administration of Atazanavir sulphate with food enhances bioavailability and reduces pharmacokinetic variability. Oral bioavailability is 60-68%.

Protein binding- 86%

Route of elimination- Atazanavir sulphate dose was excreted urine.

Metabolism- Metabolisedto Atazanavir sulphate by liver.

Half life- 6.5 hours

Adverse Reaction:

Protease inhibitors commonly cause paresthesias, nausea, vomiting, and diarrhea disturbances in glucose and lipid metabolism also occur, including diabetes, hypertriglyceridemia, and hypercholesterolemia.

Dose : Reyataz, latazanavir,zrivada 400 mg tablets.

Ritonavir

Structure:
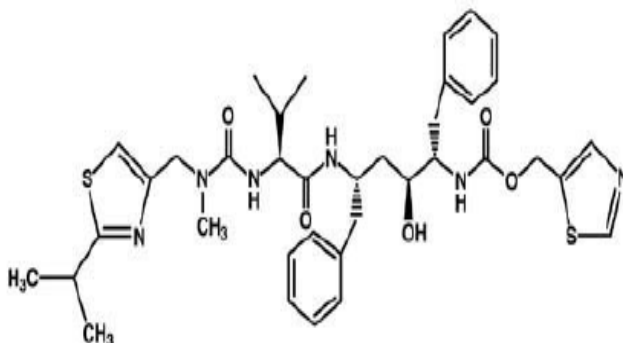
IUPAC NAME: 1,3-thiazol-5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl-2-{[methyl({[2-(propan-2-yl)-1,3-thiazol-4-yl]methyl})carbamoyl]amino}butanamido]-1,6-diphenylhexan-2-yl]carbamate[21,22].

Molecular Formula: $C_{37}H_{48}N_6O_5S_2$

Molecular Weight : 720.95

Categories: Ritonavir is an antiretroviral drug of the protease inhibitor class. Like other antiretrovirals, it is used to treat infection of human immunodeficiency virus (HIV).

Description: White-to-off white powder.

Solubility: Freely soluble in methanol and in ethanol (95%) soluble in 2-propanol.

Melting point: 120-123 °C

pKa value: strongest acidic-13.68, strongest basic-2.84.

Pharmacodynamics:

Ritonavir is a protease inhibitor with activity against Human Immunodeficiency Virus Type 1. Protease inhibitors block the part of HIV called protease. HIV-1 protease is an enzyme required for the proteolytic cleavage of the viral polyprotein precursors into the individual functional proteins found in infectious HIV-1. Ritonavir binds to the protease active site and

inhibits the activity of the enzyme. This inhibition prevents cleavage of the viral polyproteins resulting in the formation of immature non-infectious viral particles. Protease inhibitors are almost always used in combination with at least two other anti-HIV drugs.

Mechanism of action:

Ritonavir inhibits the HIV viral proteinase enzyme which prevents cleavage of the gag-pol polyprotein, resulting in noninfectious, immature viral particles.

Pharmacokinetics:

Absorption-The absolute bioavailability of ritonavir has not been determined.

Metabolism- Hepatic.

Protein binding- 98-99%

Route of elimination-Ritonavir dose was excreted urine.

Metabolism- Metabolised to Ritonavir by liver.

Half-life- 3 to 5 hours.

Adverse Reactions /Side Effects:

Protease inhibitors commonly cause paresthesias, nausea, vomiting, and diarrhea disturbances in glucose and lipid metabolism also occur, including diabetes, hypertriglyceridemia, and hypercholesterolemia.

Dose: Norvir sofgel, Norvir100 mg tablets.

Chapter 5

EXPERIMENTAL WORK

5. EXPERIMENTAL WORK

5.1 MATERIALS AND METHODS

Equipments used:

S.No	Name	Make
1	Weighing Balance	SHIMADZU
2	pH meter	ELCHEM
3	Sonicator	SAISONIC
4	HPLC	SHIMADZU LC-2010 with UV DETECTOR
5	UV-Visible Spectroscopy	PERKIN ELMER with LAMDA25

Chemicals used:

S.No	Name	Make
1	Phosphate buffer	HPLC grade (MERCK)
2	Acetonitrile	HPLC grade (MERCK)
3	Methanol	HPLC grade (MERCK)
4	Orthophosphoric acid	HPLC grade (RANCHEM)
5	Purified water	HPLC grade (MILLI-Q)

Active pharmaceutical Ingredient (pure drug)

S.No	Name	Specification
1	Atazanavir sulphate and Ritonavir	Reference Standard

Marketed Formulation

S.No	Name	Manufacturer
1	Atazotec Tablets	Sai Mirra Innopharm

SOLUBILITY

Solubility of drug was observed by dissolving it in different solvents and it was found that drug having good solubility in followings.

Solubility of drugs in different solvents

SOLVENT	SOLUBILITY Atazanavir sulphate and Ritonavir
Water	-
Acetonitrile	+
Methanol	+
0.1N NaoH	-

5.2 METHOD DEVELOPMENT

Determination of LAMBDA Max of Atazanavir sulphate by UV-Visible spectrophotometry

Selection of wavelength:

Accurately Weighed and transferred Atazanavir sulphate equivalent to 25 mg into 50 mL clean, dry, volumetric flask, 50 mL of methanol was added, sonicated for 5 minutes, and filtered through 0.45 µm nylon membrane filter. Pipetted out 5 mL of the above solution, and diluted with 50 mL of methanol in 50 mL volumetric flask, and scanned between 200 to 400 by UV spectroscopy[23]. Shown in **Figure-1.**

Determination of LAMBDA Max of Ritonavir by UV-Visible spectrophotometry

Sample Preparation

Accurately Weighed and transferred Ritonavir equivalent to 25 mg into 50 mL clean, dry, volumetric flask, 50 mL of methanol was added, sonicated for 5 minutes, and filtered through 0.45 µm nylon membrane filter. Pipetted out 5 mL of the above solution, and diluted with 50 mL of methanol in 50 mL volumetric flask, and scanned between 200 to 400 by UV spectroscopy. Shown in **Figure-2.**

Determination of ISOBESTIC point of Atazanavir sulphate and Ritonavir by UV-Visible spectrophotometry

Sample Preparation

Accurately Weighed and transferred Atazanavir sulphate and Ritonavir equivalent to 25 mg and 25 mg into 50 mL clean dry volumetric flask, 50 mL of methanol was added, sonicated for 5 minutes, and filtered through 0.45 µm nylon membrane filter. Pipette out both 5 mL of each solutions transferred into 50 mL volumetric flask, and diluted to 50 mL with methanol in 50 mL volumetric flask, and scanned between 200 to 400 by UV spectroscopy. Shown in **Figure-3.**

Selection of mobile phase:

The method development and validation of Atazanavir sulphate and Ritonavir requires greater resolution. Hence different solvent systems were tried.

The trails are using SHIMADZU LC 2010 equipment with UV detector and isocratic pump. The system controlled by LC solution software.

Selection of flow rate:

The flow rate of Atazanavir sulphate and Ritonavir were tried from 0.8 mL to 1.5 mL.

Trial-1

*Buffer preparation***:**

About 6.8 g of potassium dihydrogen orthophosphate was dissolved in 1000 mL of HPLC grade water and pH 3.0 was adjusted with orthophosphoric acid. It was filtered through 0.45 µm nylon membrane filter and degassed with sonicator. It was used as a diluent for the preparation of sample and standard solution.

Preparation of mobile phase:

Mobile phase consist of acetonitrile: methanol: buffer of pH 3.0 (65:5:30) was taken sonicated degas for 10 min and filtered through 0.45 µm nylon membrane filter.

Standard Preparation:

Weigh accurately 68 mg of Standard Atazanavir sulphate and 20 mg of Standard Ritonavir into a 100 mL volumetric flask, dissolve and make up the volume with methanol. Transfer 5 mL of the solution into a 50 mL volumetric flask and make up the volume with the mobile phase. The solution contains 68 µg/ mL and 20 µg/ mL concentration of Atazanavir sulphate and Ritonavir respectively. The chromatogram was shown in **Figure-4.**

Chromatographic conditions:

Column : Nucleodur C_8 column(100 mm x 1.8 mm x5µm)

Mobile phase : Acetonitrile: Methanol: buffer pH 3.0 (65:5:30)

Diluent : HPLC grade methanol

Flow rate : 0.8 mL/ min

Detector wavelength : 210 nm

Injection mode : Auto injector (vial)

Injection volume : 20 µl

Trial-2

Buffer preparation:

About 6.8 g of potassium dihydrogen orthophosphate was dissolved in   1000 mL of HPLC grade water and pH 3.0 was adjusted with orthophosphoric acid. It was filtered through 0.45 µm nylon membrane filter and degassed with sonicator. It was used as a diluent for the preparation of sample and standard solution.

Preparation of mobile phase:

Mobile phase consist of acetonitrile: methanol: buffer of pH 3.0 (25:5:70) was taken sonicated degas for 10 min and filtered through 0.45 µm nylon membrane filter.

Standard Preparation:

Weigh accurately 68 mg of Standard Atazanavir sulphate and 20 mg of Standard Ritonavir into a 100 mL volumetric flask, dissolve and make up the volume with methanol. Transfer 5 mL of the solution into a 50 mL volumetric flask and make up the volume with the mobile phase. The solution contains 68 µg/ mL and 20 µg/ mL concentration of Atazanavir sulphate and Ritonavir respectively. The chromatogram was shown in **Figure-5.**

Chromatographic conditions:

Column   :   Nucleodur C_8 column (100 mm x 1.8 mm x 5µm)

Mobile phase   :   Acetonitrile: Methanol: buffer pH 3.0 (25:5:70)

Diluent   :   HPLC grade methanol

Flow rate   :   0.9 mL/ min

Detector wavelength   :   210 nm

Injection mode   :   Auto injector (vial)

Injection volume   :   20 µl

Trial-3

Buffer preparation:

About 6.8 g of potassium dihydrogen orthophosphate was dissolved in 1000 mL of HPLC grade water and pH 3.0 was adjusted with orthophosphoric acid. It was filtered through 0.45 µm nylon membrane filter and degassed with sonicator. It was used as a diluent for the preparation of sample and standard solution.

Preparation of mobile phase:

Mobile phase consist of acetonitrile: methanol: buffer of pH 3.0 (60:10:30) was taken sonicated degas for 10 min and filtered through 0.45 µm nylon membrane filter.

Standard Preparation:

Weigh accurately 68 mg of Standard Atazanavir sulphate and 20 mg of Standard Ritonavir into a 100 mL volumetric flask, dissolve and make up the volume with methanol. Transfer 5 mL of the solution into a 50 mL volumetric flask and make up the volume with the mobile phase. The solution contains 68 µg/ mL and 20 µg/ mL concentration of Atazanavir sulphate and Ritonavir respectively. The chromatogram was shown in **Figure-6.**

Chromatographic conditions:

Column   :   Nucleodur C_{18} column(150 mm x 4.6 mm x5µm)

Mobile phase   :   Acetonitrile: Methanol: buffer pH 3.0 (60:10:30)

Diluent   :   HPLC grade methanol

Flow rate   :   1.0 mL/ min

Detector wavelength   :   210 nm

Injection mode   :   Auto injector (vial)

Injection volume   :   20 µl

Trial-4

Buffer preparation:

About 6.8 g of potassium dihydrogen orthophosphate was dissolved in   1000 mL of HPLC grade water and pH 3.0 was adjusted with orthophosphoric acid. It was filtered through 0.45 µm nylon membrane filter and degassed with sonicator. It was used as a diluent for the preparation of sample and standard solution.

Preparation of mobile phase:

Mobile phase consist of acetonitrile: methanol: buffer of pH 3.0 (50:10:40) was taken sonicated degas for 10 min and filtered through 0.45 µm nylon membrane filter.

Standard Preparation:

Weigh accurately 68 mg of Standard Atazanavir sulphate and 20 mg of Standard Ritonavir into a 100 mL volumetric flask, dissolve and make up the volume with methanol. Transfer 5 mL of the solution into a 50 mL volumetric flask and make up the volume with the mobile phase. The solution contains 68 µg/ mL and 20 µg/ mL concentration of Atazanavir sulphate and Ritonavir respectively. The chromatogram was shown in **Figure-7.**

Chromatographic conditions:

Column	:	Nucleodur C_{18} column(150 mm x 4.6 mm x5µm)
Mobile phase	:	Acetonitrile: Methanol: buffer pH 3.0 (50:10:40)
Diluent	:	HPLC grade methanol
Flow rate	:	1.2 mL/ min
Detector wavelength	:	210 nm
Injection mode	:	Auto injector (vial)
Injection volume	:	20 µl

OPTIMIZED METHOD:

Buffer preparation:

About 6.8 g of potassium dihydrogen orthophosphate was dissolved in 1000 mL of HPLC grade water and pH 3.0 was adjusted with orthophosphoric acid. It was filtered through 0.45 µm nylon membrane filter and degassed with sonicator. It was used as a diluent for the preparation of sample and standard solution.

Preparation of mobile phase:

Mobile phase consist of acetonitrile: methanol: buffer of pH 3.0 (44:11:45) was taken sonicated degas for 10 min and filtered through 0.45 µm nylon membrane filter.

Standard Preparation:

Weigh accurately 68 mg of Standard Atazanavir sulphate and 20 mg of Standard Ritonavir into a 100 mL volumetric flask, dissolve and make up the volume with methanol. Transfer 5 mL of the solution into a 50 mL volumetric flask and make up the volume with the mobile phase. The solution contains 68 µg/ mL and 20 µg/ mL concentration of Atazanavir sulphate and Ritonavir respectively. The chromatogram was shown in **Figure-8.**

Chromatographic conditions:

Column	:	Nucleodur C_{18} column(150 mm x 4.6 mm x5µm)
Mobile phase	:	Acetonitrile: Methanol: buffer pH 3.0 (44:11:45)
Diluent	:	HPLC grade methanol
Flow rate	:	1.5 mL/ min
Detector wavelength	:	210 nm
Injection mode	:	Auto injector (vial)
Injection volume	:	20 µl

5.2.1 ASSAY OF ATAZANAVIR SULPHATE AND RITONAVIR:

Standard preparation:

Weigh accurately 68 mg of Standard Atazanavir sulphate and 20 mg of Standard Ritonavir into a 100 mL volumetric flask, dissolve and make up the volume with methanol. Transfer 5 mL of the solution into a 50 mL volumetric flask and make up the volume with the mobile phase. The solution contains 68 µg/ mL and 20 µg/ mL concentration of Atazanavir sulphate and Ritonavir respectively. The results were tabulated in **Table-6** & the chromatogram was shown in **Figure-9.**

Sample preparation:

Weigh and powder 20 tablets and weigh powder equivalent to 218 mg of the sample was taken and 70 mL diluent. Sonicate for 30 minutes and make up to volume with the methanol. Filter Transfer 5 mL of the solution into a 50 mL volumetric flask and make up the volume with the mobile phase. With optimized Chromatographic condition a steady base line was recorded with mobile phase 20 µl quantity of sample solution was injected and the Chromatogram was recorded. The results was tabulated in **Table-7** & the chromatogram is shown in **Figure-10**

Procedure:

Inject 20 µl of the standard, sample into the chromatographic system and measure the areas for the Atazanavir sulphate and Ritonavir peaks.

Amount of drug in tablet was calculated using following formula

Amount present:

$$\frac{\text{Sample area X standard weight}}{\text{Standard area X sample weight}} \text{X dilution factor X average weight}$$

Percentage purity:

Amount present / label claim X 100

5.3 METHOD VALIDATION

The chromatographic conditions were validated by evaluating linearity, accuracy, method precision, limit of detection (LOD), limit of quantitation (LOQ), ruggedness and robustness in accordance with ICH guidelines[24].

5.3.1 SPECIFICITY:

Specificity is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the sample matrix. It is a measure of the degree of interference from such things as other active ingredients, excipients, impurities, and degradation products, ensuring that a peak response is due to only to a single component; that is, that no co-elution exists. Specificity is measured and documented in a separation by the resolution, plate count, and tailing factor.

Preparation of solutions:

a) Placebo interference:

Sample was prepared by taking the placebo equivalent to about the weight in portion of test preparation as per the test method and injected into the HPLC system. The chromatogram was shown in **Figure-11.**

Acceptance criteria:

Chromatogram of placebo should not show any peak at the retention time of analyte peak.

b) Blank Interference

Mobile phase was prepared as per the test method and injected and into the HPLC system. The chromatogram was shown in **Figure-12.**

Acceptance criteria:

Chromatogram of blank should not show any peak at the retention time of analyte peak.

Standard preparation:

Weigh accurately 68 mg of Standard Atazanavir sulphate and 20 mg of Standard Ritonavir into a 100 mL volumetric flask, dissolve and make up the volume with methanol. Transfer 5 mL of the solution into a 50 mL volumetric flask and make up the volume with the mobile phase. The solution contains 68 µg/ mL and 20 µg/ mL concentration of Atazanavir sulphate and Ritonavir respectively. The chromatogram was shown in **Figure-13-14.**

5.3.2 SYSTEM SUITABILITY:

System Suitability was the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factor, resolution, and reproducibility are determined and compared against the specification set for the method. These parameters are measured during the analysis of a system suitability sample that is a mixture of main components and expected by-product. The results was tabulated in **Table-8**

5.3.3 LINEARITY AND RANGE:

Preparation of stock solution:

Weigh accurately 68 mg of Atazanavir sulphate Standard and 20 mg of Ritonavir Standard into a 100 mL volumetric flask, dissolve and make up the volume with methanol. The solution contains 680 µg/ mL and 200 µg/ mL concentration of Atazanavir sulphate and Ritonavir respectively.

Preparation of linearity solution (50%):

2.5 mL of stock solution was taken in 50 mL of volumetric flask dilute up to the mark with diluent. The solution contains 34 µg/ mL and 10 µg/ mL concentration of Atazanavir sulphate and Ritonavir respectively.

Preparation of linearity solution (80%):

4.0 mL of stock solution was taken in 50 mL of volumetric flask dilute up to the mark with diluent.The solution contains 54 µg/ mL and 16 µg/ mL concentration of Atazanavir sulphate and Ritonavir respectively.

Preparation of linearity solution (100%):

5.0 mL of stock solution was taken in 50 mL of volumetric flask dilute up to the mark with diluent. The solution contains 68 µg/ mL and 20 µg/ mL concentration of Atazanavir sulphate and Ritonavir respectively.

Preparation of linearity solution (120%):

6.0 mL of stock solution was taken in 50 mL of volumetric flask dilute up to the mark with diluent. The solution contains 82 µg/ mL and 24 µg/ mL concentration of Atazanavir sulphate and Ritonavir respectively.

Preparation of linearity solution (150%):

7.5 mL of stock solution was taken in 50 mL of volumetric flask dilute up to the mark with diluent. The solution contains 102 µg/ mL and 30 µg/ mL concentration of Atazanavir sulphate and Ritonavir respectively.

Procedure:

Each level of the above solutions was injected into the chromatographic system for five replicate and the peak area was measured. A graph was plotted (peak area versus concentration) and the correlation coefficient (r^2) was calculated. The results were tabulated in **Table 9** & the chromatograms were shown in **Figure 15-21.**

5.3.4 PRECISION:

Precision was the measure of the degree of repeatability of an analytical method under normal operation and it was normally expressed as the relative standard deviation for a statistically number of samples. Precision should be performed at three different levels: repeatability, intermediate precision and reproducibility.

Standard preparation:

Weigh accurately 68 mg of Standard Atazanavir sulphate and 20 mg of Standard Ritonavir into a 100 mL volumetric flask, dissolve and make up the volume with methanol. Transfer 5 mL of the solution into a 50 mL volumetric flask and make up the volume with the

mobile phase. The solution contains 68 µg/ mL and 20 µg/ mL concentration of Atazanavir sulphate and Ritonavir respectively.

Sample preparation:

Weigh and powder 20 tablets and weigh powder equivalent to 218 mg of the sample was taken and 70 mL diluent. Sonicate for 30 minutes and make up to volume with the methanol. Filter Transfer 5 mL of the solution into a 50 mL volumetric flask and make up the volume with the mobile phase. The results were tabulated in **Table 9** & the chromatograms were shown in **Figure 22-27**.

Procedure:

Six replicate Sample solutions were prepared as per the test method and injected as per the test procedure.

5.3.5 ACCURACY:

Accuracy is the measure of exactness of an analytical method, or closeness of agreement between the measured value and the value that is accepted either as a conventional, true value or an accepted reference value. Accuracy is measured as the percentage of analyte recovered by assay, spiking samples in a blind study.

Preparation of stock solution:

Weigh accurately 68 mg of Standard Atazanavir sulphate and 20 mg of Standard Ritonavir into a 100 mL volumetric flask, dissolve and make up the volume with methanol. The solution contains 680 µg/ mL and 200 µg/ mL concentration of Atazanavir sulphate and Ritonavir respectively.

80% Sample preparation:

From the above stock solution 4 mL containing of Atazanavir sulphate and Ritonavir was taken into a 50 mL volumetric flask and diluted up to the mark with diluent. The solution was sonicated for 15 min and filtered through 0.45 µm nylon membrane filter. The above solution

were inject into the HPLC column same procedure was repeated for three replicate. The results are tabulated in **Table 11-12** & the chromatograms were shown in **Figure 28-30**.

100% Sample preparation:

From the above stock solution 5 mL containing of Atazanavir sulphate and Ritonavir was taken into a 50 mL volumetric flask and diluted up to the mark with diluent. The solution was sonicated for 15 min and filtered through 0.45 µm nylon membrane filter. The above solution were inject into the HPLC column same procedure was repeated for three replicate. The results are tabulated in **Table 11-12** & the chromatograms were shown in **Figure 31-33**.

120% Sample preparation:

From the above stock solution 6 mL containing of Atazanavir sulphate and Ritonavir was taken into a 50 mL volumetric flask and diluted up to the mark with diluent. The solution was sonicated for 15 min and filtered through 0.45 µm nylon membrane filter. The above solution were inject into the HPLC column same procedure was repeated for three replicate. The results are tabulated in **Table 11-12** & the chromatograms were shown in **Figure 34-36**.

Procedure:

The standard solution was injected in triplicate for Accuracy -80%, Accuracy -100% and Accuracy -120% solutions.

Calculate the Amount found and Amount added for Atazanavir sulphate & Ritonavir and calculate the individual recovery and mean recovery values.

$$\% \textbf{ Recovery } = \frac{\text{Sample peak area x weight of standard}}{\text{Standard peak area x weight of sample}} \times 100$$

5.3.6 INTERMEDIATE PRECISION (RUGGEDNESS):

Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions i.e. different analysts, laboratories, instruments, reagents, assay temperatures, small variations in mobile phase, different days etc. (i.e. from laboratory to laboratory, from analyst to analyst). Acceptance criteria for ruggedness, the % RSD for the area of five standard injections should not be more than 2%.

Standard preparation:

Weigh accurately 68 mg of Standard Atazanavir sulphate and 20 mg of Standard Ritonavir into a 100 mL volumetric flask, dissolve and make up the volume with methanol. Transfer 5 mL of the solution into a 50 mL volumetric flask and make up the volume with the mobile phase. The solution contains 68 µg/ mL and 20 µg/ mL concentration of Atazanavir sulphate and Ritonavir respectively.

Sample preparation:

Weigh and powder 20 tablets and weigh powder equivalent to 218 mg of the sample was taken and 70 mL diluent. Sonicate for 30 minutes and make up to volume with the methanol. Filter Transfer 5 mL of the solution into a 50 mL volumetric flask and make up the volume with the mobile phase. The results are tabulated in **Table 13-14** & the chromatograms were shown in **Figure 37-48**.

5.3.7 ROBUSTNESS:
As part of the robustness, deliberate change in the flow rate and mobile phase composition was made to evaluate the impact on the method. The mixed standard solution is injected in two replicate and % RSD was calculated.

Standard preparation:

Weigh accurately 68 mg of Standard Atazanavir sulphate and 20 mg of Standard Ritonavir into a 100 mL volumetric flask, dissolve and make up the volume with methanol. Transfer 5 mL of the solution into a 50 mL volumetric flask and make up the volume with the mobile phase. The solution contains 68 µg/ mL and 20 µg/ mL concentration of Atazanavir

sulphate and Ritonavir respectively. The results are tabulated in **Table 15-18** & the chromatograms were shown in **Figure 49-60**.

5.3.8 LIMIT OF DETECTION:

The limit of detection (LOD) is defined as the lowest concentration of the analyte in a sample that can be detected, though not necessarily quantitated. It was a limit test that specifies whether or not an analyte is above or below a certain value. It was expressed in a concentration at a specified signal-to-noise ratio, usually a 2- or 3-to-1 ratio. The results were tabulated in **Table 19 &** the chromatogram was shown in **Figure-61.**

$$LOD = 3.3 * \sigma \text{ / slope}$$

5.3.9 LIMIT OF QUANTITATION

The limit of Quantification (LOD) defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. The chromatogram was shown in **Figure-62.**

$$LOD = 10 * \sigma \text{ / slope}$$

Chapter 6

RESULTS & DISCUSSIONS

6. RESULTS AND DISCUSSION

6.1 METHOD DEVELOPMENT:

6.1.1 Selection of wavelength:

λ Max of Atazanavir sulphate by UV-Visible spectrophotometry
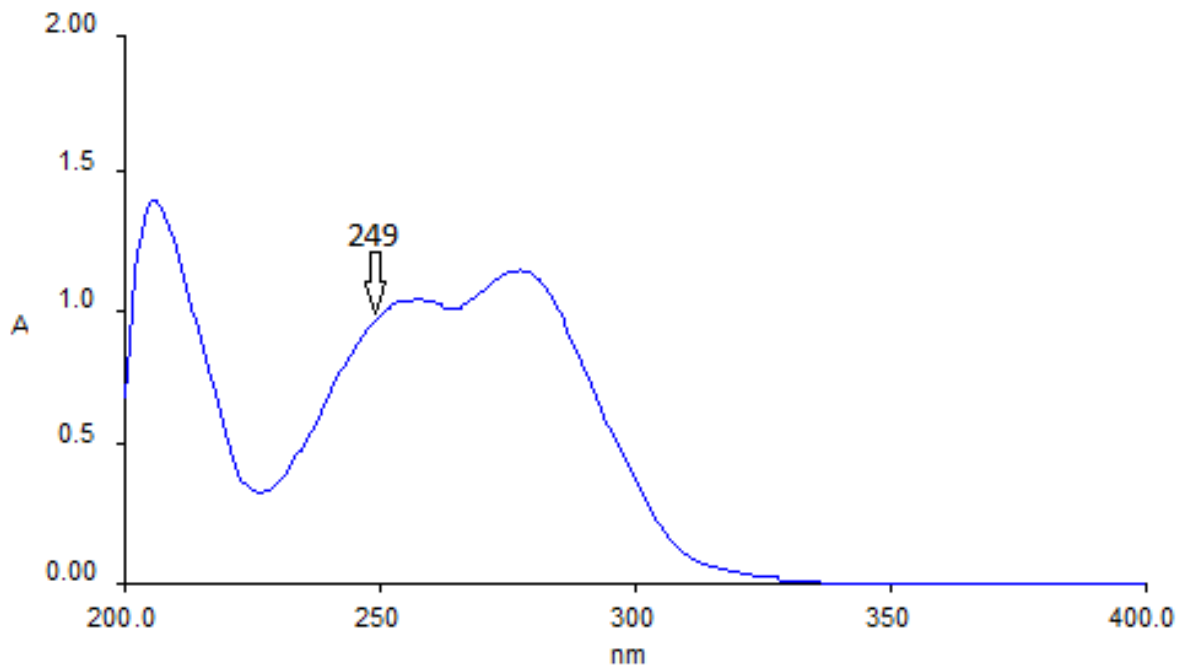
Fig. 1: λ max for Atazanavir sulphate: 249 nm

Observation:

It was concluded that Atazanavir sulphate shows maximum absorbance at   249 nm.

λ Max of Ritonavir by UV-Visible spectrophotometry
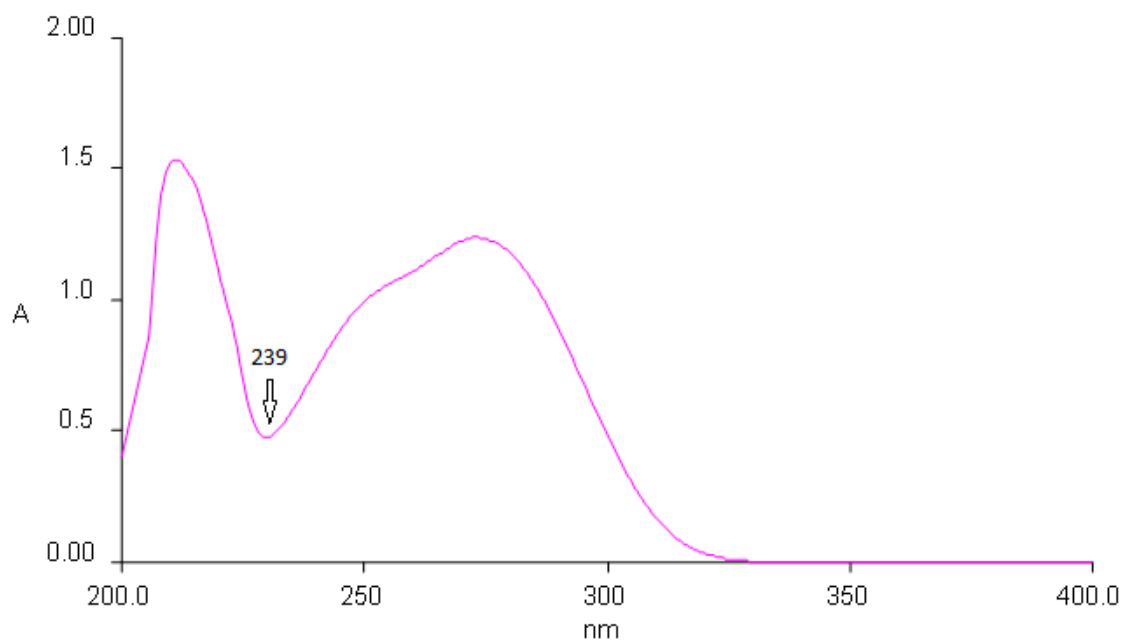
Fig. 2: λ max for Ritonavir: 239 nm

Observation:

It was concluded that Ritonavir shows maximum absorbance at 239 nm.
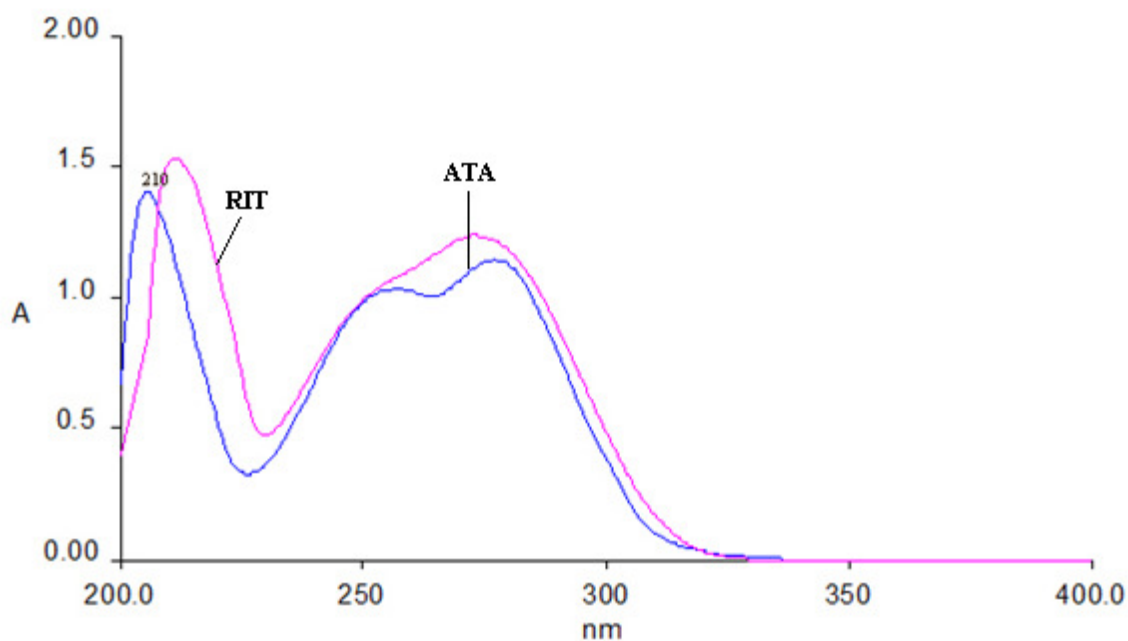
Fig. 3: Overlaid spectrum of Atazanavir sulphate and Ritonavir

Observation:

The overlaid spectrum of Atazanavir sulphate and Ritonavir shows maximum absorbance at 210 nm and was used for method development.

6.1.2 DEVELOPMENT TRAILS:

Selection of flow rate:

The flow rate was changed from 0.8-1.5 mL/ min^{-1} and the chromatogram was developed. The drugs eluted with small differences in fronting and tailing except at 1.5 mL/ min^{-1} and it was selected for analysis.

Trial-1

Table 1: Chromatographic condition

Parameters	Description
Column	Nucleodur C_8 column (100 mm x 1.8 mm x 5 µm)
Mobile Phase	Acetonitrile : Methanol: Phosphate buffer pH 3.0 (65:5:30)
Diluent	HPLC grade methanol
Flow rate	0.8 mL min^{-1}
Wavelength	210 nm
Injection mode	Auto injector (vial)
Injection volume	20 µL
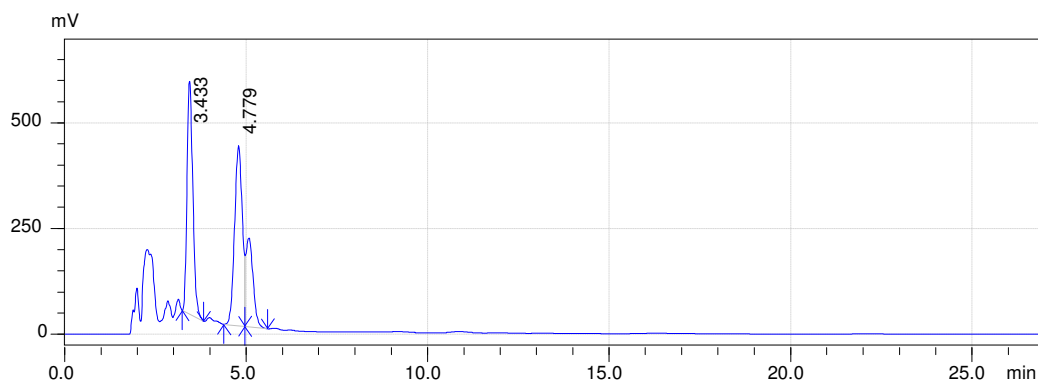
Fig. 4: Chromatogram of Atazanavir sulphate and Ritonavir

Observation:

Both Atazanavir sulphate and Ritonavir was eluted but resolution was not properly. So the flow rate and mobile phase ratio has been changed for next trial.

Trial-2

Table 2: Chromatographic condition

Parameters	Description
Column	Nucleodur C_8column (100 mm x 1.8 mm x 5 µm)
Mobile Phase	Acetonitrile : Methanol: Phosphate buffer pH 3.0 (25:5:70)
Diluent	HPLC grade methanol
Flow rate	0.9 mL min^{-1}
Wavelength	210 nm
Injection mode	Auto injector (vial)
Injection volume	20 µL
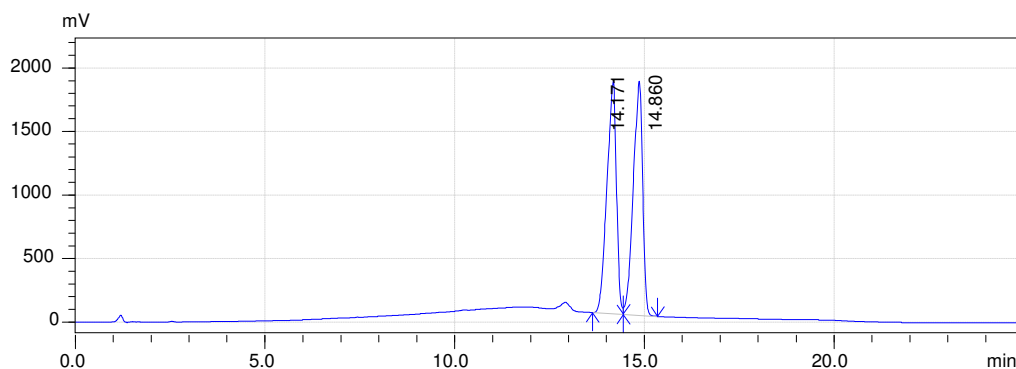
Fig. 5: Chromatogram of Atazanavir sulphate and Ritonavir

Observation:

The number of Theoretical plates for the Atazanavir sulphate and Ritonavir peaks is NLT 2000. So the flow rate and column has been changed for next trial.

Trial-3

Table 3: Chromatographic condition

Parameters	Description
Column	Nucleodur C_{18}column (150 mm x 4.6 mm x 5 µm)
Mobile Phase	Acetonitrile : Methanol: Phosphate buffer pH 3.0 (60:10:30)
Diluent	HPLC grade methanol
Flow rate	1.0 mL min^{-1}
Wavelength	210 nm
Injection mode	Auto injector (vial)
Injection volume	20 µL
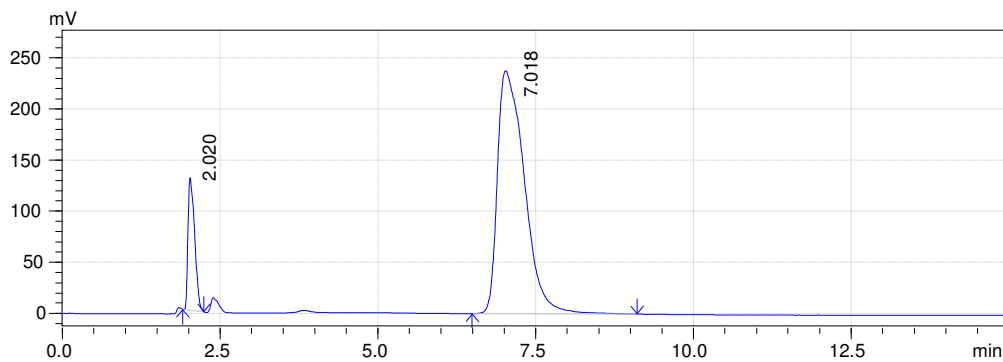
Fig. 6: Chromatogram of Atazanavir sulphate and Ritonavir

Observation:

Peak broading obtain at Ritonavir. So the mobile phase ratio and flow rate has been changed for next trial.

Trial-4

Table 4: Chromatographic condition

Parameters	Description
Column	Nucleodur C_{18}column (150 mm x 4.6 mm x 5 µm)
Mobile Phase	Acetonitrile : Methanol: Phosphate buffer pH 3.0 (50:10:40)
Diluent	HPLC grade methanol
Flow rate	1.2 mL min^{-1}
Wavelength	210 nm
Injection mode	Auto injector (vial)
Injection volume	20 µL
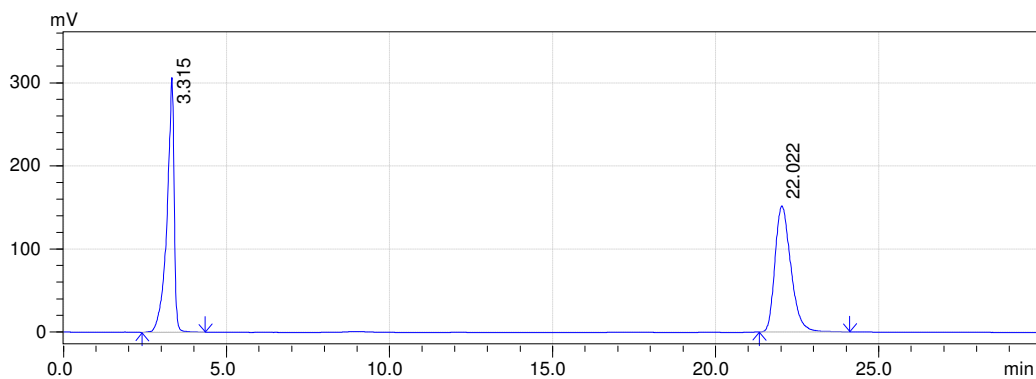
Fig. 7: Chromatogram of Atazanavir sulphate and Ritonavir

Observation:

Atazanavir sulphate was eluted properly but Ritonavir retention time greated. So the mobile phase ratio has been changed for next trial.

OPTIMIZED METHOD

Table 5: Chromatographic condition

Parameters	Description
Column	Nucleodur C_{18}column (150 mm x 4.6 mm x 5 µm)
Mobile Phase	Acetonitrile : Methanol: Phosphate buffer pH 3.0 (44:11:45)
Diluent	HPLC grade methanol
Flow rate	1.5 mL min^{-1}
Wavelength	210 nm
Injection mode	Auto injector (vial)
Injection volume	20 µL
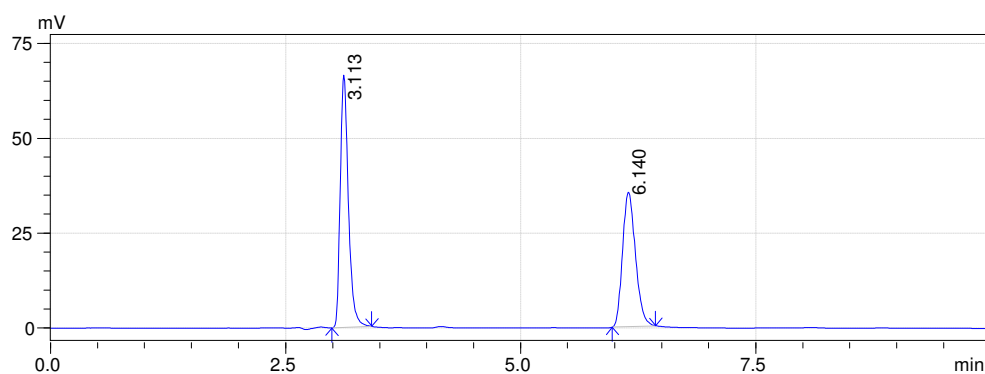
Fig. 8: Chromatogram of Atazanavir sulphate and Ritonavir

Observation:

Resolution, theoretical plates and symmetric factor were found to be within the limit. So this method is finalized as the optimized method.

ASSAY:

Table 6: Assay data for standard Atazanavir sulphate and Ritonavir

S.No.	Standard	
	Atazanavir sulphate	Ritonavir
1	1970033	568671
2	1974636	560152
3	1945756	560190
Avg	1963475	560181

Table 7: Assay data for tablets of Atazanavir sulphate and Ritonavir

S.No.	Standard	
	Atazanavir sulphate	Ritonavir
1	1972526	555272
2	1976472	555303
3	1977694	553068
Avg	1975564	554547
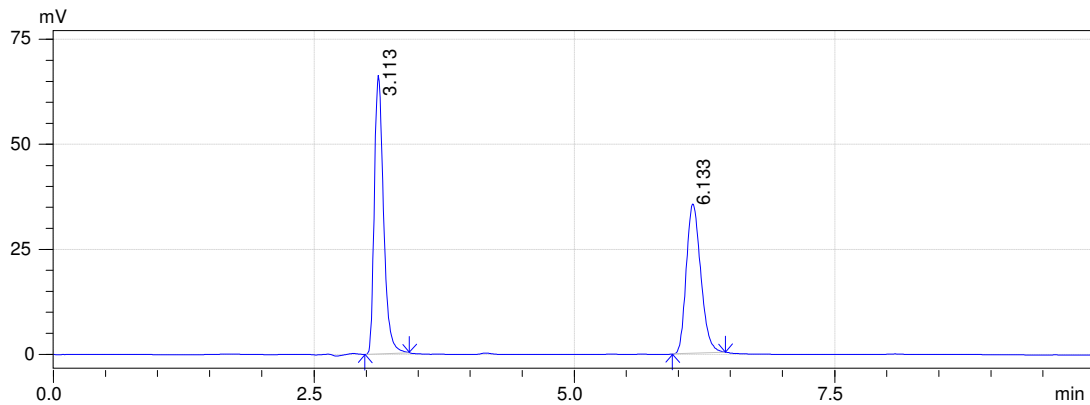
Fig. 9: Standard chromatogram of Atazanavir sulphate and Ritonavir.
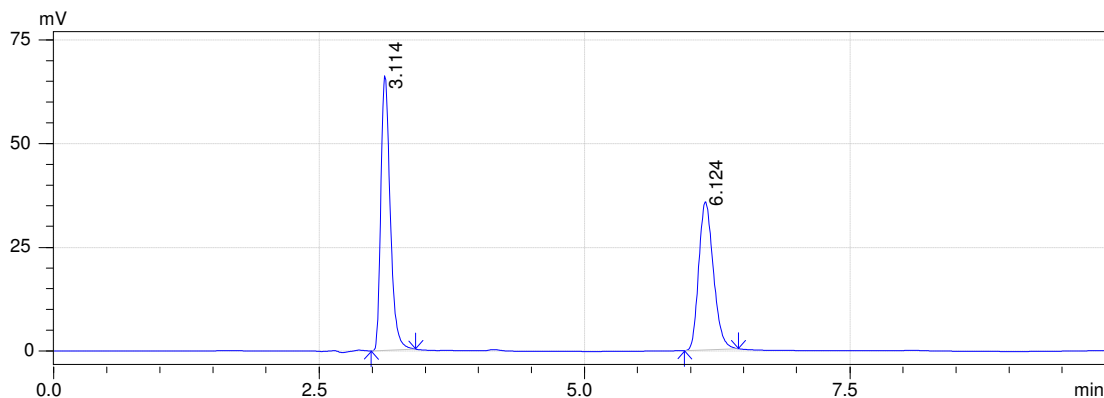
Fig. 10: Chromatogram of marketed formulation of Atazanavir sulphate and Ritonavir

Formula:

Amount present: Sample area X standard weight

$$\text{————————————— X dilution factor X average weight}$$

Standard area X sample weight

Percentage purity: Amount present/label claim X 100

For Atazanavir sulphate:

$$= \frac{1975564 \text{ X } 63.5 \text{ X } 5 \text{ X } 100 \text{ X } 50 \text{ X } 1025.5}{1963475 \text{ X } 100 \text{ x } 50 \text{ X } 220.5 \text{ X } 5}$$

= 297.10 mg

Percentage purity:

= 297.10/300 X 100

= 99.05 %

For Ritonavir:

$$= \frac{554547 \text{ X } 21.5 \text{ X } 5 \text{ X } 100 \text{ X } 50}{560181 \text{ X } 100 \text{ x } 50 \text{ X } 220.1 \text{ X } 5} \text{ X } 1025.5$$

= 99.20 mg

Percentage purity:

= 99.20/100X 100

= 99.20 %

6.2. METHOD VALIDATION

6.2.1 SPECIFICITY:

Specificity is the ability to assess unequivalently the analyte in the presence of components, which may be expected to be present. Typically these might include impurities, matrix, degradants etc. It is evaluated by injecting the blank and the control sample solution prepared as per the proposed method to check for the interference if any peak at the retention time of Atazanavir sulphate and Ritonavir.

The specificity of the HPLC method was complete separation of Atazanavir sulphate and Ritonavir was noticed in presence of tablet excipients. In addition there was no interference at the retention time of Atazanavir sulphate and Ritonavir in the chromatogram of placebo solution.

Name of the solution	Retention time in min
Blank	No peak
Placebo	No peak
Standard	3.06 and 6.18
Sample	3.13 and 6.10
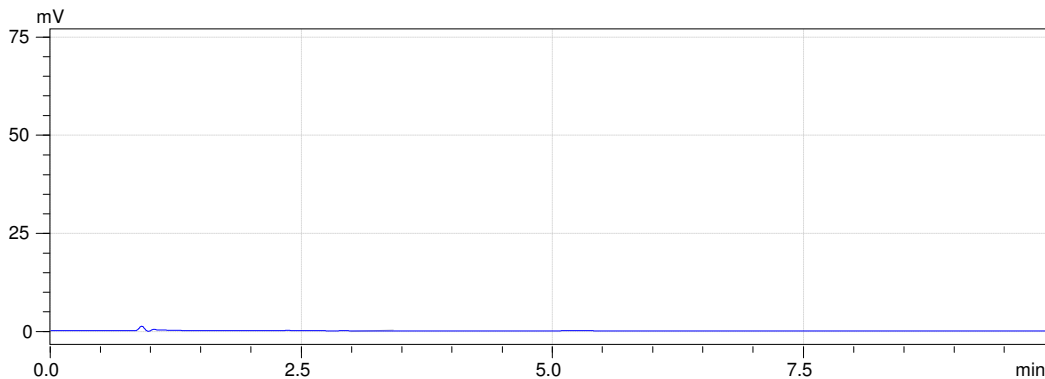
Fig. 11: Chromatogram of placebo solution
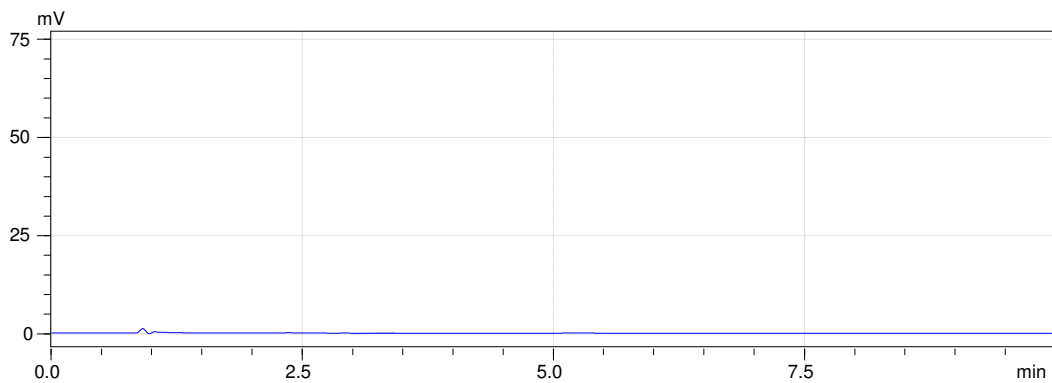
Fig. 12: Chromatogram of blank solution
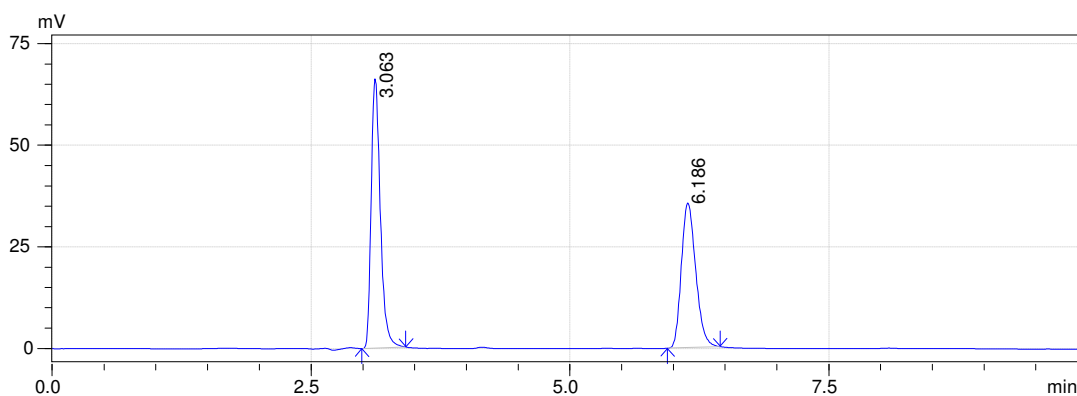
Fig. 13: Chromatogram of standard of Atazanavir sulphate and Ritonavir
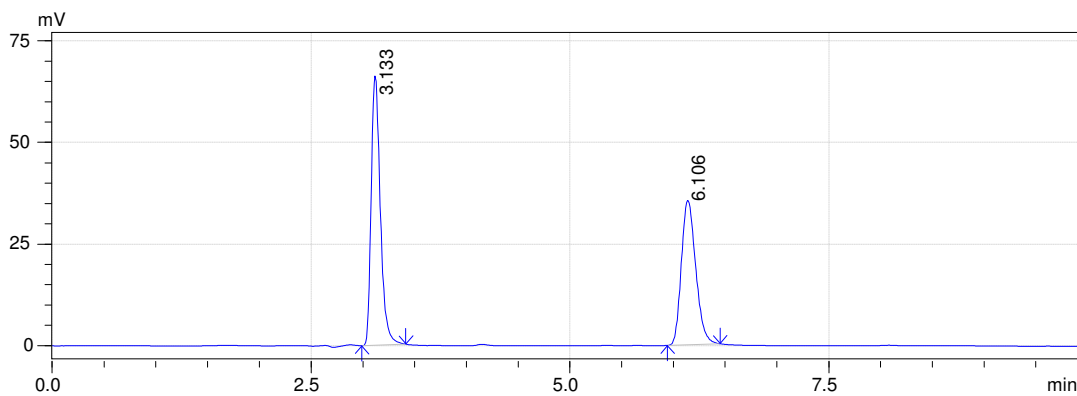
Fig. 14: Chromatogram of marketed formulation of Atazanavir sulphate and Ritonavir

Result:

The method was found to be specific because it did not show any extra peaks and there is no interference from excipients.

6.2.2 SYSTEM SUITABILITY PARAMETERS:

The system is suitable for analysis if the relative standard deviation (RSD) of area counts in the six replicate injections for each peak should not be more than 2.0%. The USP plate count of peak should not be less than 2000 theoretical plates for HPLC. The tailing factor for each peak should not be more than 2.0 and the resolution for two peaks should not be less than 2.0. The results obtained indicate the good precision of the developed method. The system suitability parameters were presented in table

Table 8: System Suitability Parameters

S. No.	Parameters	Atazanavir sulphate	Ritonavir
1	Theoretical plates	6336	7379
2	Symmetric factor	1.05	1.04
3	Resolution	6.65	6.65
4	Tailing factor	1.15	1.32

Acceptance criteria:

Theoretical plate's ≥ 2000, symmetric factor ≤ 2, tailing factor ≤ 2.

Result:

From the above table it was concluded that result obtained was within the limit only.

6.2.3 LINEARITY:

Linearity is determined by calculating the regression line using a mathematical treatment of the results (i.e. least mean squares) vs. analyte concentration. The range of the method is the interval between the upper and lower levels of an analyte that have been determined with acceptable precision, accuracy and linearity. It is determined on either a linear or nonlinear response curve (i.e. where more than one range is involved) and is normally expressed in the same units as the test results.

Table 9: Linearity of Atazanavir sulphate and Ritonavir

S.No.	Atazanavir		Ritonavir	
	Conc. (µg mL^{-1})	Peak Area	Conc. (µg mL^{-1})	Peak Area
1	34	842922	10	292661
2	54	1310821	16	455824
3	68	1658694	20	577054
4	82	1996529	24	691459
5	102	2468854	30	861146
Slope		8221		1471
Y-Intercept		24201		28693
Correlation coefficient		0.999		0.999
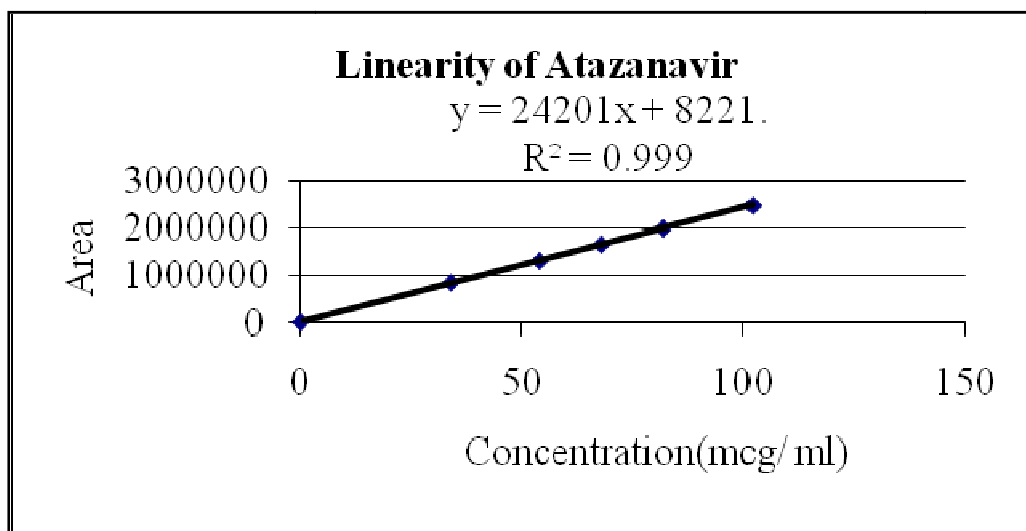
Fig. 15: Calibration plot of Atazanavir sulphate
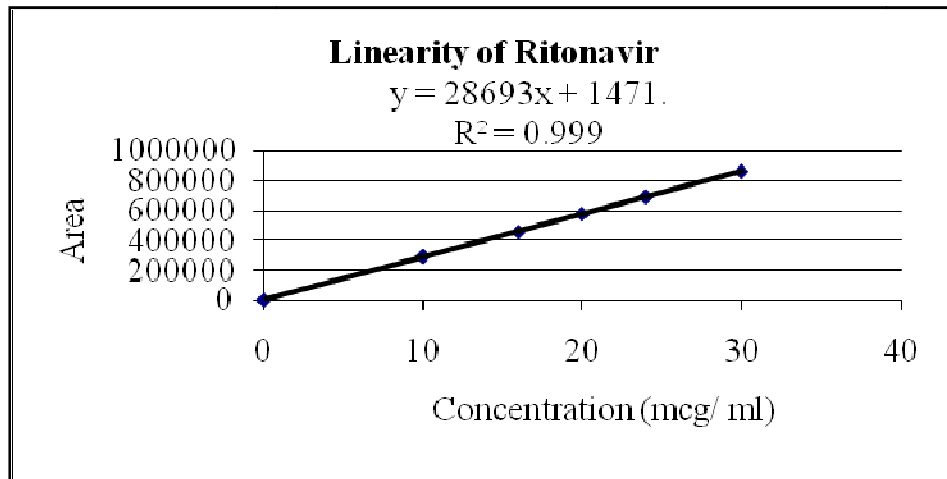
Fig. 16: Calibration plot of Ritonavir
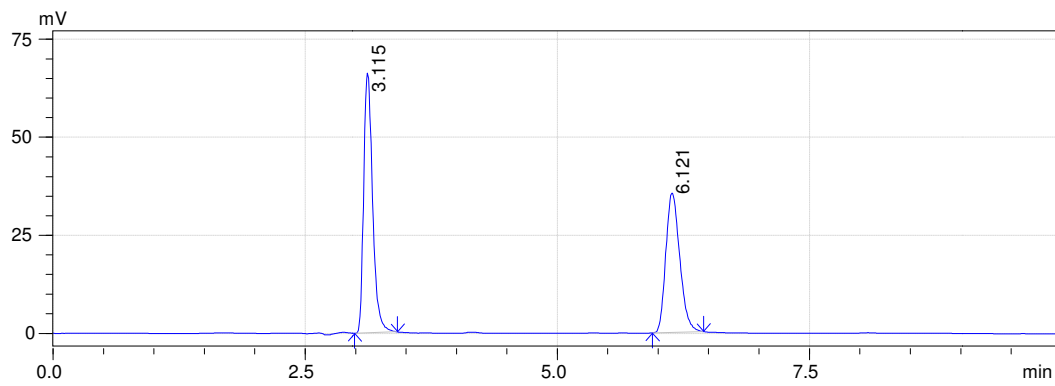
Fig. 17: Chromatogram of Atazanavir sulphate and Ritonavir for Linearity-1
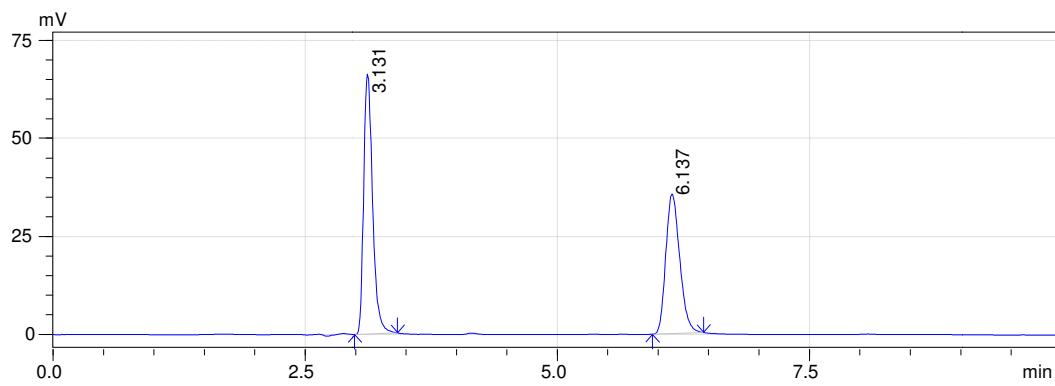
Fig. 18: Chromatogram of Atazanavir sulphate and Ritonavir for Linearity-2
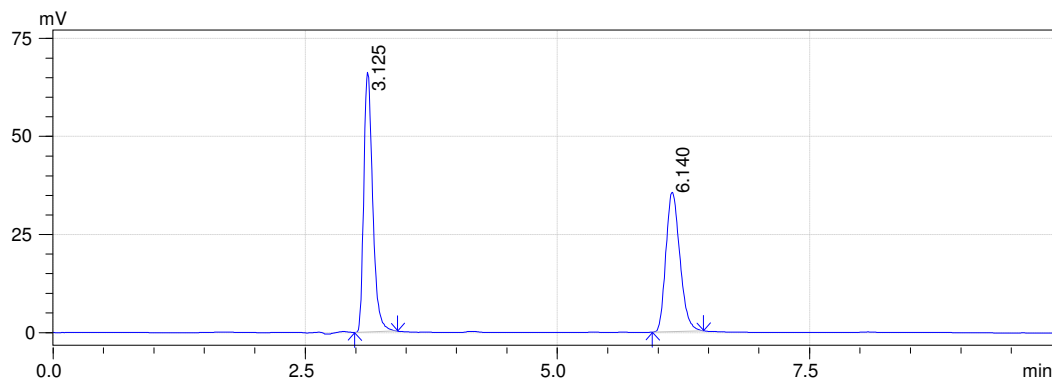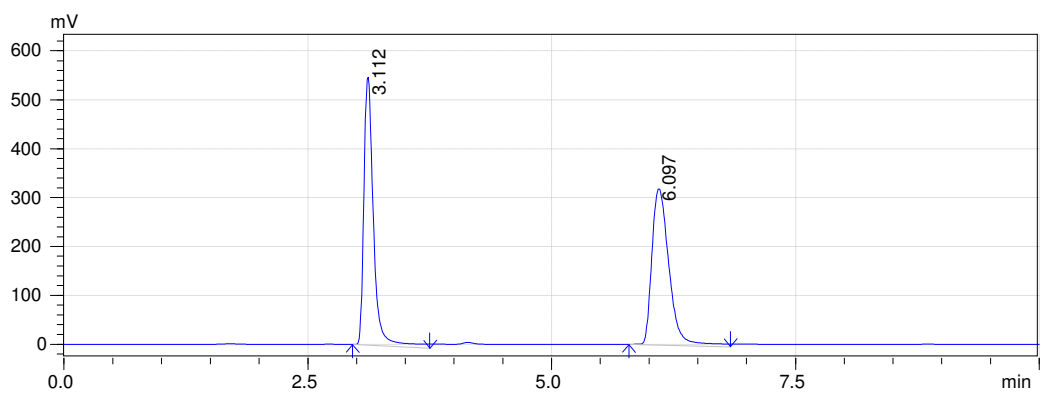
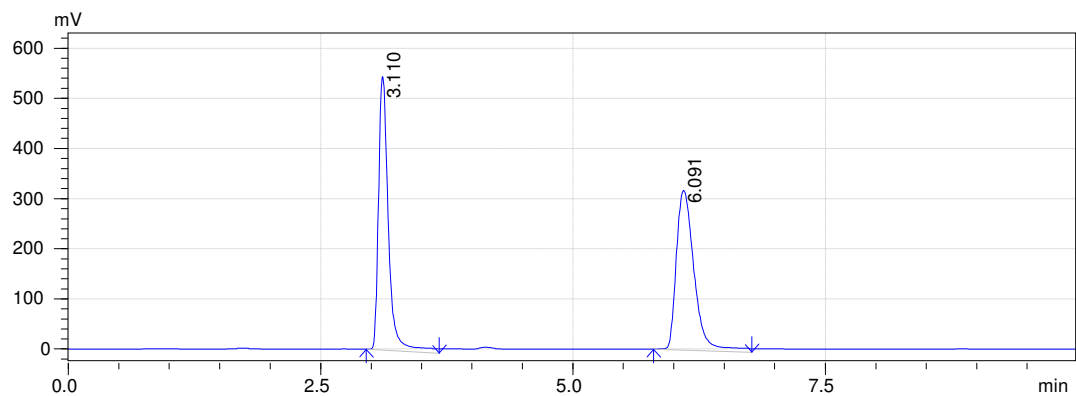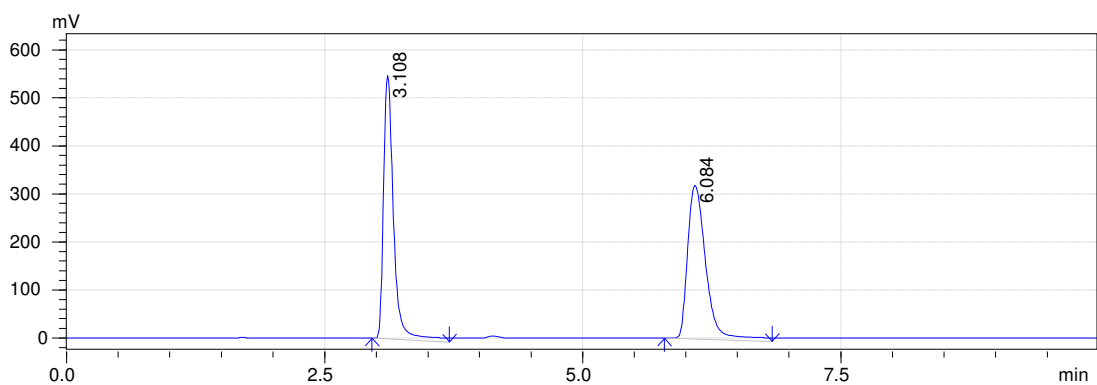Fig. 19: Chromatogram of Atazanavir sulphate and Ritonavir for Linearity-3
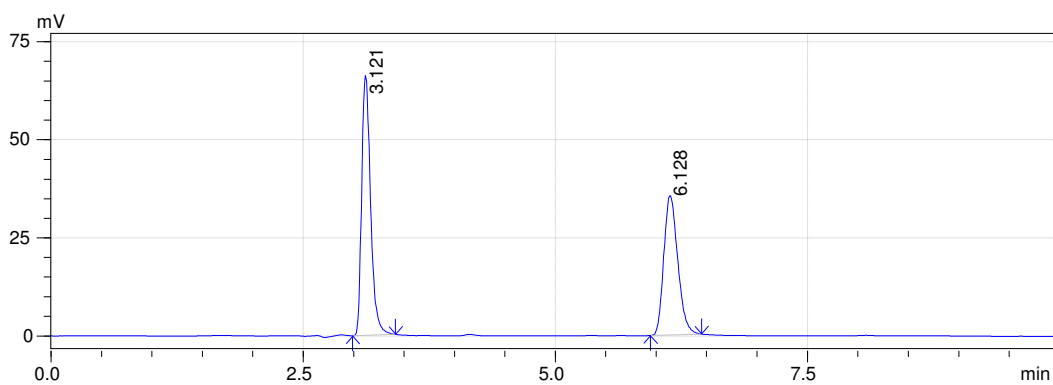
Fig. 20: Chromatogram of Atazanavir sulphate and Ritonavir for Linearity-4
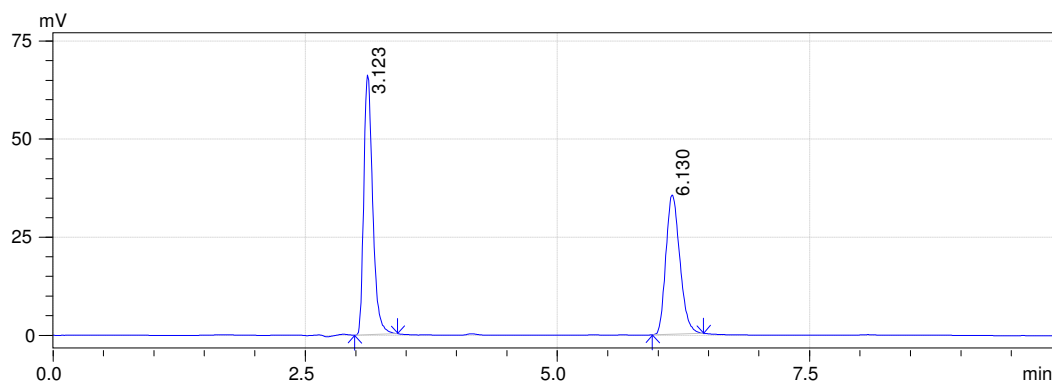
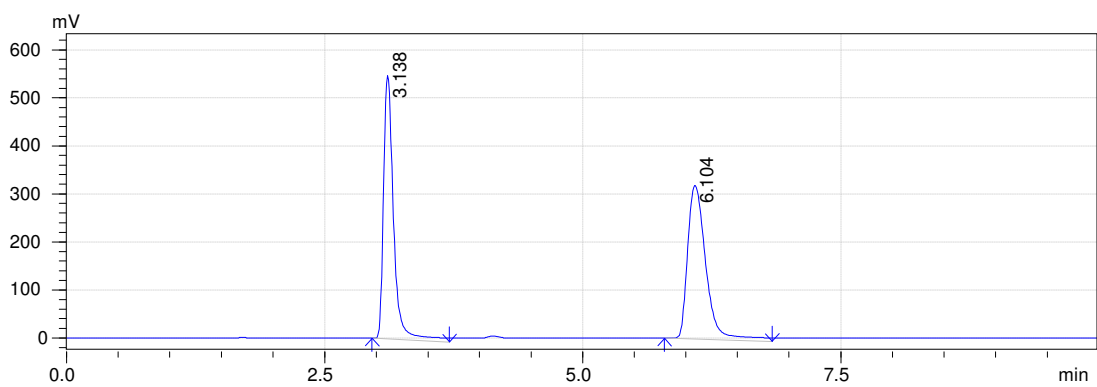Fig. 21: Chromatogram of Atazanavir sulphate and Ritonavir for Linearity-5

Acceptance criteria:

Correlation Coefficient should be not less than 0.9990

Result:

The relationship between the concentration of Atazanavir sulphate and Ritonavir was linear in the specific range and the correlation coefficient was found to be within limit only. The correlation coefficient of Atazanavir sulphate and Ritonavir was found to be 0.999 and 0.999.

6.2.4 PRECISION:

The precision of an analytical method is the degree of agreement among individual test results obtained when the method is applied to multiple sampling of a homogenous sample. Precision is a measure of the reproducibility of the whole analytical method (including sampling, sample preparation and analysis) under normal operating circumstances. Precision is determined by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision is then expressed as the relative standard deviation.

Table 10: Method precision:

S.No	ATAZANAVIR SULPHATE		RITONAVIR	
	Rt	Peak area	Rt	Peak area
1	3.10	1975564	6.03	534547
2	3.10	1976472	6.08	535303
3	3.11	1977694	6.09	533068
4	3.11	1975450	6.09	531885
5	3.10	1980537	6.08	550278
6	3.13	1973346	6.10	545065
Mean		1976511		538358
SD		2435.14		7494.10
%RSD		0.12		1.39
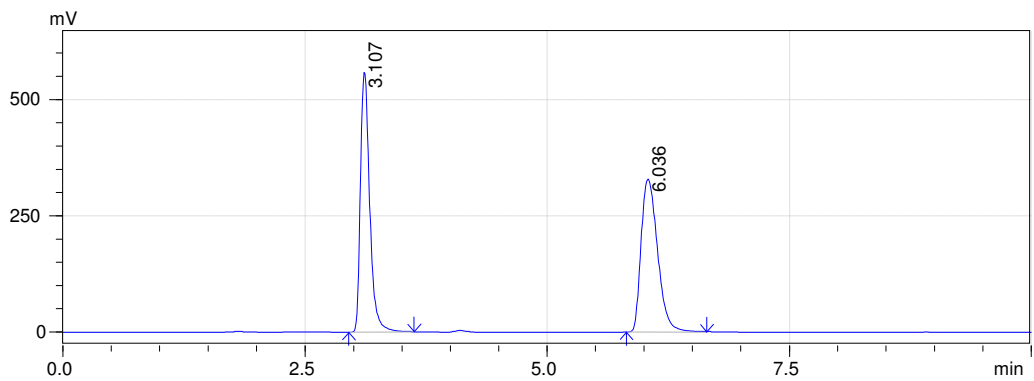
Fig. 22: Chromatogram of Atazanavir sulphate and Ritonavir for precision-1
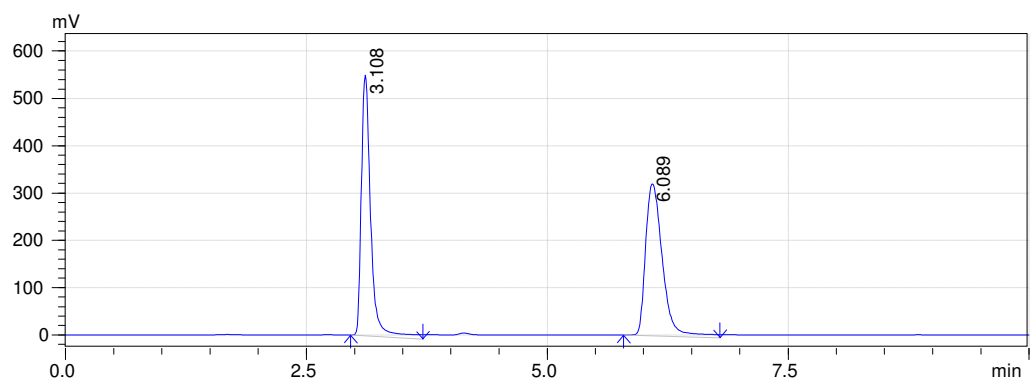
Fig. 23: Chromatogram of Atazanavir sulphate and Ritonavir for precision -2
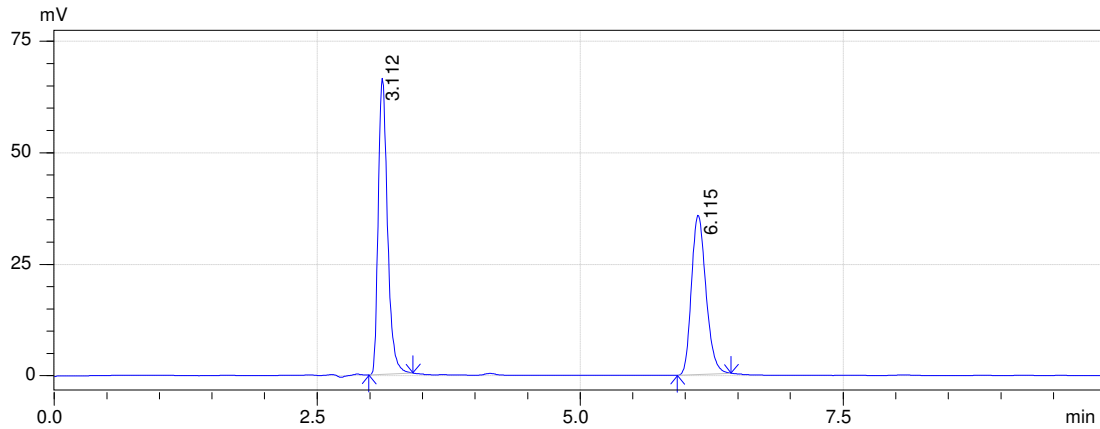

Fig. 24: Chromatogram of Atazanavir sulphate and Ritonavir for precision -3

Fig. 25: Chromatogram of Atazanavir sulphate and Ritonavir for precision -4
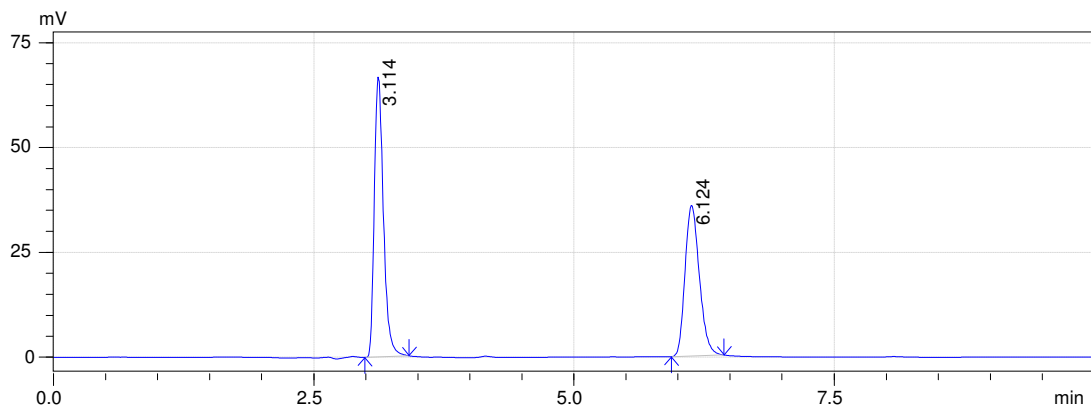

Fig. 26: Chromatogram of Atazanavir sulphate and Ritonavir for precision -5
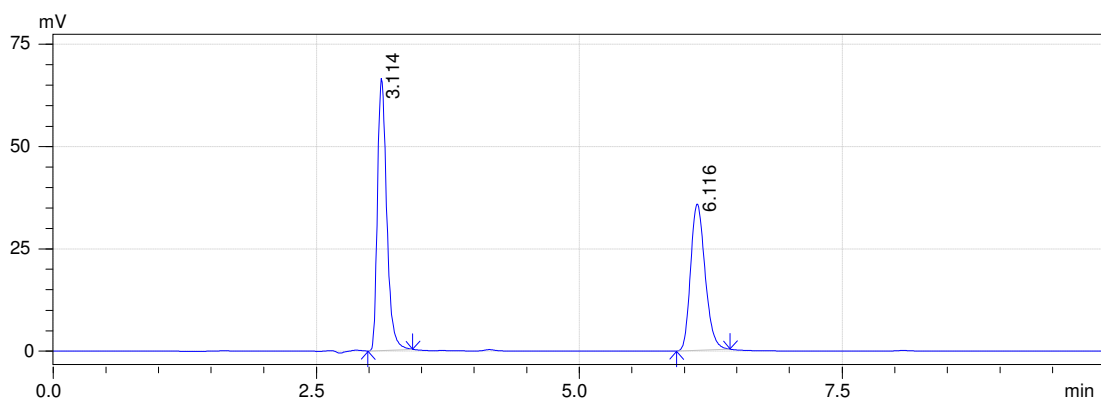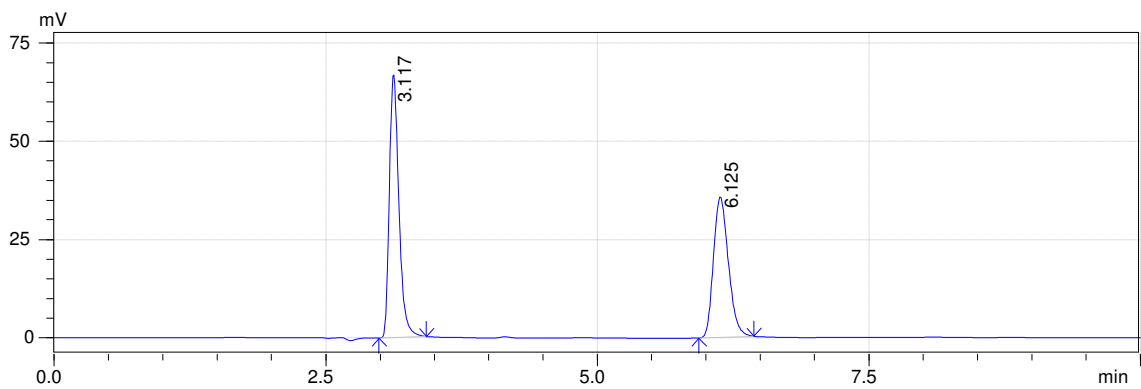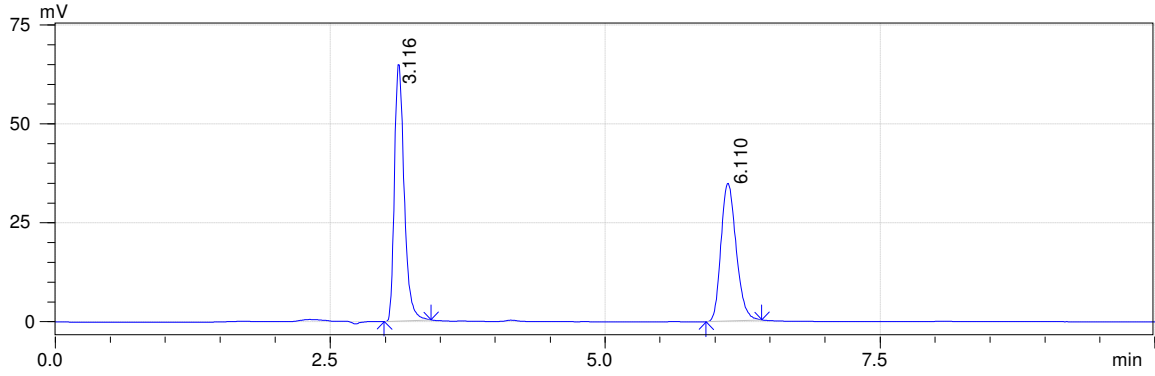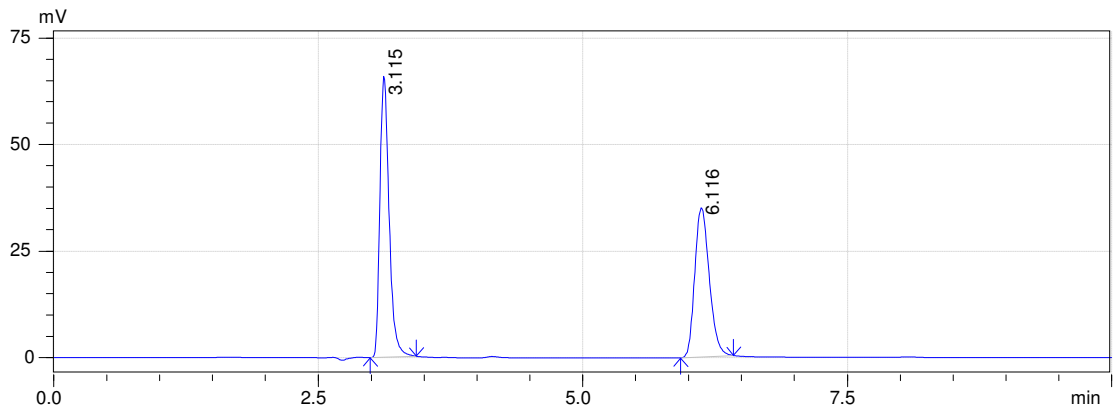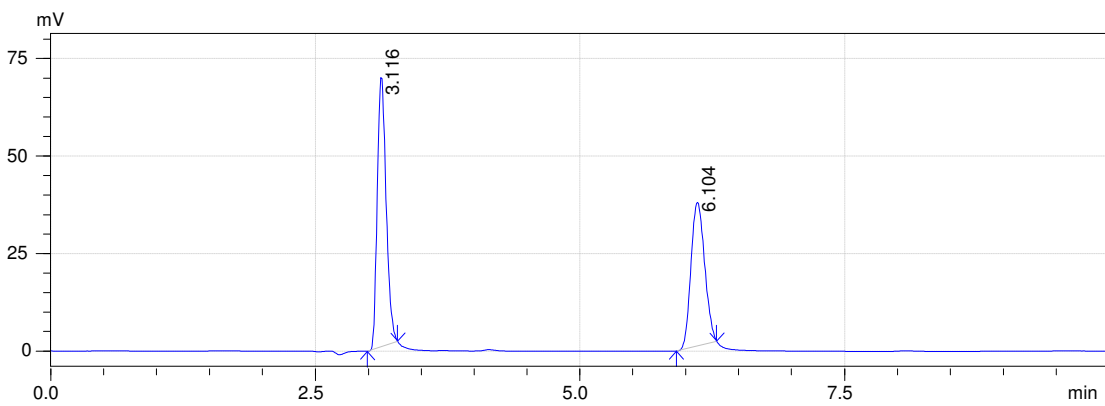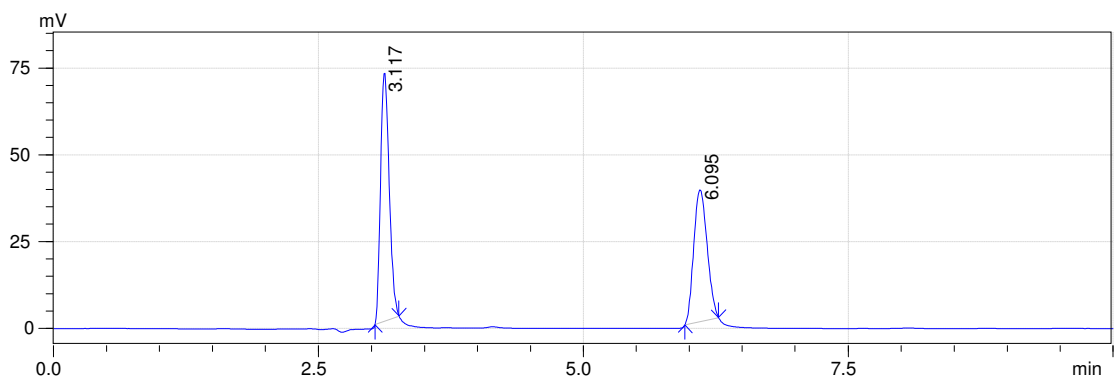

Fig. 27: Chromatogram of Atazanavir sulphate and Ritonavir for precision -6

Acceptance Criteria:

% RSD of the sample replicate should not be more than 2.

Result:

The % RSD for Atazanavir sulphate and Ritonavir it was found to be 0.12 and 1.39 in AUC, respectively. The % RSD value indicates a good degree of precision within the specified range.

6.2.5 ACCURACY:

Accuracy is a measure of the closeness of test results obtained by a method to the true value. Accuracy indicates the deviation between the mean value found and the true value. It is determined by applying the method to samples to which known amounts of analyte have been added. These should be analyzed against standard and blank solutions to ensure that no interference exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay.

Table-11: Accuracy for Atazanavir Sulphate

AMOUNT OF SPIKED	Peak Area	Amount of added (mg)	Amount recovered (mg)	% Recovery	Average recovery	% RSD
80%	1559712	240.59	240.31	99.88	100.01	0.17
	1566912	239.49	239.80	100.13		
	1563075	239.69	240.48	100.33		
100%	1986505	299.88	301.13	100.42	100.40	0.31
	1979715	299.29	299.53	100.08		
	1986012	299.98	302.06	100.69		
120%	2316272	361.70	360.71	99.73	99.91	0.24
	2317572	358.92	359.51	100.16		
	2327359	362.01	361.38	99.83		

Table-12: Accuracy for Ritonavir

AMOUNT OF SPIKED	Peak Area	Amount of added (mg)	Amount recovered (mg)	% Recovery	Average recovery	% RSD
80%	422000	79.51	79.51	100.00	100.31	0.27
	423557	78.88	79.27	100.49		
	423937	79.41	79.76	100.43		
100%	541035	100.04	100.29	100.25	100.12	0.61
	539736	100.23	99.86	99.63		
	542332	100.38	100.87	100.49		
120%	629877	120.49	119.95	99.55	100.31	0.29
	632736	119.46	120.03	100.48		
	635575	119.61	120.68	100.89		

Fig. 28: Chromatogram of Atazanavir sulphate and Ritonavir for 80%spiking-1

Fig. 29: Chromatogram of Atazanavir sulphate and Ritonavir for 80% spiking-2

Fig. 30: Chromatogram of Atazanavir sulphate and Ritonavir for 80%spiking-3

Fig. 31: Chromatogram of Atazanavir sulphate and Ritonavir for 100% spiking-1

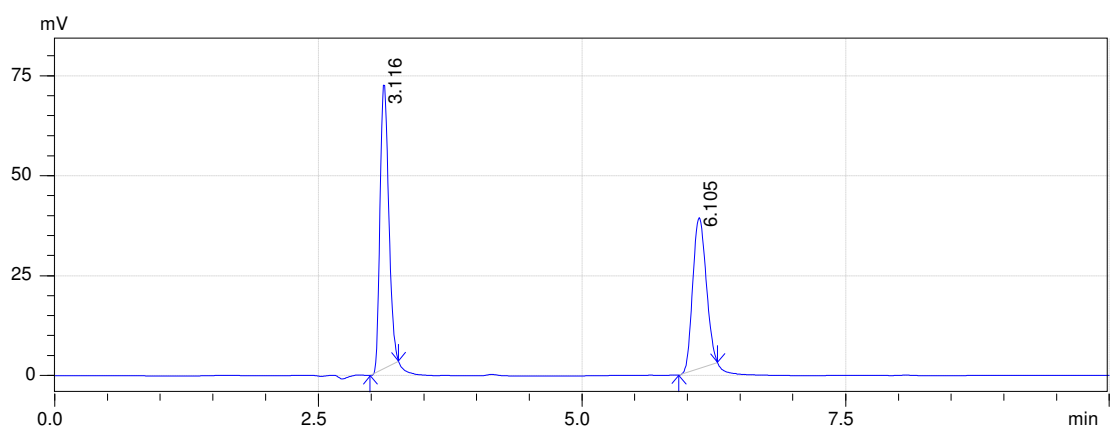Fig. 32: Chromatogram of Atazanavir sulphate and Ritonavir for 100% spiking-2

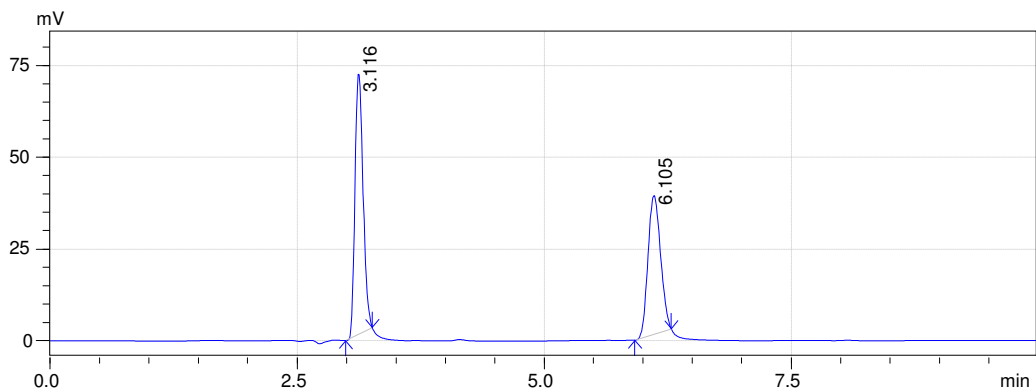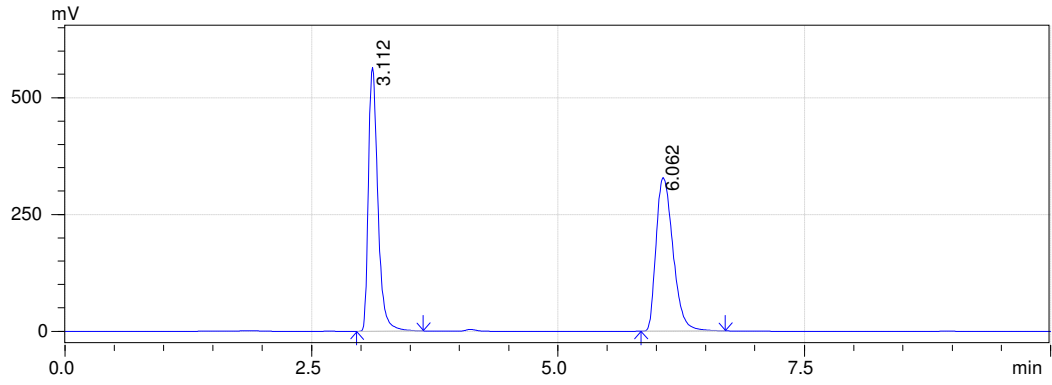Fig. 33: Chromatogram of Atazanavir sulphate and Ritonavir for 100% spiking-3

Fig. 34: Chromatogram of Atazanavir sulphate and Ritonavir for 120% spiking-1

Fig. 35: Chromatogram of Atazanavir sulphate and Ritonavir for 120% spiking-2

Fig. 36: Chromatogram of Atazanavir sulphate and Ritonavir for 120% spiking-3

Acceptance criteria:

The mean percentage recovery of the Atazanavir sulphate and Ritonavir at each spike level should be not less than 98.0% and not more than 102.0%.

Result:

From the Accuracy table it was found that % Recovery of the drug was found to be in the range of 99.91-100.40 % and 100.12-100.31 % for Atazanavir sulphate and Ritonavir respectively. This indicates that the method was accurate.

6.2.6 RUGGEDNESS:

Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions i.e. different analysts, laboratories, instruments, reagents, assay temperatures, small variations in mobile phase, different days etc. (i.e. from laboratory to laboratory, from analyst to analyst). Acceptance criteria for ruggedness, the % RSD for the area of five standard injections should not be more than 2%.

Table-13: Intraday Precision and Intermediate precision analyst-1

Parameter	Inj	Atazanavir Sulphate			Ritonavir		
		Rt	AUC	%ASSAY	Rt	AUC	%ASSAY
Intraday Precision Data Analyst-1	1	3.116	1987964	99.78	6.105	544358	100.42
	2	3.112	1986984	99.14	6.062	546722	100.26
	3	3.107	1979854	99.56	6.047	541098	100.00
	4	3.127	1985269	99.60	6.027	544037	100.32
	5	3.106	1985060	99.41	6.040	546826	100.65
	6	3.107	1998324	99.80	6.041	539046	98.94
	Mean						
	% R.S.D		0.31	0.25		0.57	0.60

Fig.37: Chromatogram of Atazanavir sulphate and Ritonavir for ruggedness -1

Fig. 38: Chromatogram of Atazanavir sulphate and Ritonavir for ruggedness -2
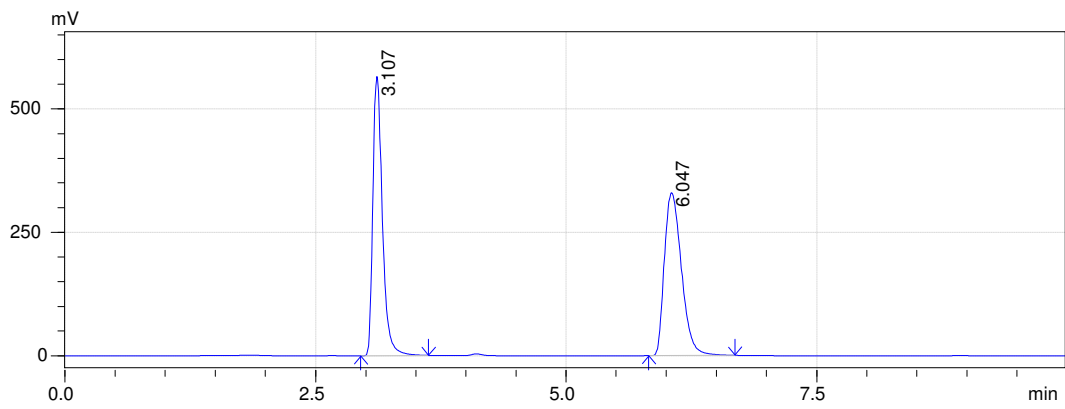

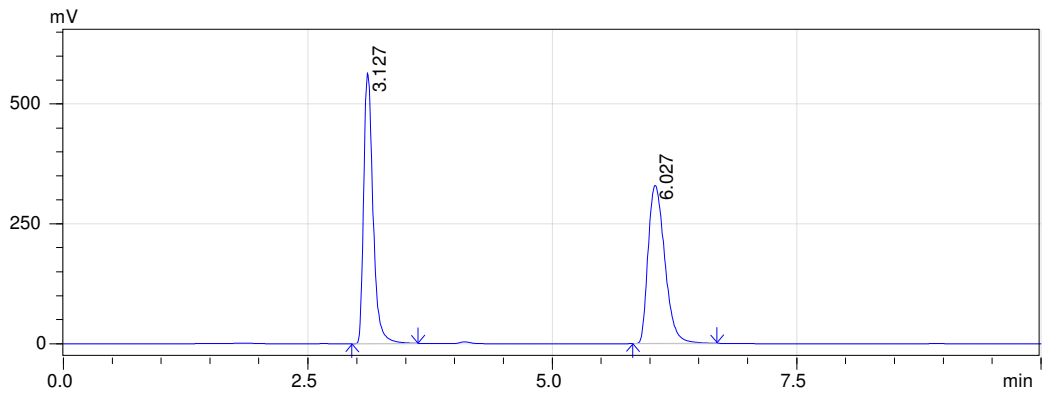Fig. 39: Chromatogram of Atazanavir sulphate and Ritonavir for ruggedness -3

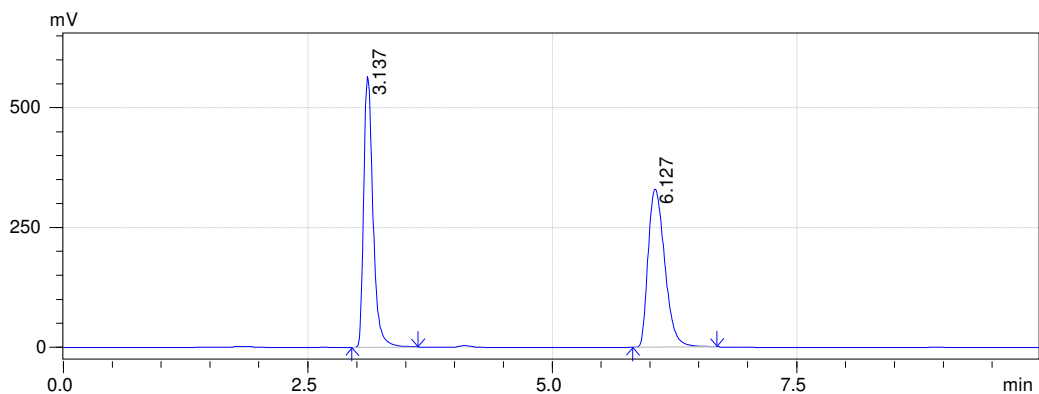Fig. 40: Chromatogram of Atazanavir sulphate and Ritonavir for ruggedness -4

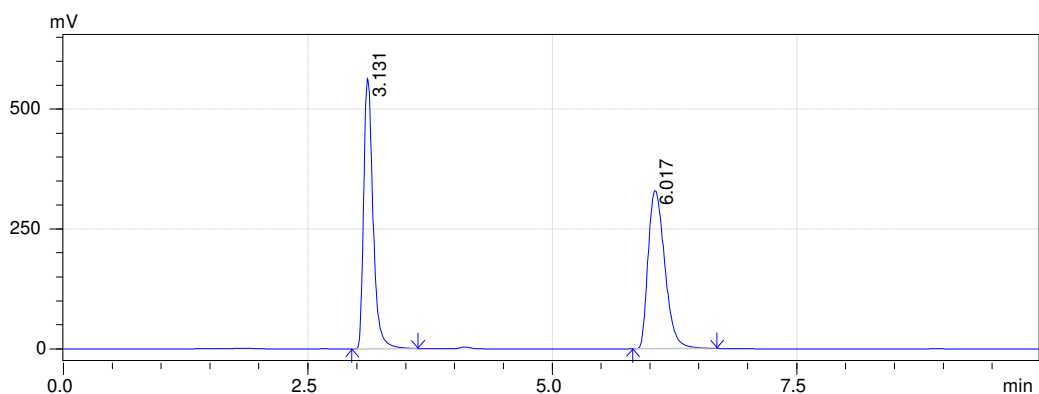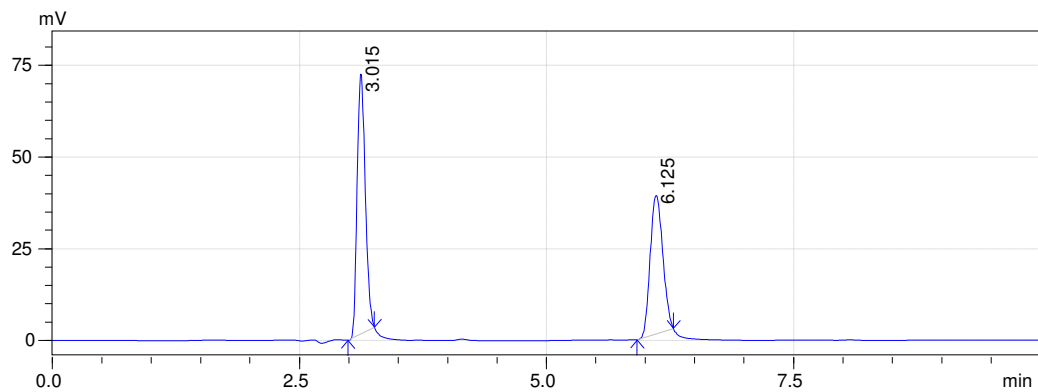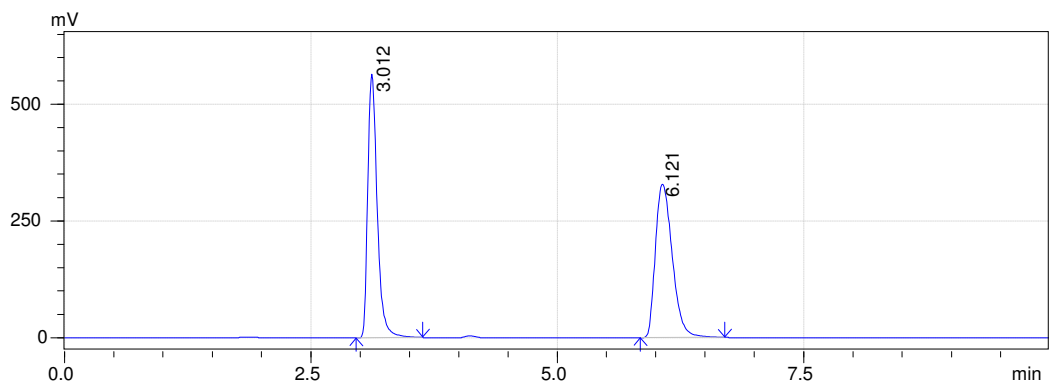Fig. 41: Chromatogram of Atazanavir sulphate and Ritonavir for ruggedness -5

Fig. 42: Chromatogram of Atazanavir sulphate and Ritonavir for ruggedness -6

Table-14: Intermediate precision analyst-2

Parameter	Inj	Atazanavir Sulphate			Ritonavir		
		Rt	AUC	%ASSAY	Rt	AUC	%ASSAY
Intraday Precision Data Analyst-2	1	3.015	1993291	100.05	6.125	542741	100.12
	2	3.012	1988365	99.21	6.121	543104	99.60
	3	3.112	1986281	99.88	6.101	549121	101.49
	4	3.001	1996202	100.15	6.031	547160	100.89
	5	3.006	1974107	98.86	6.141	545397	100.38
	6	3.017	1997980	99.78	6.121	547218	100.44
	Mean						
	% R.S.D		0.44	0.51		0.46	0.64

Fig.43: Chromatogram of Atazanavir sulphate and Ritonavir for ruggedness -1

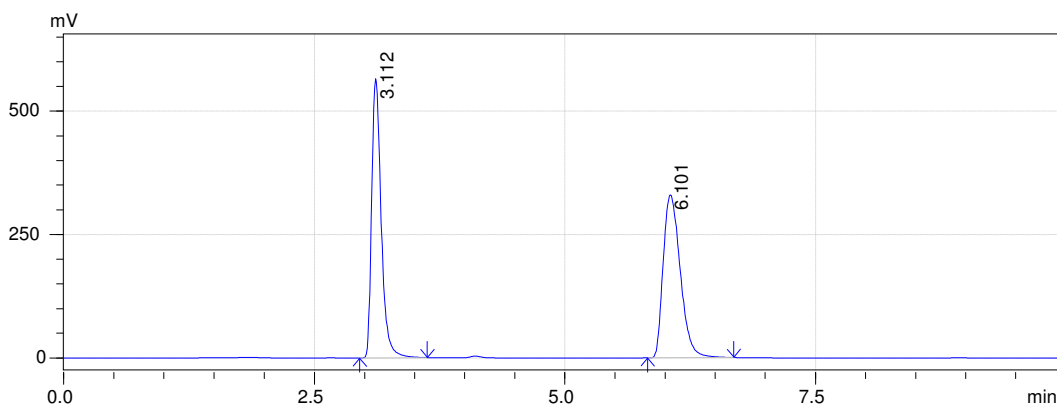Fig. 44: Chromatogram of Atazanavir sulphate and Ritonavir for ruggedness -2

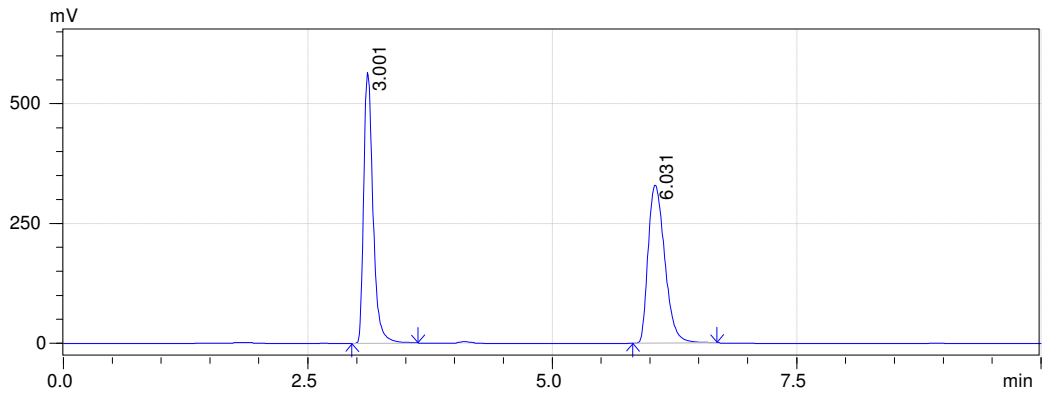Fig. 45: Chromatogram of Atazanavir sulphate and Ritonavir for ruggedness -3

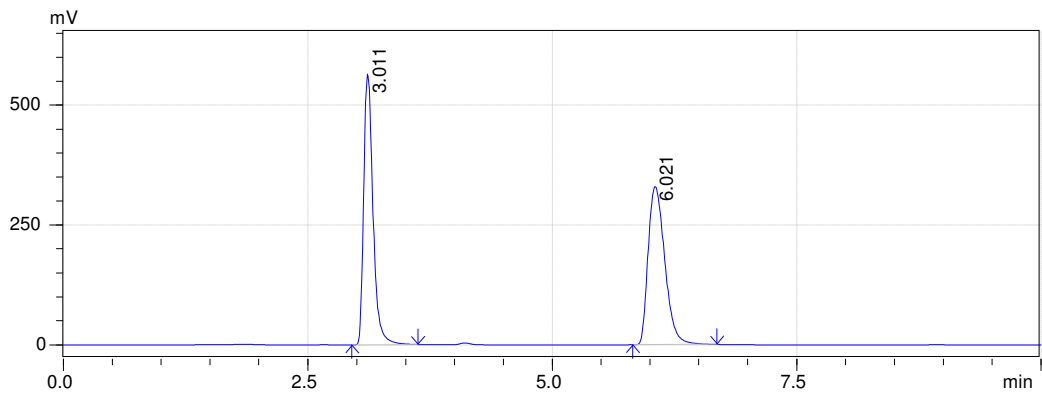Fig. 46: Chromatogram of Atazanavir sulphate and Ritonavir for ruggedness -4

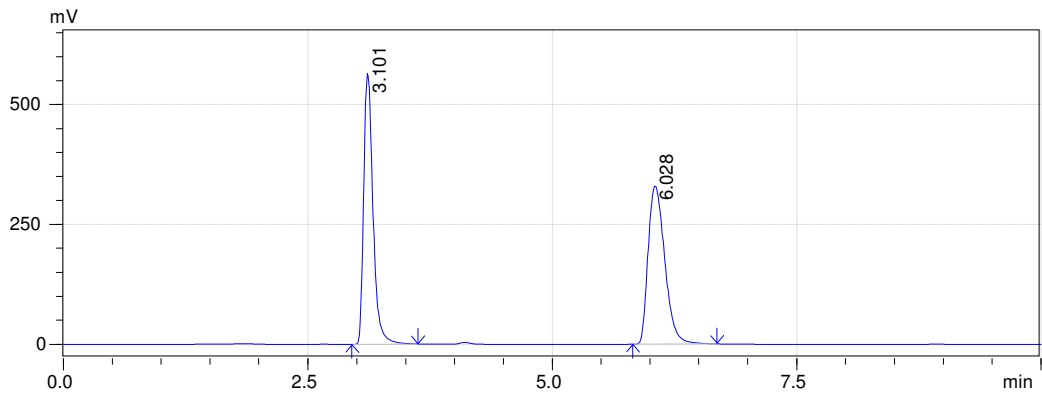Fig. 47: Chromatogram of Atazanavir sulphate and Ritonavir for ruggedness -5

Fig. 48: Chromatogram of Atazanavir sulphate and Ritonavir for ruggedness -6

Result:

Individual % RSD of Assay was found to be 0.25 % and 0.60 %. The Relative standard deviation of % Assay of Analyst 1 found to be for Atazanavir sulphate 0.31 % and Ritonavir 0.57 % and the sample passed the test. The relative standard deviation of % Assay of Analyst 2 found to be for Atazanavir sulphate 0.44 % and Ritonavir 0.46 % respectively.

6.2.7 ROBUSTNESS:

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by a small, but deliberate variation in the method parameters and provides an indication of its reliability during normal usage. Robustness of the method was investigated under a variety of conditions including deliberate changes in the temperature, mobile phase composition and change in flow rate. The degree of reproducibility of the results obtained as a result of small variations in the method parameters has proven that the method is robust. The relative standard deviation for the assay value of Atazanavir sulphate and Ritonavir obtained under deliberately modified chromatographic conditions should be less than 2%. The results obtained in the present study also indicate the method is robust.

Change in flow rate: ± 0.1

Table 15: Flow rate changes for Atazanavir Sulphate

S.NO	FLOW RATE 1.2mL/min		FLOW RATE 1.3mL/min		FLOW RATE 1.4mL/min	
	Rt	Peak area	Rt	Peak area	Rt	Peak area
1	3.12	1995819	3.13	1991097	3.21	1993678
2	3.10	1972906	3.12	1999055	3.24	1990321
Mean		1984363		1995076		1992000
SD		16201.9		5627.1		2373.8
% RSD		0.81		0.28		0.12

Table 16: Flow rate changes for Ritonavir

S.NO	FLOW RATE 1.2mL/min		FLOW RATE 1.3mL/min		FLOW RATE 1.4mL/min	
	Rt	Peak area	Rt	Peak area	Rt	Peak area
1	6.13	542654	6.14	559592	6.23	551280
2	6.13	543459	6.13	556273	6.24	550695
Mean		543056		559432		550988
SD		569.2		225.5		413.7
% RSD		0.10		0.04		0.07
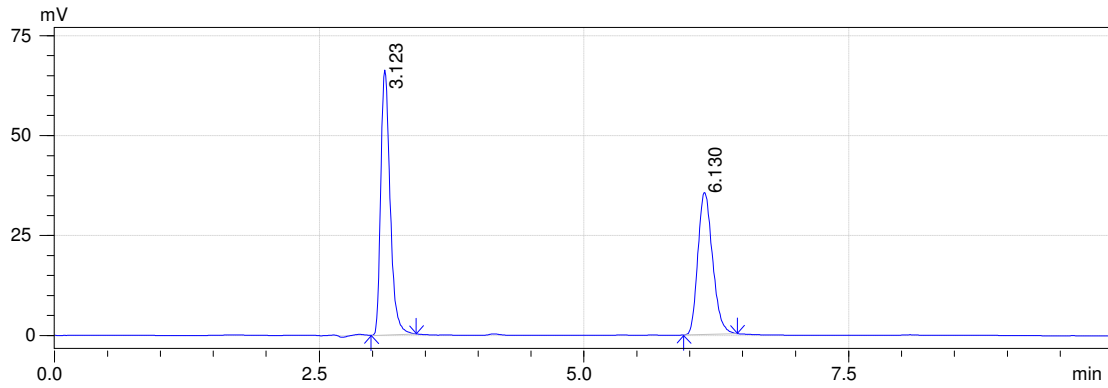
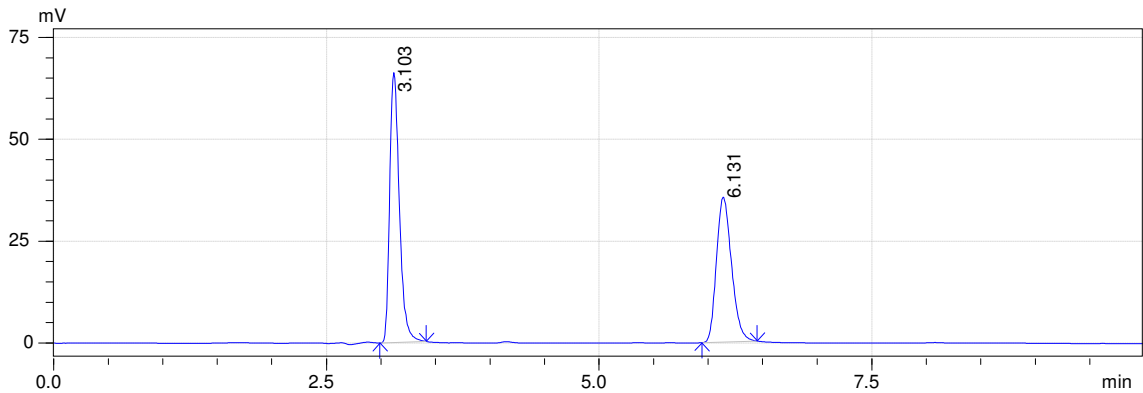Fig. 49: Flow rate changes (Flow rate 1.2mL)

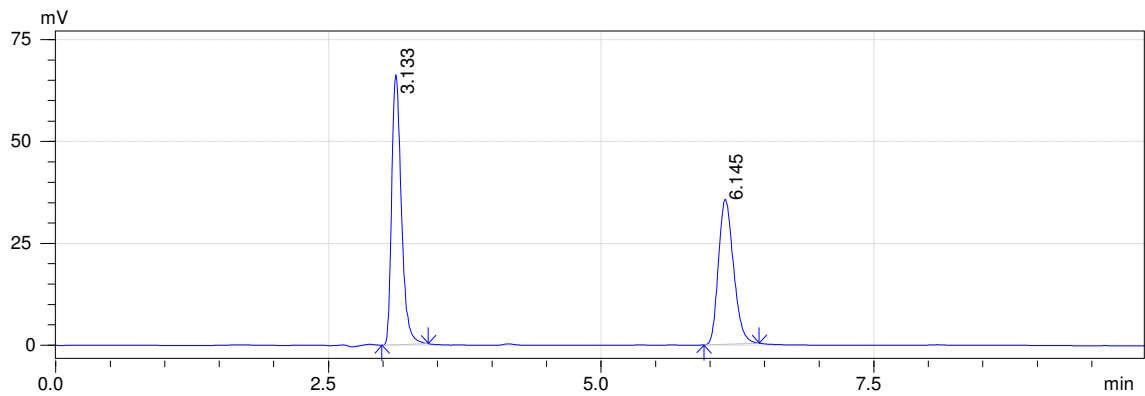Fig. 50: Flow rate changes (Flow rate 1.2mL)
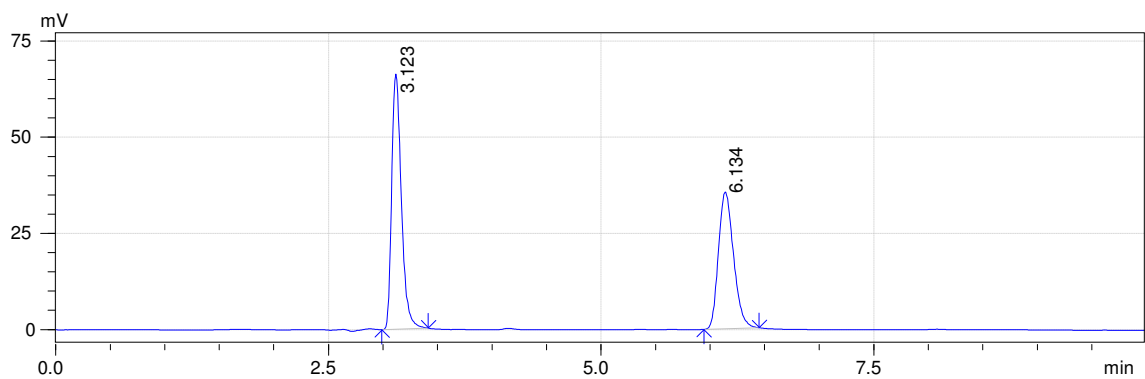
Fig. 51: Flow rate changes (Flow rate 1.3mL)
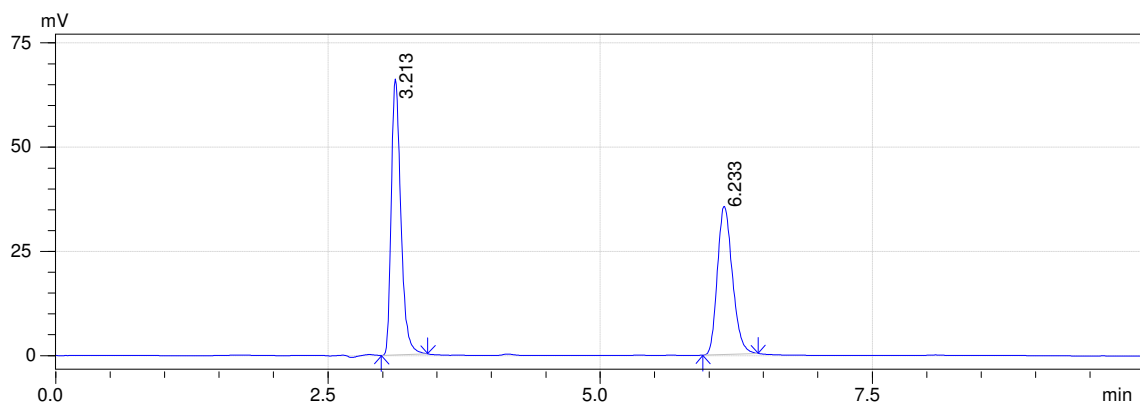

Fig. 52: Flow rate changes (Flow rate 1.3mL)
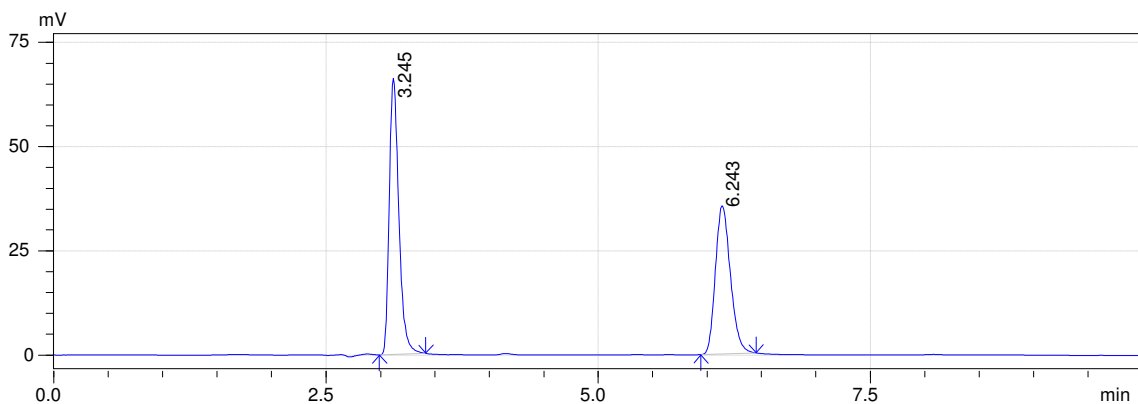

Fig. 53: Flow rate changes (Flow rate 1.4mL)

Fig. 54: Flow rate changes (Flow rate 1.4mL)

Table 17: Mobile phase ratio changes for Atazanavir Sulphate

S.NO	MOBILE PHASE 50:10:40		MOBILE PHASE CONSTANT 44:11:45		MOBILE PHASE 45:15:40	
	Rt	**Peak area**	**Rt**	**Peak area**	**Rt**	**Peak area**
1	3.13	1982858	3.10	1979925	3.13	1984227
2	3.14	1983273	3.11	1983419	3.11	1983419
Mean		1983066		1981672		1983823
SD		293.45		2470.63		571.34
% RSD		0.01		0.12		0.03

Table 18: Mobile phase ratio changes for Ritonavir

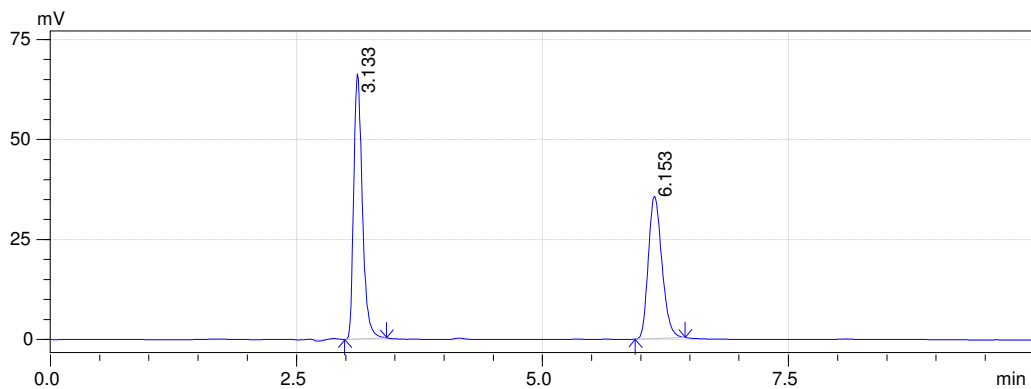| S.NO	MOBILE PHASE 50:10:40		MOBILE PHASE CONSTANT 44:11:45		MOBILE PHASE 45:15:40	
	Rt	Peak area	Rt	Peak area	Rt	Peak area
1	6.15	549370	6.13	541350	6.14	543884
2	6.18	542393	6.13	543884	6.13	549614
Mean		545882		542617		546749
SD		4933.48		1791.81		4051.72
% RSD		0.90		0.33		0.74

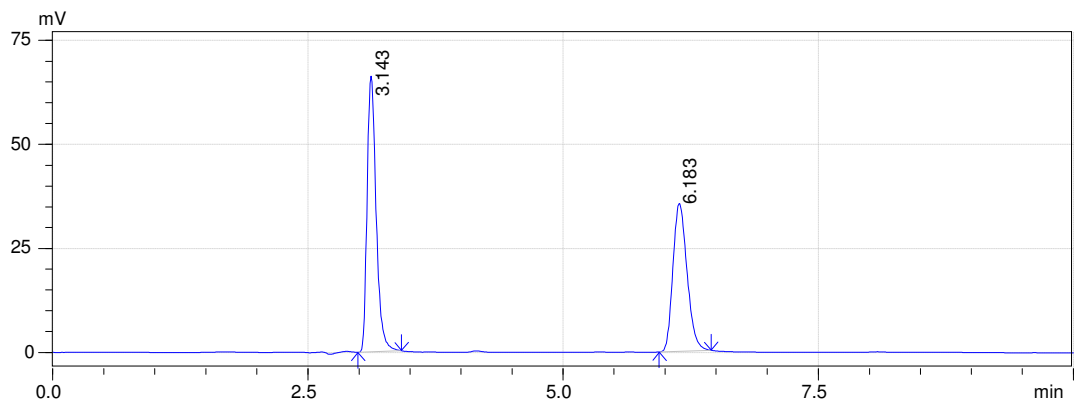Fig. 55: Mobile phase ratio changes (ACN 50: Methanol 10: Buffer 40)

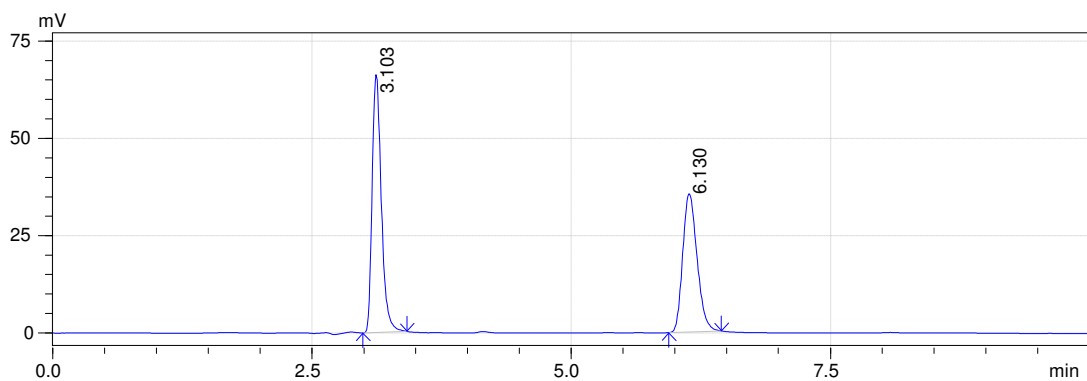Fig. 56: Mobile phase ratio changes (ACN 50: Methanol 10: Buffer 40)

Fig. 57: Mobile phase ratio changes (ACN 44: Methanol 11: Buffer 45)
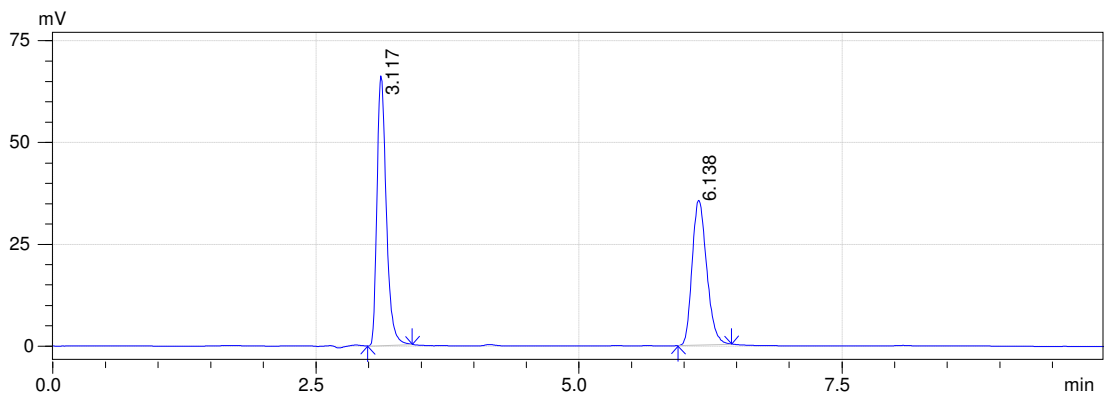

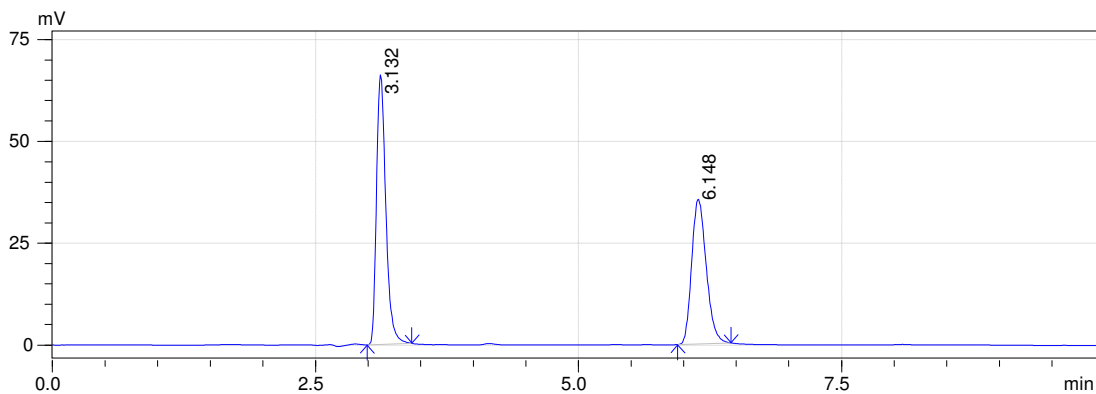Fig. 58: Mobile phase ratio changes (ACN 44: Methanol 11: Buffer 45)

Fig. 59: Mobile phase ratio changes (ACN 45: Methanol 15: Buffer 40)
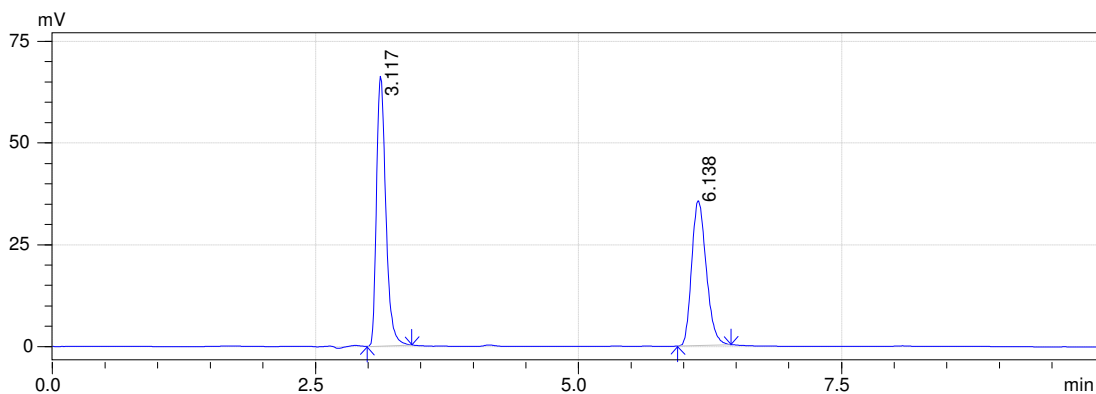
Fig. 60: Mobile phase ratio changes (ACN 45: Methanol 15: Buffer 40)

Result:

When flow rate was altered RT has no changed significantly, when mobile phase was altered there was no change in the RT significantly. The method was found to be robust as per ICH Guidelines.

6.2.8 LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTITATION (LOQ):

LOD and LOQ were calculated by using instrumental method.

- ➢ Limit of Detection for Atazanavir sulphate $= 0.0023 \mu g \ mL^{-1}$
- ➢ Limit of Quantitation for Atazanavir sulphate $= 0.0161 \mu g \ mL^{-1}$
- ➢ Limit of Detection for Ritonavir $= 0.0006 \mu g \ mL^{-1}$
- ➢ Limit of Quantitation for Ritonavir $= 0.0047 \mu g \ mL^{-1}$

Table 19: Limit of Detection and Quantitation

Contents	Atazanavir	Ritonavir
LOD (S/N)	3.1	3.1
LOQ (S/N)	9.9	10.1

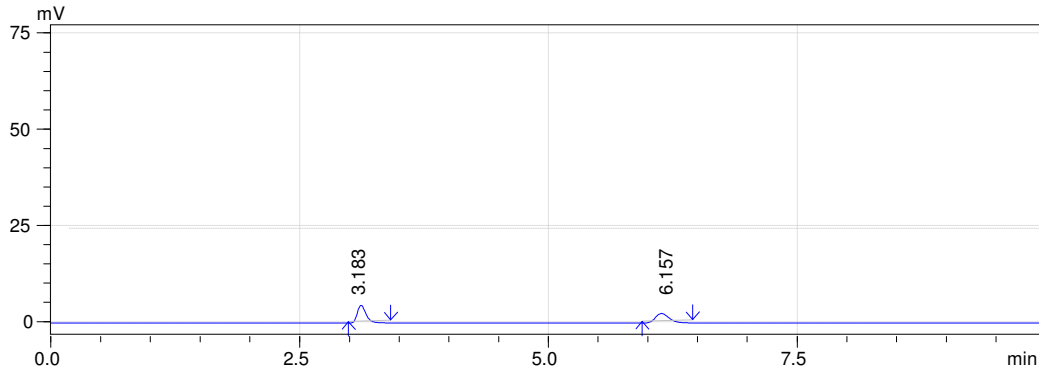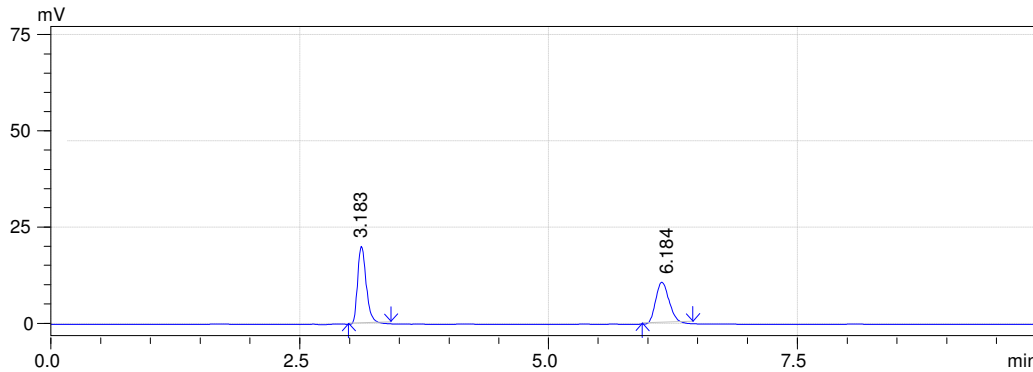Fig. 61: LOD chromatogram of Atazanavir sulphate and Ritonavir

Fig. 62: LOQ chromatogram of Atazanavir sulphate and Ritonavir.

Acceptance criteria:

LOD should not exceed LOQ.

Result:

LOD and LOQ of Atazanavir sulphate was found to be 0.0023 µg mL^{-1} and 0.016 µg mL^{-1}

LOD and LOQ of Ritonavir was found to be 0.0006 µg mL^{-1} and 0.0047 µg mL^{-1}.

Chapter 7

SUMMARY & CONCLUSIONS

7. SUMMARY AND CONCLUSIONS

In the present study, new RP-HPLC method for simultaneous estimation of Atazanavir sulphate and Ritonavir in bulk and pharmaceutical dosage form was developed. The developed method was validated for various parameters such as accuracy, precision, ruggedness, linearity, robustness, system suitability, specificity, limit of detection and limit of quantification as per ICH guidelines.The results of the studies are summarized as follows

A) Method development:

➢ Trial 5 was optimized for the method development of deliberately changing the chromatographic conditions.

➢ Column used was C18 Nucleodur (150 mm X 4.6 mm X 5 µm), mobile phase composition of ACN: Methanol: phosphate buffer pH 3.0 was adjusted with orthophosphoric acid in the ratio (44:11:45 V/V/V). The Flow rate 1.5 mL min^{-1} with UV detection was carried out at 210 nm.

➢ Assay percentage was found to be 99.05 % for Atazanavir sulphate and 99.20 % for Ritonavir.

B) Validation Parameters:

➢ The calibration was linear with correlation coefficient 0.999 for Atazanavir sulphate and 0.999 for Ritonavir.

➢ In precision it was found that % RSD is less than 2% which indicates that the proposed method has good reproducibility.

➢ The system suitability parameter indicates good resolution of both the peaks > 2.

➢ From the Accuracy was found that % Recovery of the drug was found to be in the range of 99.91-100.40 % and 100.12-100.31 % for Atazanavir sulphate and Ritonavir respectively.

➢ Robustness, When flow rate was altered RT has no changed significantly, when mobile phase was altered there was no change in the RT significantly.

➢ LOD and LOQ, the limit of detection (LOD) was 0.002 µg mL^{-1} for Atazanavir sulphate and 0.001 µg mL^{-1} for Ritonavir and the limit of quantitation (LOQ) was 0.016 µg mL^{-1} for Atazanavir sulphate and 0.004 µg mL^{-1} for Ritonavir.

Conclusions:

The results of the present study indicated that the developed method is simple, precise and cost effective for the simultaneous estimation of Atazanavir sulphate and Ritonavir for routine quality control analysis of these either in bulk and pharmaceutical formulation.

The developed and validated RP-HPLC method outlined is very obvious, affordable, dynamic, low cost, rapid easy to perform with small sample volume and good repeatability. It can be adopted for the routine quality control analysis of simultaneous determination of Atazanavir sulphate and Ritonavir because of good resolution of the chromatographic peaks.

Chapter 8

REFERENCES

8. REFERENCES

1. Skoog, W., Fundamental of analytical chemistry; Saunders College, 1992, 7thEdn; pp 1-3.

2. Kasture, A. V.; Wadodkar, S. G.; Mahadik, K. R., Textbook of pharmaceutical analysis II; Published by NiraliPrakashan, 2005, 13th Edn; pp 1-2.

3. Seth, P. D., HPLC Quantitative analysis pharmaceutical formulations; CBS publishers and distributors, New delhi: India, 2001, pp 3-137.

4. Beckett A. H., Stanlake J. B., Practical pharmaceutical chemistry; Part-II; CBS publishers and distributors, 2002, 4thEdn; pp 157-174.

5. Christine, F. R., Use of HPLC for in-process testing, separation science and technology, 2007, pp 407-423.

6. Berry, R. I.; Nash, A. R., Pharmaceutical process validation, analytical method validation. Marcel Dekker Inc. New work. 1993, pp 411-428.

7. Williard, H. H.; Merritt, L. L.; Instrumental method of Analysis, CBS publishers, 7th Edn, 1988, pp 580-608.

8. Nagaraju, P. T.; Sreekanthreddy, K. P.; Chinnaganganna, P.; Venugopal, N.; Ravindranath, S.; BhanuPriya, B.; Satyanandam, S., RP-HPLC method for determination of Ritonavir in pharmaceutical preparations. Int. J.Pharm. Sci. Rev. Res. 2012, 13(2), 55-57.

9. Arun, R.; Anton, A. S., Development and Validation of Analytical method for Lopinavir and Ritonavir by HPLC. Int. J. Drug Dev. Res. 2013, 5(2), 1166-1171.

10. Dario, C.; Franco, M.; Diego, R.; Norberto, P., Determination of Atazanavir in Human Plasma by High-Performance Liquid Chromatography with UV Detection. J. Chromgra. Sci. 2008, 46, 485-488.

11. Manoj, G.; Anil, B.; Bhanubhai, S.; Ishwarsinh, R.; Urvish, D.; Arpit,P.; Ketan,V., Simultaneous Determination of Ritonavir and Atazanavir in combined tablet dosage form by HPTLC. Asian. J. Biomed. Pharm. Sci. 2012, 2(15), 15-19.

12. Salinthip, J.; Kittisak, S.; Hutimamatayat, S. K.; Prapin, W., Simultaneous determination of plasma Lopinavir and Ritonavir by chemometrics- assisted spectrophotometry and comparisonwith HPLC method. Maejo Int. J. Sci. Tech. 2013, 7(02), 248-257.

13. Anusha, T.; Ashwini, G.; Annapurna, R. C.; Aravind, S.; Abbarajuprasana, L.;Avinash, K., Method development and validation for the simultaneous estimation of Atazanavir and Ritonavir in pharmaceutical dosage form by RP-HPLC. Int. J. Pharm. Chem. Bio. Sci. 2012, 03 (01), 44-54.

14. Swetha M. A.; Ravindrareddy,Y., Method development and validation of Atazanavir and Ritonavir in a combined dosage form By RP-HPLC Method. Int. J. Pharm. Tech. 2011, 3(03), 3316-3334.

15. Nilesh, A. B.; Shailendra, P. K.; Shailesh, N. S.; Saroj, V. S.; Vishnu, P. C., Spectrophotometric simultaneous determination of Atazanavir and Ritonavir in combined tablet dosage form by ratio derivative and area under curve method. Der. Pharma. Chemica. 2012, 4(01), 208-213.

16. Sathish, K, K.; Sujana, K.; Prameela, R. A., New validated RP-HPLC method for the determination of Atazanavir Sulphate in bulk and dosage form. Der. Pharma. Chemica. 2012, 4(03), 1305-1310.

17. Srinivasu , K.; Venkateswara, R. J.; Appala, R.N.; Mukkanti, K. A.,Validated RP- HPLC method for the determination of Atazanavir in pharmaceutical dosage form. J. Chem. 2011, 8(01), 453-456.

18. Nanda, R. K.; Kulkarni, A. A.; Yadav, P. B., Simultaneous spectrophotometric estimation of Atazanavir sulfate and Ritonavir in tablet. Der. Pharma. Chemica. 2011, 3(03), 84-88.

19. http://www.drugbank.ca/drugs/DB01072.

20. http://en.wikipedia.org/wiki/Atazanavir_sulfate.

21. http://www.drugbank.ca/drugs/DB00503

22. http://en.wikipedia.org/wiki/Ritonavir.

23. ICH Q8 (R2), Pharmaceutical Development, 2009.

24. ICH Q2 (R1), Validation of analytical procedures: Text and methodology, 2005, Geneva, Switzerland.

Chapter 9

APPENDIX

9. APPENDIX

%	-	Percentage.
µg	-	Microgram
µl	-	Microlitre
µm	-	Micrometer
ACN	-	Acetonitrile
AUC	-	Area under curve
cm	-	Centimeter
HPLC	-	High performance liquid chromatography
HPTLC	-	High performance thin layer chromatography
LOD	-	Limit of detection
LOQ	-	Limit of Quantitation
Mg	-	Milligram
RP-HPLC	-	Reverse phase high performance liquid chromatography
ICH	-	International conference of harmonization
min	-	Minute
FDA	-	Food and drug administration
WHO	-	World health organization
USFDA	-	United state food and drug administration
NP-HPLC	-	Normal phase high performance liquid chromatography
mL	-	Millilitre
mm	-	Millimetre
nm	-	Nanometer
Avg wt	-	Average weight
RSD	-	Relative standard deviation
r^2	-	Regression coefficient
ATA	-	Atazanavir Sulphate
RIT	-	Ritonavir
C_{18}	-	Carbon 18
V/V	-	Volume by volume