DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF CABOTEGRAVIR AND RILPIVIRINE IN BULK AND TABLET DOSAGE FORM

A Dissertation submitted to

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY,

CHENNAI- 600 032

In partial fulfilment of the award of the degree of

MASTER OF PHARMACY

IN

Branch V–PHARMACEUTICAL ANALYSIS

Submitted by

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Under the Guidance of

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OCTOBER – 2021

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CERTIFICATES

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF CABOTEGRAVIR AND RILPIVIRINE IN BULK AND TABLET DOSAGE FORM",** submitted by the student bearing **Reg. No: 261930204** to "**The Tamil Nadu Dr. M.G.R. Medical University – Chennai**", in partial fulfilment for the award of Degree of **Master of Pharmacy** in **Pharmaceutical Analysis** was evaluated by us during the examination held on……

Internal Examiner **External Examiner**

CERTIFICATE

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DECLARATON

I do hereby declared that the dissertation **"DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF CABOTEGRAVIR AND RILPIVIRINE IN BULK AND TABLET DOSAGE FORM",** submitted to "**The Tamil Nadu Dr. M.G.R Medical University - Chennai",** for the partial fulfilment of the degree of **Master of Pharmacy** in **Pharmaceutical Analysis,** is a bonafide research work has been carried out by me during the academic year 2020-2021, under the guidance and supervision of **Mr. D. KAMALAKANNAN, M.Pharm., (Ph.D).,** Assistant Professor, Department of Pharmaceutical Analysis, J.K.K. Nattraja College of Pharmacy, Kumarapalayam.

I further declare that this work is original and this dissertation has not been submitted previously for the award of any other degree, diploma, associate ship and fellowship or any other similar title. The information furnished in this dissertation is genuine to the best of my knowledge.

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Dedicated to

Almighty

My Beloved Parents,

&

My Family Members

ABSTRACT

A simple, Accurate, precise method was developed for the simultaneous estimation of the Cabotegravir and Rilpivirine in bulk and Tablet dosage form. Chromatogram was run through Denali C18 (150 x 4.6 mm, 5μ). Mobile phase containing Buffer 1.0% OPA: Acetonitrile taken in the ratio 60:40 was pumped through column at a flow rate of 1.0 mL/min. Buffer used in this method was 0.1% OPA buffer. Temperature was maintained at 30°C. Optimized wavelength selected was 260.0 nm. Retention time of Cabotegravir and Rilpivirine were found to be 3.020 min and 2.281 min. %RSD of the Cabotegravir and Rilpivirine were and found to be 0.9 and 0.6 respectively. %Recovery was obtained as 100.60% and 100.54% for Cabotegravir and Rilpivirine respectively. LOD, LOQ values obtained from regression equations of Cabotegravir and Rilpivirine were 0.91, 2.74 and 3.88, 11.72 respectively. %Assay was obtained as 100.32% and 100.42% for Cabotegravir and Rilpivirine respectively. Regression equation of Cabotegravir is y =4099.x + 5230, y = 6957.x + 26409 of Rilpivirine. Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

Key Words: Cabotegravir, Rilpivirine, RP-HPLC

CONTENTS

S. No.	DESCRIPTION	Page.no.
1	**INTRODUCTION**	01
2	**DRUG PROFILE**	41
3	**LITERATURE REVIEW**	46
4	**AIM AND OBJECTIVE**	53
5	**MATERIALS AND METHODS**	55
6	**RESULTS AND DISCUSSION**	62
7	**SUMMARY AND CONCLUSION**	108
8	**BIBLIOGRAPHY**	110

LIST OF TABLES

Table No	TITLE	PAGE NO.
1.1	Different types of chromatographic techniques	03
1.2	Physical properties of common HPLC solvents	16
1.3	System Suitability Parameters and their recommended limits	35
1.4	Characteristics to be validated in HPLC	40
6.1	System Suitability Parameters of Cabotegravir and	67
6.2	Linearity table for Cabotegravir and Rilpivirine.	72
6.3	System precision table of Cabotegravir and Rilpivirine	80
6.4	Repeatability table of Cabotegravir and Rilpivirine	83
6.5	Intermediate precision table of Cabotegravir and Rilpivirine	86
6.6	Accuracy table of Cabotegravir	89
6.7	Accuracy table of Rilpivirine	90
6.8	Sensitivity table of Cabotegravir and Rilpivirine	94
6.9	Robustness data for Cabotegravir and Rilpivirine.	95
6.10	Assay Data of Cabotegravir	102
6.11	Assay Data of Rilpivirine	102
6.12	Degradation Data of Cabotegravir	104
6.13	Degradation Data of Rilpivirine	104
7.1	Summary Table	108

LIST OF FIGURES

Figure No	TITLE	PAGE NO.
1.1	HPLC Basic Instrumentation	09
1.2	Injection System	12
1.3	Showing Retention Factor	19
1.4	Showing Number Of Theoretical Plates	20
1.5	Showing Asymmetric Factor	22
1.6	Showing Resolution Factor	23
1.7	Showing Selectivity	24
2.1	Cabotegravir	41
2.2	Rilpivirine	43
6.1	Trial Chromatogram 1	62
6.2	Trial Chromatogram 2	63
6.3	Trial Chromatogram 3	64
6.4	Trial Chromatogram 4	65
6.5	Optimized Chromatogram	66
6.6	System Suitability Chromatogram	69
6.7	Chromatogram Of Blank.	70
6.8	Chromatogram Of Placebo	70
6.9	Typical Chromatogram	71
6.10	Calibration curve of Cabotegravir	72
6.11	Calibration curve of Rilpivirine	73
6.12	Linearity 25% Chromatogram of Cabotegravir and	74
6.13	Linearity 50% Chromatogram of Cabotegravir and	75
6.14	Linearity 75% Chromatogram of Cabotegravir and	76
6.15	Linearity 100% Chromatogram of Cabotegravir and	77
6.16	Linearity 125% Chromatogram of Cabotegravir and	78

6.17	Linearity 150% Chromatogram of Cabotegravir and	79
6.18	System precision chromatogram	82
6.19	Repeatability chromatogram	85
6.20	Inter Day precision Chromatogram	88
6.21	Accuracy 50% Chromatogram of Cabotegravir and	91
6.22	Accuracy 100% Chromatogram of Cabotegravir and	92
6.23	Accuracy 150% Chromatogram of Cabotegravir and	93
6.24	LOD Chromatogram of Standard	94
6.25	LOQ Chromatogram of Standard	94
6.26	Flow minus Chromatogram of Cabotegravir and Rilpivirine.	96
6.27	Flow plus Chromatogram of Cabotegravir and Rilpivirine.	97
6.28	Mobile phase minus Chromatogram of Cabotegravir and	98
6.29	Mobile phase Plus Chromatogram of Cabotegravir and	99
6.30	Temperature minus Chromatogram of Cabotegravir and	100
6.31	Temperature plus Chromatogram of Cabotegravir and	101
6.32	Chromatogram of working standard solution	103
6.33	Chromatogram of working sample solution	103
6.34	Acid chromatogram of Cabotegravir and Rilpivirine	105
6.35	Base chromatogram of Cabotegravir and Rilpivirine	105
6.36	Peroxide chromatogram of Cabotegravir and Rilpivirine	106
6.37	Thermal chromatogram of Cabotegravir and Rilpivirine	106
6.38	UV chromatogram of Cabotegravir and Rilpivirine	107
6.39	Water chromatogram of Cabotegravir and Rilpivirine	107

LIST OF ABRIVATIONS

API	:	Active Pharmaceutical Ingredient
AR	:	Analytical Reagent
AU	:	Angstrom Unit
EMR	:	Electro Magnetic Radiation
FDA	:	Food and Drug Administration
gm	:	Gram
HPLC	:	High Performance Liquid Chromatography
TLC	:	Thin Layer Chromatography
HPTLC	:	High Performance Thin Layer Chromatography
MS	:	Mass Spectroscopy
ICH	:	International Conference On Harmonization
I.D.	:	Internal Diameter
IUPAC	:	International Union of Pure and Applied Chemistry
LC	:	Liquid Chromatography
LOD	:	Limit of Detection
LOQ	:	Limit of Quantitation
mg	:	Milli Gram
min	:	Minute
mL	:	Milli Litre

mm : Milli Meter

mM : Milli Micron

mV : Milli Volt

N : Theoretical Plates

ng : Nano Gram

nm : Nano Meter

pKa : Acid Dissociation Constant

QA : Quality Assurance

QC : Quality Control

R^2 : Correlation Coefficient

RP-HPLC : Reversed Phase High Performance Liquid Chromatography

RSD : Relative Standard Deviation

SD : Standard Deviation

S/N : Signal / Noise

T : Tailing Factor

TLC : Thin Layer Chromatography

R_t : Retention Time

USP : United States Pharmacopoeia

UV : Ultra Violet

v/v : Volume / Volume

WHO : World Health Organization

^{0}C : Centigrade Temperature

µg : Micro Gram

µL : Micro Litre

µm : Micro Meter

λ_{max} : Absorbance Maximum

% T : % Transmittance

1. INTRODUCTION [1-24]

The quality of a drug plays an important role in ensuring the safety and efficacy of the drugs. Quality assurance and control of pharmaceutical and chemical formulations is essential for ensuring the availability of safe and effective drug formulations to consumers. Hence Analysis of pure drug substances and their pharmaceutical dosage forms occupies a pivotal role in assessing the suitability to use in patients. The quality of the analytical data depends on the quality of the methods employed in generation of the data (1). Hence, development of rugged and robust analytical methods is very important for statutory certification of drugs and their formulations with the regulatory authorities.

The quality and safety of a drug is generally assured by monitoring and controlling the assay and impurities effectively. While assay determines the potency of the drug and impurities will determine the safety aspect of the drug. Assay of pharmaceutical products plays an important role in efficacy of the drug in patients.

The wide variety of challenges is encountered while developing the methods for different drugs depending on its nature and properties. This along with the importance of achieving the selectivity, speed, cost, simplicity, sensitivity, reproducibility and accuracy of results gives an opportunity for researchers to come out with solution to address the challenges in getting the new methods of analysis to be adopted by the pharmaceutical industry and chemical laboratories. Different physico-chemical methods (1) are used to study the physical phenomenon that occurs as a result of chemical reactions. Among the physico-chemical methods, the most important are optical (refractometry, polarimetry, emission and fluorescence methods of analysis), photometry (photo colorimetry and spectrophotometry covering UV-Visible, IR Spectroscopy and nepheloturbidimetry) and chromatographic (column, paper, thin layer, gas liquid and high performance liquid chromatography) methods.

Methods such as nuclear magnetic resonance (NMR) and para magnetic resonance (PMR) are becoming more and more popular. The combination of mass spectroscopy (MS) with gas chromatography is one of the most powerful tools available. The chemical methods include the gravimetric and volumetric procedures which are based on complex formation; acid-base, precipitation and redox reactions. Titrations in non-aqueous media and complexometry have also been used in pharmaceutical analysis. The number of new drugs is constantly growing. This requires new methods for controlling their quality. Modern pharmaceutical analysis must need the following requirements.

1. The analysis should take a minimal time.
2. The accuracy of the analysis should meet the demands of Pharmacopoeia.
3. The analysis should be economical.
4. The selected method should be precise and selective.

1.1 CHROMATOGRAPHY

Chromatography (Chroma means ‘color’ and graphein means to ‘write’) is the collective term for a set of laboratory techniques for the separation of mixtures. It involves passing a mixture dissolved in a "mobile phase" through a stationary phase,(2-4) which separates the analyte to be measured from other molecules in the mixture based on differential partitioning between the mobile and stationary phases. Differences in compounds partition coefficient results in differential retention on the stationary phase and thus changing the separation.

Table No. 1.1 Different types of chromatographic techniques

Sl. No	Basic principle involved	Type of Chromatography
1.	Techniques of Chromatographic bed shape	Column chromatography
		Paper chromatography
		Thin layer chromatography
2	Techniques by physical state of mobile phase	Gas chromatography
		Liquid chromatography
3	Affinity chromatography	Supercritical fluid chromatography
4	Techniques by separation mechanism	Ion exchange chromatography
		Size exclusion chromatography
5	Special techniques	Reversed phase chromatography
		Simulated moving bed chromatography
		Pyrolysis gas chromatography
		Fast protein liquid chromatography
		Counter current chromatography
		Chiral chromatography

Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for further use (and is thus a form of purification). Analytical chromatography is done normally with smaller amounts of material and is for measuring the relative proportion of analytes in a mixture**.**

1.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Liquid chromatography (3) is an analytical chromatographic technique that is useful for separating ions or molecules that are dissolved in a solvent. If the sample solution is in contact with a second solid or liquid phase to differing degrees due to differences in adsorption, ion exchange, partitioning or size. These differences will allow the mixture components to be separated from each other by using these differences to determine the time of the solutes through a column. During 1970's, most chemical separations were carried out using a variety of techniques including open-column chromatography, paper chromatography and thin layer chromatography (TLC). However, these chromatographic techniques were inadequate for quantification of compounds and resolution between similar compounds. During this time pressure liquid chromatography began to be used to decreased flow through time, thus reducing separation time of compounds being isolated by column chromatography. However, flow rates were inconsistent, and the question of whether it was better to have constant flow rate or constant pressure debated. Additional convenience of on-line detectors became rapidly a powerful separation technique and is today called as High Performance Liquid Chromatography (HPLC).

CLASSIFICATION OF HPLC [4-10]

Based on modes of chromatography:

- Normal phase chromatography
- Reverse phase chromatography

Based on principle of separation:

- Adsorption chromatography
- Partition chromatography
- Ion exchange chromatography

- Size exclusion chromatography
- Affinity chromatography
- Chiral phase chromatography

Based on elution technique:

- Isocratic separation
- Gradient separation

Based on the scale of operation:

- Analytical HPLC
- Preparative HPLC

Normal Phase - High Performance Liquid Chromatography (NP-HPLC)

NP-HPLC explores the differences in the strength of the polar interactions of the analytes in the mixture with the stationary phase. The stronger the analyte-stationary phase interaction, the longer the analyte retention. Analyte molecules compete with the mobile phase molecules for the adsorption sites on the surface of the stationary phase. The stronger the mobile phase interactions with the stationary phase, the lower the difference between the stationary phase interactions and the analyte interactions, and thus the lower the analyte retention. Mobile phases in NP-HPLC are based on non-polar solvents (such as hexane, heptanes, etc.) with the small addition of polar modifier (i.e., methanol, ethanol).

Packing materials traditionally used in NP-HPLC are usually porous oxides such as silica (SiO_2) or alumina (Al_2O_3). Surface of these stationary phases is covered with the dense population of OH groups, which makes these surfaces highly polar. Chemically modified stationary phases can also be used in NP-HPLC. Silica modified with trimethoxy glycidoxypropyl silanes (common name: diol-phase) is typical packing material with decreased surface polarity. Since NP-HPLC uses mainly non-polar solvents, it is the method

of choice for highly hydrophobic compounds (which may show very stronger interaction with non-polar mobile phases), which are insoluble in polar or aqueous solvents.

Reversed Phase - High Performance Liquid Chromatography (RP-HPLC)

As opposed to NP-HPLC, RP-HPLC employs mainly dispersive forces (hydrophobic or vanderwal's interactions). The polarities of mobile and stationary phases are reversed, such that the surface of the stationary phase in RP-HPLC is hydrophobic and mobile phase is polar, where mainly water-based solutions are employed. RP-HPLC is by far the most popular mode of chromatography. Almost 90 % of all analyses of low-molecular-weight samples are carried out using RP-HPLC. Dispersive forces employed in this separation mode are the weakest intermolecular forces, thereby making the overall background interaction energy in the chromatographic system very low compared to other separation techniques. This low background energy allows for distinguishing very small differences in molecular interactions of closely related analytes. Adsorbents employed in this mode of chromatography are porous rigid materials with hydrophobic surfaces. The majority of packing materials used in RP-HPLC are chemically modified porous silica.

Adsorption chromatography:

The analyte interact with solid stationary surface and are displaced with eluent for active sites on surface.

Partition chromatography:

This method results from a thermodynamic distribution of analytes between two liquid phases. On the basis of relative polarities of stationary and mobile phase, partition chromatography can be divided in to normal phase and reverse phase chromatography. In normal phase chromatography, the stationary phase bed is strongly polar in nature (e.g. silica

gel) and the mobile phase is non polar (such as n-hexane or tetrahydrofuran). Polar sample are thus retained on polar surface of the column packing longer than polar material while in reverse phase chromatography, the stationary bed is non polar (hydrophobic in nature, while the mobile phase is polar liquid, such as mixture of water and methanol or acetonitrile).

Size exclusion chromatography:

This involves a solid stationary phase with controlled pole size. Solids are separated according to molecular size, with the large molecule unable to enter the pores eluted first.

Ion exchange chromatography (IEC):

IEC is based on the differences in affinities of the analyte ions for the oppositely charged ionic centre in the resin or adsorbed counter ions in the hydrophobic stationary phase. Consider the exchange of two ions **A+** and **B+** between the solution and exchange resin

E−: A•E + B+ ↔B•E + A+

The equilibrium constant for this process is shown in Eq. below:

K = ([A+][BE])/([AE][B+])

This essentially determines the relative affinity of both cations to the exchange centres on the surface. If the constant is equal to 1, no discriminating ability is expected for this system. The higher the equilibrium constant (provided that, it is greater than 1), the greater the ability of cation B+ to substitute A on the resin surface. Depending on the charge of the exchange centres on the surface, the resin could be either anion-exchanger (positive ionic centers on the surface) or cation-exchanger (negative centres on the surface). Cross linked

styrene-divinyl benzene is the typical base material for ion exchange resin. Exchange groups are attached to the Phenyl rings in the structure and the degree of cross linkage is between 5 % and 20 %. The higher the cross linkage, the harder the material and the less susceptible it is to swelling, but the material usually shows lower ion-exchange capacity. Four major types of ion-exchange centres are usually employed:

- SO_3-—Strong cation-exchanger
- CO_2-—Weak cation-exchanger
- Quaternary amine—Strong anion-exchanger
- Tertiary amine—Weak anion-exchanger

Analyte retention and selectivity in ion exchange chromatography are strongly dependent on the pH and ionic strength of the mobile phase.

Size exclusion chromatography (SEC):

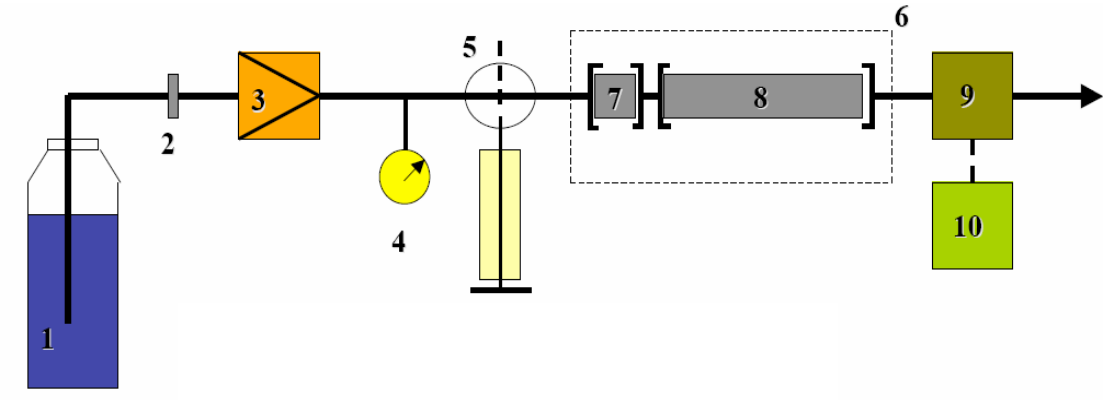
SEC is the method for dynamic separation of molecules according to their size. The separation is based on the exclusion of the molecules from the porous space of packing material due to their steric hindrance. Hydrodynamic radius of the analyte molecule is the main factor determining its retention. This is the only chromatographic separation method where any positive interaction of the analyte with the stationary phase should be avoided.

In SEC, the higher the molecular weight of the molecule, the greater its hydrodynamic radius results in faster elution. At the same time, if an analyte molecule interacts (undesired) with the stationary phase, thus increasing the retention of larger molecules, which may conform separation of molecules based solely on their hydrodynamic radius. The adsorbent pore size distribution plays the dominant role in the adsorbent ability to discriminate

molecules according to their molecular weight. Hydrodynamic radius of the polymer is also dependent on the analyte interaction with the solvent. Polymer conformation and degree of the salvation varies with the variation of the solvent properties.

1.3 INSTRUMENTATION OF HPLC

HPLC is a special branch of Column Chromatography in which the mobile phase is forced through the column at high speed. As a result, the analysis time is reduced by 1-2 orders of magnitude relative to classical Column Chromatography and the use of much smaller particles of the absorbent or support becomes possible increasing the column efficiency substantially. The Basic HPLC Instrumentation (5-9) was shown in the Fig. No. 1



1 = eluent reservoir
2 = filter
3 = high pressure pump with pulse dampener
4 = pressure gauge
5 = sample injection valve with syringe
6 = column oven
7 = guard column
8 = column
9 = detector
10 = recorder (integrator, PC etc.)

Figure .No.1.1 HPLC basic instrumentation

I. Solvent delivery system:

The most important component of HPLC in solvent delivery system is the pump, because its performance directly effects the retention time, reproducibility and detector sensitivity. Among the several solvent delivery systems, (direct gas pressure, pneumatic intensifier, reciprocating etc.) reciprocating pump with twin or triple pistons is widely used, as this system gives less baseline noise, good flow rate reproducibility etc.

The pumping systems used in HPLC can be categorized in three different ways.

- The first classification is according to the eluent flow rate that the pump is capable of delivering.
- The second classification is according to the construction materials.
- The final classification is according to the mechanism by which the pump delivers the eluent.

Each of these classifications is considered below.

Pump classification according to flow rate*:*

When classified in terms of flow rate, pumps may be defined as micro bore or preparative.

- **Standard bore systems** are the most commonly used pumping systems for analytical HPLC because they provide reliable operation at flow rates ranging from 100 µL / min to 10 µL / min.
- **Micro bore systems** are intended for use with column diameters ranging up to 2 mm. The narrow column diameter and small size of the packing material causes relatively low flow rates for the pumping system, from 1 to 250 µL / min as the minimum head size for reciprocating pumps is around 25 µL, smooth, reliable operation at flow rates less than 10 µL / min is difficult.

Pump classification according to materials of construction:

Pumps may also be classified according to the primary construction materials.

The pumps are classified as

- Metallic
- Non-metallic, depending on the material used for the eluent flow path.
- The most commonly used material for HPLC pumping systems is 316 stainless steel, because of its mechanical strength, corrosion resistance, good thermal stability and malleability. Only a handful of HPLC solvents such as Hydrochloric acid will cause damage to 316 stainless steel. Therefore pumps are also constructed from non-metallic materials such as PEEK (poly ethyl ethyl ketone), Teflon (poly tetra fluoro ethylene) and Ceramics.

Pump classification according to mechanism of eluent displacement:

The third classification of pumps is according to the mechanism by which the liquid is forced through the chromatograph. The pumps are classified into two types. They are

- Syringe pumps and
- Reciprocating-piston pump

HPLC systems are also provided an online degassing system which continuously removes the dissolved gases from the mobile phase.

Solvent degassing system

- The constituents of the mobile phase should be degassed and filtered before use. Several methods can be applied to remove the dissolved gases in the mobile phase. They include

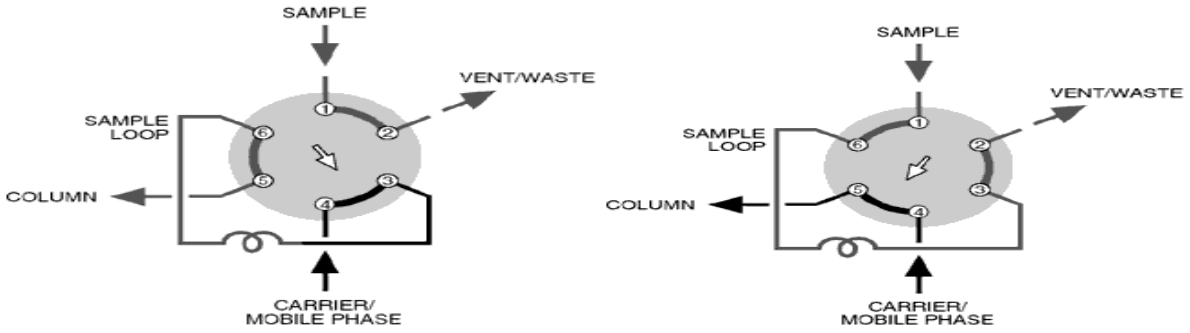
- Heating and stirring,
- Vacuum degassing with an aspirator,
- Filtration through 0.45µm filters,
- Vacuum degassing with an air-soluble membrane,
- Helium purging ultra-signification or purging or combination of these methods.

II. Sample introduction system

Two means for analyte introduction on the column are injection into a flowing stream and a stop flow injection. These techniques can be used with a syringe or an injection valve. Automatic injector is a microprocessor-controlled version of the manual universal injector.

Injector

Injectors should provide the possibility of injecting the liquid sample within the range of 0.1 to 100 ml of volume with high reproducibility and under high pressure (up to the 4000 psi). They should also produce minimum band broadening and minimize possible flow disturbances. The most useful and widely used sampling device for modern LC is the micro sampling injector valve. With these sampling valves, samples can be introduced reproducibly into pressurized columns without significant interruption of flow even at elevated temperatures.



LOAD (the sample loop) INJECT (move the sample loop In the mobile phase

Figure .No.1.2 Injection system

III. Columns

The heart of the system is the column. Analytical column is the most important part of the HPLC which decides the efficiency of separation. The choice of common packing material and mobile phases depends on the physical properties of the drug.

The following properties of the column stationary phases play an important role in giving different selectivity for separations.

i) Particle size, ii) Particle shape, iii) Pore size / Pore volume, iv) Specific surface area, v) End capping vi) % carbon loading.

The following are the most widely used columns with stationary phases for separation and quantification of wide variety of drugs.

i) Pure silica and hybrid silica columns.

ii) Silica based columns with different bonding phases like C4, C6, C8, C18, C20 and bonding phases having functional groups like cyano, phenyl, naphthyl and amino.

iii) Silica based columns with polar embedded phases within chains of C8, C18, and NH2.

iii) Hybrid silica based columns like C4, C6, C8, C18, C20 and bonding phases having functional groups like cyano, phenyl, naphthyl and Amino.

iv) Strong cation exchange (SCX) and strong anion exchange (SAX) columns.

v) Size Exclusion chromatography (SEC) or gel permeation chromatography (GPC) columns.

vi) Silica based monolith columns.

vii) Fused core silica columns with bonding phases like C8, C18, CN, and phenyl.

viii) Metal oxide columns like zirconia based and alumina based.

ix) Chiral columns.

Column-packing materials:

Silica ($SiO_2.X\ H_2O$) is the most widely used substance for the manufacture of packing materials it consist of a network of siloxane linkages(Si-O-Si) in a rigid three dimensional structure containing inter connected pores. Thus a wide range of commercial products are available with surface areas ranging from 100 to 800 m^2/g and particle sizes from 3 to 50 µm. The silanol groups on the surface of silica give it a polar character, which is exploited in adsorption chromatography using non-polar organic eluents. Silica can be drastically altered by reaction with organo chloro silanes or organo alkoxy silanes giving Si-O-Si-R linkages with the surface. The attachment of hydrocarbon chain to silica produces a non-polar surface suitable for reversed phase chromatography where mixtures of water and organic solvents are used as eluents. The most popular material is Octa decyl silica (ODS) which contains C_{18}chains, but material with C_2, C_6, C_8 and C_{22} chains are also available. During manufacture, such materials can be reacted with a small mono functional silane (e.g.: trimethylchlorosilane) to reduce further number of silanol groups remaining on the surface (end capping). There is a vast range of materials which have intermediate surface polarities arising from the bonding to silica of other organic compounds which contain groups such as phenyl, nitro, amino and hydroxyl. Strong ion exchange is also available in which sulphonic acid groups and quaternary ammonium groups are bonded to Silica. The useful pH range for columns is 2 to 8, since Siloxane linkages are cleaved below pH 2 while at pH values above 8 Silica may dissolve.

In HPLC, generally two types of columns are used, normal phase column and reversed phase column. Using normal phase chromatography, particularly of non-polar and moderately polar drugs can make excellent separation and was originally believed that separation of compounds in mixtures takes place slowly by differential adsorption on a stationary silica phase. However, it now seems that partition plays an important role, with the

compounds interacting with the polar silanol groups on the silica or with bound water molecules. While in normal phase, seems the passage of a relatively non-polar mobile phase over a polar stationary phase, reversed phase chromatography is carried out using a polar mobile phase such as methanol, acetonitrile, water, buffer etc. over a non polar stationary phase. A range of stationary phases (C_{18}, C_8, -NH_2, -CN, -Phenyl etc.) are available and very selective separation can be achieved.

The most popular brands of LC columns are Inertsil, Hypersil, X-terra, X-bridge, Sun-fire, Atlantis, Aquity-BEH, Zorbax, Lichrosphere, Purosphere, Sperisorb, Luna, Kromasil, ACE, YMC, Symmetry, Chiralcel and Chiralpak. These LC columns are supplied in different dimensions, viz., lengths of 10 mm, 50 mm, 100mm, 150mm, 250mm, 300mm, 500mm and internal diameters of 2.1mm, 3.0mm, 4.0mm, 4.6mm. LC columns with stationary phases having different particle sizes like 5.0 µm, 4.0 µm 3.5 µm, 3.0 µm, 2.5 µm, 2.0 µm, 1.9 µm, 1.8 µm, 1.7 µm and 1.3 µm are available.

IV. Mobile phase

Mobile phases used for HPLC are typically mixtures of organic solvents and water or aqueous buffers.

The following points should also be considered when choosing a mobile phase:

- ✓ It is essential to establish that the drug is stable in the mobile phase for at least the duration of the analysis.
- ✓ Excessive salt concentrations should be avoided. High salt concentrations can result in precipitation which can damage HPLC equipment. Reduce cost and toxicity of the mobile phase by using methanol instead of acetonitrile whenever possible.
- ✓ Minimize the absorbance of buffer. Since trifluoroacetic acid or formic acid absorb at shorter wavelengths. They may prevent detection of products without chromophores above 220 nm. Carboxylic acid modifiers can be frequently replaced by phosphoric

acid which does not absorb above 200 nm.

Table No. 1.2 Physical properties of common HPLC solvents

Solvent	**MW**	**BP**	**RI (25 ºC)**	**UV Cut-off (nm)**	**Density g / ml (25 ºC)**	**Viscosity cP (25 ºC)**	**Dielectric Constant**
Acetonitrile	41.0	82	1.342	190	0.787	0.358	38.8
Dioxane	88.1	101	1.420	215	1.034	1.26	2.21
Ethanol	46.1	78	1.359	205	0.789	1.19	24.5
Ethyl acetate	88.1	77	1.372	256	0.901	0.450	6.02
Methanol	32.0	65	1.326	205	0.792	0.584	32.7
CH_2Cl_2	84.9	40	1.424	233	1.326	0.44	8.93
Isopropanol	60.1	82	1.375	205	0.785	2.39	19.9
n-propanol	60.1	97	1.383	205	0.804	2.20	20.3
Water	18.0	100	1.333	170	0.998	1.00	78.5

V. Detectors

When a chromophore is present, the wavelength of detection for a drug should be based on its UV Spectrum in the mobile phase and not in pure solvents. The most selective wavelength for detecting a drug is frequently the longest wavelength maximum to avoid interference from solvents, buffers and excipients. Other methods of detection can be useful are required in some instances. The detection of UV light absorbance offers both convenience and sensitivity for molecules.

- Solute specific detectors (UV-Vis, fluorescence, electrochemical, infra-red, radio activity)

- Bulk property detectors (refractive index, viscometer, conductivity)
- Desolvation detectors (flame ionization etc.)
- LC-MS detectors
- Reaction detectors

Applications of HPLC in pharmaceutical research [9-13]

• Separation:

This can be accomplished using HPLC by utilizing the fact that, certain compounds have different migration rates given a particular column and mobile phase. The extent or degree of separation is determined by the choice of stationary phase and mobile phase along with parameters like flow, temperature and gradient programme.

- **Identification:**

For this purpose a clean peak of known sample has to be observed from the chromatogram. Selection of column mobile phase and flow rate matter to certain level in this process. Identification is generally by comparing with reference compound based on retention time and also based on UV-Vis spectra in some cases. Identification can be assured by combining two or more detection methods, where necessary.

- **Quantification:**

Analyte concentrations are estimated by measuring the responses known reference standards followed by unknown samples. Quantification of known and unknown components are done by various methods like - area normalization method, internal standard method, external standard method and diluted standard method along with relative response factors.

- **Isolation:**

It refers to the process of isolation and purification of compounds using analytical

scale or preparative scale HPLC. Volatile buffers and solvents are preferred choice as mobile phases as it reduces the effort on purification. Solute purity and throughput is the key challenge in isolation and purification processes.

1.4) HPLC theory [14-16]

System suitability parameters:

High performance liquid chromatography is defined as a separation of mixtures of compounds due to differences in their distribution equilibrium between two phases, the stationary phase packed inside columns and the mobile phase, delivered through the columns by high pressure pumps. Components whose distribution into the stationary phase is higher, are retained longer, and get separated from those with lower distribution into the stationary phase. The theoretical and practical foundations of this method were laid down at the end of 1960s and at the beginning of 1970s. The theory of chromatography has been used as the basis for system- suitability tests, which are set of quantitative criteria that test the suitability of the chromatographic system to identify and quantify drug related samples by HPLC at any step of the pharmaceutical analysis.

Retention time (RT), capacity factor k' and relative retention time (RRT):

The time elapsed between the injection of the sample components into the column and their detection is known as the retention time (RT). The retention time is longer when the solute has higher affinity to the stationary phase due to its chemical nature.

Therefore, the retention time is a property of the analyte that can be used for its identification. A non-retained substance passes through the column at a time t0, called the void time.

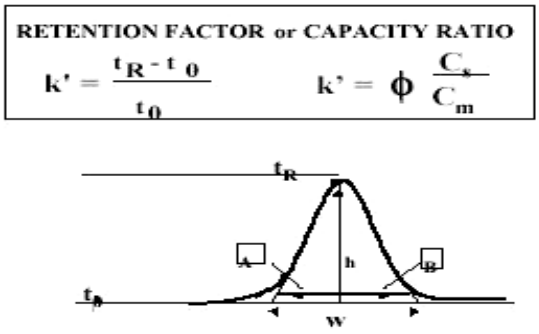


Figure .No.1.3. Showing retention factor

The capacity factor describes the thermodynamic basis of the separation and its definition is the ratio of the amounts of the solute at the stationary and mobile phases within the analyte band inside the chromatographic column: Where C_s is the concentration of the solute at the stationary phase and C_m is its concentration at the mobile phase and phi is the ratio of the stationary and mobile phase volumes all within the chromatographic band. The Retention Factor is used to compare the retention of a solute between two chromatographic systems, normalizing it to the column's geometry and system flow rate. The retention factor value should be in between 1-20. The need to determine the void time can be tricky sometimes, due to the instability of the elution time of the void time marker, t0, therefore, when the chromatogram is complex in nature, and one known component is always present at a certain retention time, it can be used as a retention marker for other peaks. In such cases the ratio between the retention time of any peak in the chromatogram and the retention time of the marker is used (tR (Peak) / tR (Marker)) and referred to as the Relative Retention Time (RRT). RRT is also used instead of the capacity ratio for the identification of the analyte as well as to compare its extent of retention in two different chromatographic systems. The sharpness of a peak relative to its retention time is a measure of the system's efficiency, calculated as N, plate count. Band-broadening phenomena in the column such as eddy diffusion, molecular diffusion, and mass-transfer kinetics and extra-column effects reduce the efficiency of the separation. The sharpness of a peak is relevant to the limit of detection and

limit of quantification of the chromatographic system. The sharper the peak for a specific area, the better is its signal-to-noise; hence the system is capable of detecting lower concentrations. Therefore, the efficiency of the chromatographic system must be established by the system suitability test before the analysis of low concentrations that requires high sensitivity of the system, such as the analysis of drug impurities and degradation products.

Efficiency: Plate count N and peak capacity Pc:

The efficiency of the separation is determined by the plate count N when working at isocratic conditions, whereas it is usually measured by Peak Capacity P_c when working at gradient conditions. The following equation for the plate count is used by the United States Pharmacopoeia (USP) to calculate N:

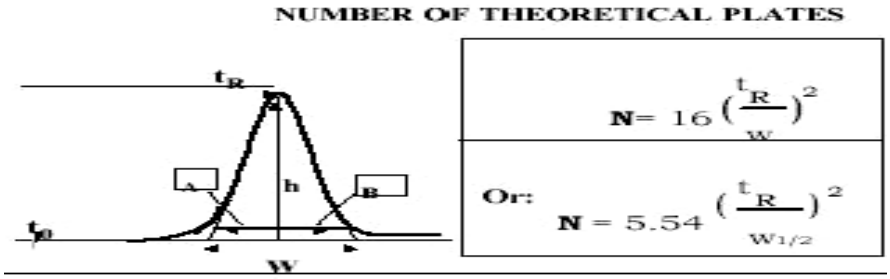


Figure. No.1.4. showing Number of Theoretical Plates

Where w is measured from the baseline peak width calculated using lines tangent to the peak width at 50 % height. European and Japanese pharmacopoeias use the peak width at 50% of the peak height, hence the equation becomes:

Peak capacity P_c is defined as number of peaks that can be separated within a retention window for a specific pre-determined resolution. In other words, it is the runtime measured in peak width units (34). It is assumed that peaks occur over the gradient chromatogram. Therefore, peak capacity can be calculated from the peak widths in the chromatogram as follows:

Where n is the number of peaks at the segment of the gradient selected for the calculation, tg. Thus peak capacity can be simply the gradient run time divided by the average peak width. The sharper the peaks the higher is the peak capacity, hence the system should be able to resolve more peaks at the selected run time as well as detect lower concentrations.

Another measure of the column's chromatographic efficiency is the height equivalent to theoretical plate (HETP) which is calculated from the following equation:

HETP = (L/N)

Where L is column length and N is the plate count. HETP is measured in micrometer.

The behaviour of HETP as function of linear velocity has been described by various equations. It is frequently called "The Van-Deemter curve", and it is frequently used to describe and characterize various chromatographic stationary phases' performance and compare them to each other. The lower are the values of HETP, the more efficient is the chromatographic system, enabling the detection of lower concentrations due to the enhanced signal-to-noise ratio of all the peaks in the chromatogram.

Peak asymmetry factor Af and tailing factor T:

- The chromatographic peak is assumed to have a Gaussian shape under ideal conditions, describing normal distribution of the velocity of the molecules populating the peak zone migrating through the stationary phase inside the column. Any deviation from the normal distribution indicates non-ideality of the distribution and the migration process therefore

might jeopardize the integrity of the peak's integration, reducing the accuracy of the quantitation. This is the reason why USP Tailing is a peak's parameter almost always measured in the system suitability step of the analysis.

- The deviation from symmetry is measured by the asymmetry factor, A_f or tailing factor T. The calculation of asymmetry factor, A_f is described by the following equation

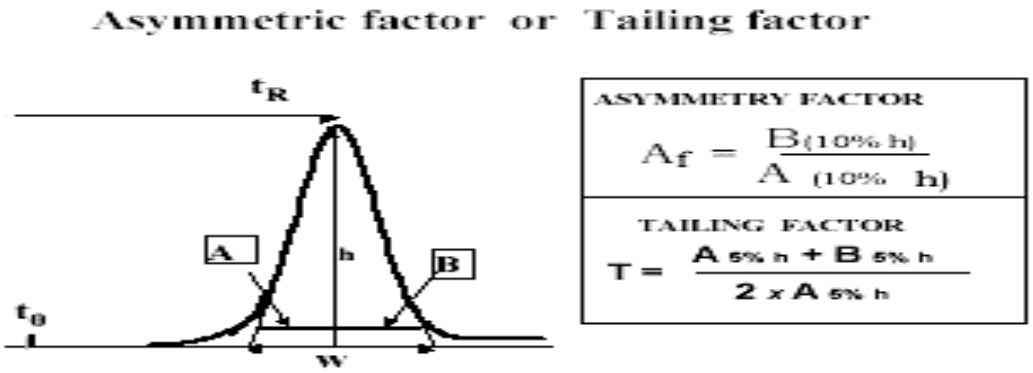


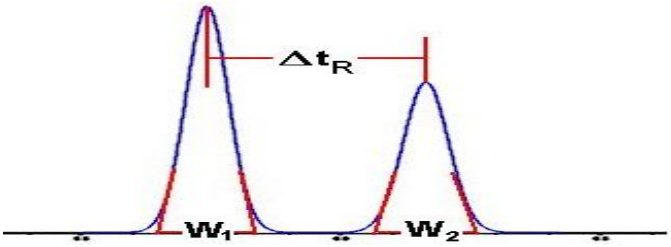
Figure. No.1.5. showing Asymmetric Factor

Where A and B are sections in the horizontal line parallel to the baseline, drawn at 10% of the peak height. The calculation of tailing Factor, T, which is more widely used in the pharmaceutical industry, as suggested by the pharmacopeia's, where A and B are sections in the horizontal line parallel to the baseline, drawn at 5% of the peak height. The USP suggests that tailing factor should be in the range of 0.5 up to 2 to assure a precise and accurate quantitative measurement.

Selectivity Factor α, and Resolution Factor Rs:

The separation is a function of the thermodynamics of the system. Substances are separated in a chromatographic column when their rate of migration differs, due to their different distribution between the stationary and mobile phases. The selectivity factor, α, and resolution factor, Rs, measure the extent of separation between two adjacent peaks. The selectivity factor accounts only for the ratio of the retention factors, k', of the two peaks

(k'2/k'1), whereas the resolution factor, Rs, accounts for the difference between the retention times of the two peaks relative to their width,



FigureNo.1.6.showing Resolution Factor

The equation that describes the experimental measurement of the resolution factor, Rs, is as follows:

$$Rs = \Delta tR / 0.5 (W1 + W2)$$

Where RT is the retention time of peaks 1 and 2 respectively and w is their respective peak width at the tangents' baseline. According to the pharmacopeia should be above 1.5 for an accurate quantitative measurement.

The resolution is a critical value when working with complex samples such as drug impurities and degradation products, or when the formulation is complex and excipients might interfere with the quantitative measurements. Therefore, it is an essential part of the system suitability measurement stage before the quantitative work of these types of samples. The sample used for the measurements of Rs during the system suitability runs is sometimes called Resolution Solution, It usually contains the components that are the most difficult to resolve. The theoretical description of the Resolution Factor Rs equation is shown in Equation. It includes some of the above parameters, the plate count N, the selectivity α and the average of the two peaks' capacity factors k':

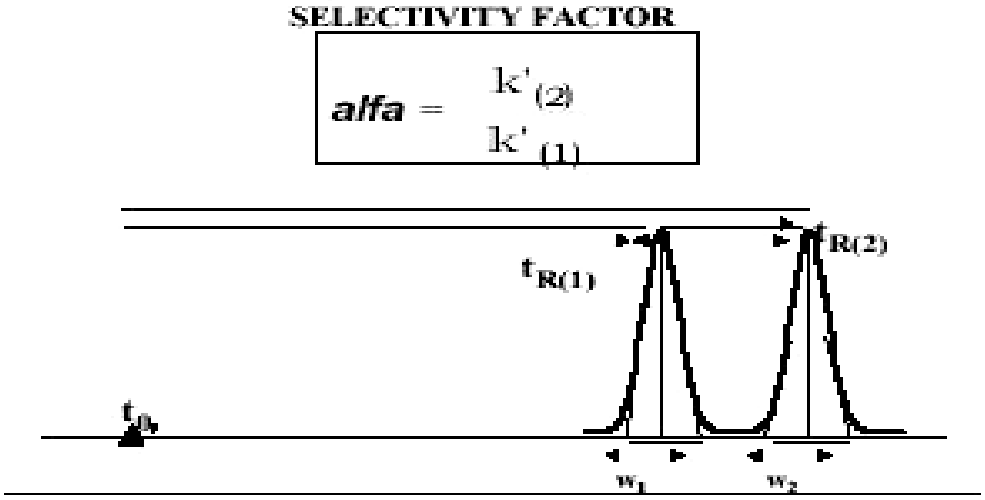


Figure. No.1.7 showing selectivity

It can be clearly seen from this equation that the plate count is the most effecting parameter in the increase of the chromatographic resolution. Since the plate count increases with the reduction in particle diameter, it explains the reduction in particle diameter of the stationary phase material during the last 3 decades of HPLC. This is also the rationale behind the recent trend in HPLC, the use of sub 2 micron particle columns and the development of a specially design of ultra-performance HPLC systems to accommodate such columns.

1.5) ANALYTICAL METHOD DEVELOPMENT (17-21)

Methods are developed for new products when no official methods are available. Alternate methods for existing (Non-Pharmacopoeias) products are developed to reduce the cost and time for better precision and ruggedness. Trial runs are conducted, method is optimized and validated. When alternate method proposed is intended to replace the existing procedure, comparative laboratory data including merits / demerits should be made available.

Steps involved in method development

Documentation starts at the very beginning of the development process. A system for full documentation of development studies must be established. All data relating to these studies must be recorded in laboratory notebook or an electronic database.

1. Analyte standard characterization

a) All known information about the analyte and its structure is collected i.e., physical and chemical properties.

b) The standard analyte (100 % purity) is obtained. Necessary arrangement is made for the proper storage (refrigerator, desiccators and freezer).

c) When multiple components are to be analyzed in the sample matrix, the number of components is noted, data is assembled and the availability of standards for each one is determined.

d) Only those methods (spectroscopic, MS, GC, HPLC etc.,) that are compatible with sample stability are considered.

2. Method requirements

The goals or requirements of the analytical method that need to be developed are considered and the analytical figures of merit are defined. The required detection limits, selectivity, linearity, range, accuracy and precision are defined.

3. Literature search and prior methodology

The literature for all types of information related to the analyte is surveyed. Solubility profile (solubility of Drug in different solvents and at different pH conditions), analytical profile (Physico-chemical properties, Eg: pKa, melting point, degradation pathways, etc) and stability profile (sensitivity of the drug towards light, heat, moisture etc)

and relevant analytical methods, books, periodicals, chemical manufacturers and regulatory agency compendia such as USP / NF, are reviewed.

4. Choosing a method

a) Using the information in the literatures and prints, methodology is adapted. The methods are modified wherever necessary. Sometimes it is necessary to acquire additional instrumentation to reproduce, modify, improve or validate existing methods for in-house analytes and samples. If there are no prior methods for the analyte in the literature, from analogy, the compounds that are similar in structure and chemical properties are investigated and are worked out.

b) There is usually one compound for which analytical method already exist that is similar to the analyte of interest.

5. Instrumental setup and initial studies

The required instrumentation is setup. Installation, operational and performance qualification of instrumentation using laboratory standard operating procedures (SOP's) are verified. Always new consumables (e.g. solvents, filters and gases) are used. For example, method development is never started on a HPLC column that has been used earlier. The analyte standard in a suitable injection / introduction solution and in known concentrations and solvents are prepared. It is important to start with an authentic, known standard rather than with a complex sample matrix. If the sample is extremely close to the standard (e.g., bulk drug), then it is possible to start work with the actual sample.

6. Optimization

During optimization one parameter is changed at a time and set of conditions are isolated, rather than using a trial and error approach. Work has been done from an organized methodical plan, and every step is documented (in a lab notebook) in case of dead ends.

7. Documentation of analytical figures of merit

The originally determined analytical figures of merit are limit of quantitation (LOQ), limit of detection (LOD), linearity, time per analysis, cost, sample preparation etc., are documented.

8. Evaluation of method development with actual samples

The sample solution should lead to unequivocal, absolute identification of the analyte peak of interest apart from all other matrix components.

9. Determination of percent recovery of actual sample and demonstration of quantitative sample analysis

Percent recovery of spiked, authentic standard analyte into a sample matrix that is shown to contain no analyte is determined. Reproducibility of recovery (average + / - standard deviation) from sample to sample and whether recovery has been optimized or not has been shown. It is not necessary to obtain 100 % recovery as long as the results are reproducible and known with a high degree of certainty. The validity of analytical method can be verified only by laboratory studies.

Therefore documentation of the successful completion of such studies is a basic requirement for determining whether a method is suitable for its intended applications.

1.6) METHOD DEVELOPMENT PROCEDURE [18]

The wide variety of equipment's, columns, eluent and operation preparations involved high performance liquid chromatography (HPLC) method development seems complex. The processes influenced by the nature of analytes and generally follow the following steps

Steps:

- Step 1 - Selection of the HPLC method and initial system
- Step 2 - Selection of initial conditions
- Step 3 - Selectivity optimization
- Step 4 - System optimization
- Step 5 - Method validation.

Depending on the overall requirements and nature of the sample and analytes, some of these steps will not be necessary during HPLC analysis. For example, a satisfactory separation may be found during step 2, thus steps 3 and 4 may not be required. The extent to which method validation (step 5) is investigated will depend on the use of the end analysis; for example, a method required for quality control will require more validation than one developed for a one-off analysis. The following must be considered when developing an HPLC method:

HPLC method development [19]

Step 1 - Selection of the HPLC method and initial system.

When developing an HPLC method, the first step is always to consult the literature to ascertain whether the separation has been previously performed and if so, under what conditions - this will save time doing unnecessary experimental work. When selecting an HPLC system, it must have a high probability of actually being able to analyse the sample; for example, if the sample includes polar analytes then reverse phase HPLC would offer both adequate retention and resolution, whereas normal phase HPLC would be much less feasible. Consideration must be given to the following:

Sample preparation:

- ✓ Does the sample require dissolution, filtration, extraction, preconcentration or clean up,
- ✓ Is chemical derivatization required to assist detection sensitivity or selectivity

Types of chromatography:

1.Reverse phase is the choice for the majority of samples, but if acidic or basic analytes are present then reverse phase ion suppression (for weak acids or bases) or reverse phase ion pairing (for strong acids or bases) should be used. The stationary phase should be C18 bonded.

2. For low/medium polarity analytes, normal phase HPLC is a potential candidate, particularly if the separation of isomers is required. Carbon bonded phases are easier to work with than plain silica for normal phase separations. For inorganic anion/cation analysis, ion exchange chromatography is best. Size exclusion chromatography would normally be considered for analysing high molecular weight compounds.

Column dimensions:

For most samples (unless they are very complex), long columns (25 cm) are recommended to enhance the column efficiency. A flow rate of 1-1.5 ml/min should be used initially. Packing particle size should be 3 or 5 μm.

Detectors:

Consideration must be given to the following:

- Do the analytes have chromophores to enable UV detection
- Is more selective/sensitive detection required
- What detection limits are necessary
- Will the sample require chemical derivatization to enhance detectability and/or improve the chromatography?

Fluorescence or electrochemical detectors should be used for trace analysis. For preparative HPLC, refractive index is preferred because it can handle high concentrations without over loading the detector. UV wavelength for the greatest sensitivity λmax should be used, which detects all sample components that contain chromophores. UV wavelengths below 200 nm should be avoided because detector noise increases in this region. Higher wavelengths give greater selectivity.

The excitation wavelength locates the excitation maximum; that is, the wavelength that gives the maximum emission intensity. The excitation is set to the maximum value then the emission is scanned to locate the emission intensity. Selection of the initial system could, therefore, be based on assessment of the nature of sample and analytes together with literature data, experience, expert system software and empirical approaches.

Step 2 - Selection of initial conditions.

This step determines the optimum conditions to adequately retain all analytes; that is, ensures no analyte has a capacity factor of less than 0.5 (poor retention could result in peak overlapping) and no analyte has a capacity factor greater than 10–15 (excessive

retention leads to long analysis time and broad peaks with poor detectability). Selection of the following is then required.

Mobile phase solvent strength:

The solvent strength is a measure of its ability to pull analytes from the column. It is generally controlled by the concentration of the solvent with the highest strength; for example, in reverse phase HPLC with aqueous mobile phases, the strong solvent would be the organic modifier; in normal phase HPLC, it would be the most polar one. The aim is to find the correct concentration of the strong solvent. With many samples, there will be a range of solvent strengths that can be used within the aforementioned capacity limits. Other factors (such as pH and the presence of ion pairing reagents) may also affect the overall retention of analytes.

Step 3 - Selectivity optimization:

The aim of this step is to achieve adequate selectivity (peak spacing). The mobile phase and stationary phase compositions need to be taken into account. To minimize the number of trial chromatograms involved, only the parameters that are likely to have a significant effect on selectivity in the optimization must be examined. To select these, the nature of the analytes must be considered. Once the analyte types are identified, the relevant optimization parameters may be selected. Note that the optimization of mobile phase parameters is always considered first as this is much easier and convenient than stationary phase optimization.

Step 4 - System parameter optimization:

This is used to find the desired balance between resolution and analysis time after satisfactory selectivity has been achieved. The parameters involved include column dimensions, column-packing particle size and flow rate. These parameters may be changed without affecting capacity factors or selectivity.

Step 5 - Method validation:

Proper validation of analytical methods is important for pharmaceutical analysis when ensure of the continuing efficacy and safety of each batch manufactured relies solely on the determination of quality. The ability to control this quality is dependent upon the ability of the analytical methods, as applied under well-defined conditions and at an established level of sensitivity, to give a reliable demonstration of all deviation from target criteria.

Analytical methods should be used within good manufacturing practice (GMP) and good laboratory practice (GLP) environments, and must be developed using the protocols set out in the international conference on harmonization (ICH) guidelines (Q2A and Q2B). The US food and drug administration (FDA) and US Pharmacopoeia (USP) both refer to ICH guidelines. The most widely applied validation characteristics are accuracy, precision (repeatability and intermediate precision), specificity, detection limit, quantitation limit, linearity, range, robustness and stability of analytical solutions. Method validation must have a written and approved protocol prior to use.

1.7. STABILITY INDICATING METHOD

It is essential that the analytical methods developed for estimation of the purity and impurities are capable enough to separate all the desired and undesired components and devoid of any interferences from the formulation matrix. When analytical methods are able to precisely and accurately quantify without missing any impurities, without underestimation or over estimation, and detect all possible impurities and degradants those can form during stability studies with adequate sensitivity and exactly reflect the quality of drug substances and drug products (formulated products of drugs), those methods are called stability indicating methods.

A stability-indicating assay method should accurately measure the active ingredients, without interference from degradation products, process impurities, excipients, or other potential impurities. If an industry uses a non-stability indicating analytical procedure for release testing, then an analytical procedure capable of qualitatively and quantitatively monitoring the impurities, including degradation products, should complement it. Analytical procedures for stability studies of assay should be stability indicating. As a result of stability testing a re-test period for the active substance or a shelf life for the pharmaceutical product can be established, and storage conditions can be recommended.

The ICH (International conference on Harmonization) guideline QIA on Stability Testing of New Drug Substances and Products emphasizes that the testing of those features which are susceptible to change during storage and are likely to influence quality, safety and/or efficacy must be done by validated stability indicating testing methods. It is also mentioned that forced decomposition studies (stress testing) at temperatures in 10 °C increments above the accelerated temperatures, extremes of pH, under oxidative and

photolytic conditions should be carried out on the drug substance and drug product so as to establish the inherent stability characteristics and degradation pathways to support the suitability of the proposed analytical procedures.

1.8 ANALYTICAL METHOD VALIDATION [16-19]

According to ICH Guidelines Method Validation can be defined as "Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics".

An assay for a major component requires a different approach and acceptance criteria than a method for a trace impurity. A final method may be performed at different sites around the world. Differences in HPLC instrumentation, laboratory equipment and reagent sources and variations in the skills and background of personnel may require specific features in the HPLC method. In addition, the development of different formulations of the same drug with varying strengths or physical forms may require flexibility in method procedures.

Method validation study include system suitability, linearity, precision, accuracy, specificity, robustness, limit of detection, limit of quantification and stability of samples, reagents, instruments.

1. System Suitability

Prior to the analysis of samples of each day, the operator must establish that the HPLC system and procedure are capable of providing data of acceptable quality. This is accomplished with system suitability experiments, which can be defined as tests to ensure

that the method can generate results of acceptable accuracy and Precision. The requirements for system suitability are usually developed after method development and validation have been completed.

Table No.1.3 System Suitability Parameters and their recommended limits

Parameter	Recommendation
Capacity Factor (K')	The peak should be well-resolved from other peaks and the void volume generally K>2
Repeatability	RSD ≤ 2% N ≥ 5 is desirable
Relative Retention	Not essential as the resolution is stated
Resolution(R_S)	R_S of > 2 between the peak of interest and the closest eluting
Tailing Factor(T)	T ≤ 2
Theoretical Plates(N)	In general should be > 2000

2. Linearity

The linearity of a method is a measure of how well a calibration plot of response vs concentration approximates a straight line. Linearity can be assessed by performing single measurements at several analyte concentrations. The data is then processed using a linear least-squares regression. The resulting plot slope, intercept and correlation coefficient provide the desired information on linearity.

3. Precision

Precision can be defined as "The degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample".

A more comprehensive definition proposed by the International Conference on Harmonization (ICH) divides precision into three types:

- Repeatability
- Intermediate precision and
- Reproducibility

Repeatability is the precision of a method under the same operating conditions over a short period of time.

Intermediate precision is the agreement of complete measurements (including standards) when the same method is applied many times within the same laboratory.

Reproducibility examines the precision between laboratories and is often determined in collaborative studies or method transfer experiments.

4. Accuracy

The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample (whose "true value" is known) is analyzed and the measured value is identical to the true value. Typically, accuracy is represented and determined by recovery studies.

There are three ways to determine accuracy:

1. Comparison to a reference standard
2. Recovery of the analyte spiked into blank matrix or
3. Standard addition of the analyte.

It should be clear how the individual or total impurities are to be determined. E.g. Weight / weight or area percent in all cases with respect to the major analyte.

5. Specificity / Selectivity

The terms selectivity and specificity are often used interchangeably. According to ICH, the term specific generally refers to a method that produces a response for a single analyte only while the term selective refers to a method which provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is said to be selective. Since there are very few methods that respond to only one analyte, the term selectivity is usually more appropriate. The analyte should have no interference from other extraneous components and be well resolved from them. A representative chromatogram or profile should be generated and submitted to show that the extraneous peaks either by addition of known compounds or samples from stress testing are baseline resolved from the parent analyte.

6. Robustness

The concept of robustness of an analytical procedure has been defined by the ICH as "a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters". A good practice is to vary important parameters in the method systematically and measure their effect on separation. The variable method parameters in HPLC technique may involves flow rate, column temperature, sample temperature, pH and mobile phase composition.

7. Limit of detection

Limit of detection (LOD) is the lowest concentration of analyte in a sample that can be detected, but not necessarily qualitated, under the stated experimental conditions. With UV detectors, it is difficult to assure the detection precision of low level compounds due to potential gradual loss of sensitivity of detector lamps with age or noise level variation by detector manufacturer. At low levels, assurance is needed that the LOD and LOQ limits are achievable with the test method each time. With no reference standard for a given impurity or means to assure detectability, extraneous peak(s) could "disappear / appear." A crude method to evaluate the feasibility of the extraneous peak detection is to use the percentage claimed for LOD from the area counts of the analyte. Several approaches for determining the LOD are possible, depending on whether the procedure is a non-instrumental or instrumental.

- Based on visual evaluation
- Based on signal-to-noise
- Based on the standard deviation of the response and the slope

The LOD may be expressed as:

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

Where,

σ = Standard deviation of Intercepts of calibration curves

S = Mean of slopes of the calibration curves

8. Limit of quantitation

Limit of quantitation (LOQ) is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. Several approaches for determining the LOQ are possible depending on whether the procedure is a non-instrumental or instrumental.

- Based on visual evaluation
- Based on signal-to-noise Approach
- Based on the standard deviation of the response and the slope

The LOQ may be expressed as:

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

Where,

σ = Standard deviation of Intercepts of calibration curves

S = Mean of slopes of the calibration curves

The slope S may be estimated from the calibration curve of the analyt

Table No.1.4: Characteristics to be validated in HPLC

Characteristics	Acceptance Criteria
Accuracy/trueness	Recovery 98-102% (individual)
Precision	RSD < 2%
Repeatability	RSD < 2%
Intermediate Precision	RSD < 2%
Specificity / Selectivity	No interference
Detection Limit	S/N > 2 or 3
Quantitation Limit	S/N > 10
Linearity	Correlation coefficient R^2 > 0.999
Range	80 –120 %

2. DRUG PROFILE [25-27]

CABOTEGRAVIR:

Description:

Cabotegravir, or GSK1265744, is an HIV-1 integrase inhibitor that is prescribed with the non-nucleoside reverse transcriptase inhibitor, rilpivirine.4,5,6 Early research into Cabotegravir showed it had lower oral bioavailability than dolutegravir.4 The development of Cabotegravir was later developed to create a long acting monthly intramuscular injection.

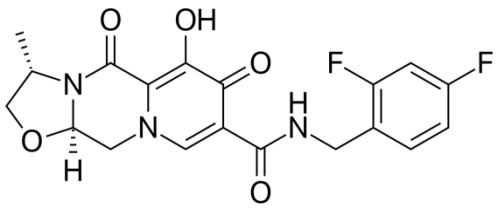


Fig. 2.1. Structure of Cabotegravir

CAS Number	: 1051375-10-0
Molecular Weight	: Average: 405.358
Molecular Formula	: $C_{19}H_{17}F_2N_3O_5$
Physical State	: Liquid
Solubility	: Soluble in DMSO and Dimethyl formamide.
Storage	: Room temperature
Melting point	: 248-251ºC
pKa	: 10.04(Strongest acid)

Pharmacodynamics:-

Cabotegravir is an inhibitor of HIV integrase, which reduces viral replication. It has a long duration of action as the oral tablet is given daily and the intramuscular suspension is given monthly. Patients should be counselled regarding the risk of hypersensitivity, hepatotoxicity, and depression.

Mechanism of action:-

Cabotegravir binds to the active site of HIV integrase, preventing strand transfer of the viral genome into the host genome, and preventing replication of the virus.

Absorption:

Oral Cabotegravir has a Tmax of 3 hours, reaches a Cmax of 8.0 µg/mL, and has an AUC of 145 µg*h/mL.5 Intramuscular extended-release Cabotegravir has a Tmax of 7 days, reaches a Cmax of 8.0 µg/mL, and has an AUC of 1591 µg*h/mL

Metabolism:

Cabotegravir is O-glucuronidated to the M1 and M2 metabolites, with 67% of glucuronidation performed by UGT1A1, and 33% by UGT1A.

Route of elimination:

An oral radio labelled dose of Cabotegravir is 58.5% recovered in the feces and 26.8% recovered in the urine.

Half-life:

The mean half-life of oral Cabotegravir is 41 hours. The mean half-life of intramuscular extended-release cabotegravir is 5.6-11.5 weeks.

Clearance:

Data regarding the clearance of cabotegravir is not readily available.3,6,8 Clearance in dogs was 0.34 mL/min/kg and in cynomolgus monkeys was 0.32 mL/min/kg.

Dosage forms: Tablet

Brand names: Vocabria

RILPIVIRINE

Description:

Rilpivirine is non-nucleoside reverse transcriptase inhibitor (NNRTI) which is used for the treatment of HIV-1 infections in treatment-naive patients. It is a diary pyrimidine, a class of molecules that resemble pyrimidine nucleotides found in DNA. Because of its flexible chemical structure, resistance of Rilpivirine is less likely to develop than other NNRTI's. FDA approved on May 20, 2011.

Structure:

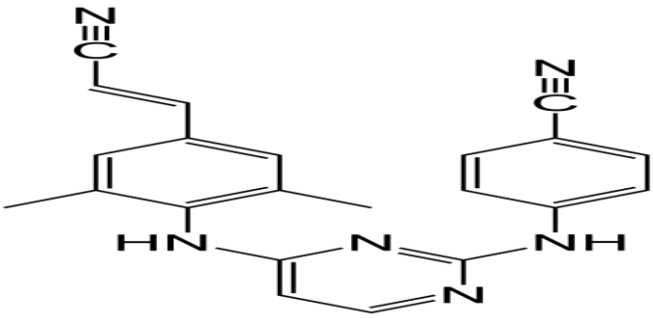


Fig. 2.2. Structure of Rilpivirine

CAS Number	: 500287-72-9
Molecular Weight	: Average: 366.4185
Molecular Formula	: $C_{22}H_{18}N_6$
Physical State	: solid
Solubility	: water solubility-0.1mg/ml
Storage	: Room temperature
Melting point	: 241-243°C
pKa	: 11.43 (strongest acid)

Indication:

Treatment of HIV-1 infections in treatment-naive patients with HIV-1 RNA ≤100,000 copies/mL in combination with at least 2 other antiretroviral agents.

Pharmacodynamics:

Rilpivirine is the most potent NNRTI and has a EC50 of 0.73 nM in vitro against HIV-1 because its chemical structure allowed for better binding to reverse transcriptase.

Mechanism of action:

Rilpivirine is an NNRTI which binds to reverse transcriptase which results in a block in RNA and DNA- dependent DNA polymerase activities. One such activity is HIV-1 replication. Intracellular phosphorylation is not necessary for its antiviral activity. Because of the structure of Rilpivirine is flexible around the aromatic rings, the molecule can have multiple conformations so that can bind to residues in the reverse transcriptase enzyme which have a lower mutation rate.

Absorption:

Rilpivirine demonstrates dose-dependent pharmacokinetics and does not change between subcutaneous, IV, and intramuscular administration. Absorption increases with meals. Tmax, oral administration = 4 hours;

Protein binding: >99% protein bound.

Metabolism:

Mainly hepatically metabolized by CYP3A4. Because it is highly protein bound, its free plasma concentration is very small thus is unlikely to inhibit cytochrome proteins to a clinically relevant degree despite being an inhibitor of CYP3A4, CYP2C19, and CYP2B6**.**

Route of elimination:

Excreted fecally (85%, 25% as unchanged drug) and urine (6%, <1% as unchanged drug)

Half-life: 34-55 hours after oral administration

SIDE EFFECTS:

- Sleep problems (insomnia), unusual dreams;
- Mild nausea, vomiting, stomach pain, diarrhea;
- Headache, dizziness;
- Mild skin rash;
- Tired feeling;
- Changes in the shape or location of body fat (especially in your arms, legs, face, neck, breasts, and waist).

Brand names: Complera

3. LITERATURE REVIEW [29-37]

Perrine Courlet *et al.,* the widespread use of highly active antiretroviral treatments has dramatically changed the prognosis of people living with HIV (PLWH). However, such treatments have to be taken lifelong raising issues regarding the maintenance of both therapeutic effectiveness and long-term tolerability. Recently approved or investigational antiretroviral drugs present considerable advantages, allowing once daily oral dosage along with activity against resistant variants (eg, Bictegravir and Doravirine) and also parenteral intramuscular administration that facilitates treatment adherence (eg, long-acting injectable formulations such as Cabotegravir and Rilpivirine). Still, there remains a risk of insufficient or exaggerated circulating exposure due to absorption issues, abnormal elimination, drug-drug interactions, and others. In this context, a multiplex ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) bioassay has been developed for the monitoring of plasma levels of Bictegravir, Cabotegravir, Doravirine, and Rilpivirine in PLWH. A simple and convenient protein precipitation was performed followed by direct injection of the supernatant into the UHPLC-MS/MS system. The four analytes were eluted in less than 3 minutes using a reversed-phase chromatography method coupled with triple quadrupole mass spectrometry detection. This bioassay was fully validated following international guidelines and achieved good performances in terms of trueness (94.7%-107.5%), repeatability (2.6%-11%), and intermediate precision (3.0%-11.2%) over the clinically relevant concentration ranges (from 30 to 9000 ng/mL for Bictegravir, Cabotegravir, and Doravirine and from 10 to 1800 ng/mL for Rilpivirine). This sensitive, accurate, and rapid UHPLC-MS/MS assay is currently applied in our laboratory for routine therapeutic drug monitoring of the oral drugs Bictegravir and Doravirine and is also

intended to be applied for the monitoring of Cabotegravir/Rilpivirine levels in plasma from PLWH receiving once monthly or every 2-month intramuscular injection of these long-acting antiretroviral drugs.

Srinivas Reddy *et al.*, new simple, sensitive, and validated stability-indicating RP-HPLC method has been developed for the simultaneous estimation of Rilpivirine and Dolutegravir in its bulk form. Chromatographic separation was achieved on a Hypersil ODS (250mm × 4.6mm i.d., 5µm) maintained at ambient temperature by a mobile phase consisted of methanol and water (80:20v/v) and a flow rate of 1.0mL/min with a load of 20µL. The detection wavelength was set at 282nm. The retention time for the drugs was found to be Rilpivirine (5.14min), Dolutegravir (6.72min). The eluted compounds were detected using a UV detector. The drugs were subjected to stress degradation as per ICH Q1A. There was the interference of degradant at RT of Rilpivirine and Dolutegravir. The developed method was successfully validated according to ICH guidelines. The calibration curve was found to be linear over a range of 10-100µg/mL. The accuracy of the method is indicated by a good recovery in the range of Rilpivirine 99-102% and of Dolutegravir 99-102%. The limit of detection and limit of quantification of Rilpivirine was found to be LOD-0.844µg/mL and LOQ-2.557µg/mL, and for Dolutegravir was found to be LOD-0.082µg/mL and LOQ-0.249µg/mL.

Rubesh Kumar *et al.*, A simple, rapid, precise, accurate and sensitive reverse phase liquid chromatographic method has been developed for the determination of Rilpivirine in bulk and pharmaceutical dosage form dosage form. The chromatographic method was standardized using Develosil ODS HG-5 RP C18, 5µm, 15cm x 4.6mm i.d. column with UV detection at 205 nm and 0.1% Ortho phosphoric acid and Acetonitrile with 65:35 ratio at a flow rate of 1.0 ml/ min. The proposed

method was successfully applied to the determination of Rilpivirine in bulk and pharmaceutical dosage form. The method was linear over the range of 20-70µg/ml. The recovery was in the range of 98% to 102% and limit of detection was found to be 0.8 µg/ml and quantification was found to be 2.4 µg/ml. Different analytical performance parameters such as precision, accuracy, limit of detection, limit of quantification and robustness were determined according to International Conference on Harmonization (ICH) guidelines.

Vejendla *et al..,* using a Symmetry C18 (4.6×150 mm, 3.5) column, a high-performance liquid chromatographic Method for quantifcation of Rilpivirine and Cabotegravir in active pharmaceutical ingredients was developed and Validated. The mobile phase is made up of bufer, acetonitrile, and 0.1 percent formic acid in a 20:80v/v ratio. The fow Rate was kept constant at 1.0 ml/min, and detection was accomplished through absorption at 231 nm with a photodi-Ode array detector. The calibration curve was linear, with a regression coefcient (R2) value of 0.999 and concentrations ranging From 30 to 450 g/ml of Rilpivirine and 20–300 g/ml of Cabotegravir. The method's LOD and LOQ were 0.375 g/ml, 1.238 g/ml, and 0.25 g/ml, 0.825 g/ml for Rilpivirine and Cabotegravir, respectively.In the forced degradationstudies, the degradants were characterized by using LCMS and FTIR. The current application was found to be simple, economical, and suitable, and validated according to ICH guidelines.

Ramolle *et al..,* the antiretroviral agent's rilpivirine (RPV) and cabotegravir (CAB) are approved as a combined treatment regimen against human immunodeficiency virus (HIV). To fully understand the biodistribution of these agents and determine their concentration levels in various parts of the body, a simple, selective and sensitive bioanalytical method is essential. In the present study, a high

performance liquid chromatography method with mass spectrometry detection (HPLC-MS) was developed for simultaneous detection and quantification of RPV and CAB in various biological matrices. These included plasma, skin, lymph nodes, vaginal tissue, liver, kidneys and spleen, harvested from female Sprague Dawley rats. The suitability of the developed method for each matrix was validated based on the guidelines of the International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) on bioanalytical method validation. Analytes were extracted from biological samples employing a simple one-step protein precipitation method using acetonitrile. Samples were analysed using an Apex Scientific Inertsil ODS-3 column (4.6 mm × 250 mm, 5 µm particle size), maintained at 40 °C, on a HPLC system coupled with a single quadrupole MS detector. RPV was detected at a mass-to-charge ratio (m/z) of 367.4 and CAB at 406.3. Separation was achieved using isocratic elution at 0.3 mL/min with a mixture of acetonitrile and 0.1% (v/v) trifluoroacetic acid in water (81:19, v/v) as the mobile phase. The run time was set at 13 min. The presented method was selective, sensitive, accurate and precise for detection and quantification of RPV and CAB in all matrices. The developed and validated bioanalytical method was successfully employed for in vivo samples with both drugs simultaneously.

Veeraswami *et al..,* developed a simple, accurate, and precise reversed-phase high-performance liquid chromatography (HPLC) method for rapid and simultaneous quantification of dolutegravir (DTG) and rilpivirine (RPV) in bulk and pharmaceutical dosage form and rat plasma. The chromatographic separation was achieved on Phenomenex C18 (150x4.6mm, 5µm). Mobile phase contained a mixture of 0.1% Ortho phosphoric acid and acetonitrile in the rato of 60:40 v/v, flow rate 1.0ml/min and ultraviolet detection at 262nm. The retention time of DTG and RPV

was 4.35 min and 7.73 min, respectively. The proposed method shows a good linearity in the concentration range of 10–150 µg/ml for DTG and 5–75 µg/ml for RPV under optimized conditions. Precision and recovery study results are in between 98 and 102%. In the entire robustness conditions, percentage relative standard deviation is <2.0%. Degradation has minimum effect in stress condition and solutions are stable up to 24 h. DTG and RPV drugs are release 98% at 2 h in rat body. Conclusion: This method is validated for different analytical performance parameters like linearity. Precision, accuracy, limit of detection, limit of quantification, robustness, and pharmacokinetic study were determined according to the International Conference of Harmonization (ICH) Q2B guidelines. All the parameters of validation were found in the acceptance range of ICH guidelines. The same method is also applied for plasma samples study in bioanalytical work.

Kavitha *et al..,* developed a simple, rapid, precise, and reliable simultaneous stability indicating RP-HPLC method was developed for the separation and estimation of three drugs Emtricitabine, Rilpivirine and Tenofovir in bulk drug mix and pharmaceutical dosage forms. Chromatography was carried on an Inertsil ODS 3V column using gradient composition of 0.02 M sodium dihydrogen orthophosphate as mobile phase A and mixture of Methanol and water in ratio of 85: 15as mobile phase B at a flow rate of 1.5 mL/min with detection at 261 nm. The retention times of the Emtricitabine, Tenofovir Disoproxil Fumarate and Rilpivirine were about 5.875, 8.800 and 12.020 mins respectively. The detector response is linear from 8-120 µg/ml, 12-180µg/mL, 20-360 µg/mL of test concentration for Emtricitabine, Tenofovir and Rilpivirine respectively. The respective linear regression equation being Y= 10175x-76883 for Emtricitabine, Y= 6280.8 x+ 219800 for Tenofovir Disoproxil Fumarate and Y= 1883.5 x+ 323060 for Rilpivirine. The limit of detection

and Limit of quantification was 0.06, 0.07 and 0.08 µg/mL and 0.14, 0.12 and 0.15 µg/mL for Emtricitabine, Tenofovir and Rilpivirine respectively. The percentage assay of Emtricitabine, Tenofovir Disoproxil Fumarate and Rilpivirine were about 99.50, 100.2 and 99.46% respectively and percentage recovery for average of five different concentrations were 99.65%, 99.62% and 100.34% respectively. The method was validated as a final verification of method development with respect to precision, linearity, accuracy, ruggedness, and robustness. The validated method was successfully applied to the commercially available pharmaceutical dosage form, yielding very good and reproducible result.

Vijaya Sri *et al.*, done development and validation of RP HPLC method for simultaneous estimation of Tenofovir Disoproxil fumarate, Emtricitabine and Rilpivirine hydrochloride in bulk and formulated in a pharmaceutical dosage form as a nanosuspension. Antiretroviral drug treatment is the primary line of therapy for treating HIV. The multicomponent system formulated as a nanosuspension evidenced increased hydrophilicity, potency and decreased side effects. The separation was carried out by using efficient BDS Hypersil C18 HPLC column with empower software. Combination method of Precipitation—ultrasonic homogenization was used for the preparation of the nanosuspension. The mobile phase used was methanol, water, acetonitrile (80:13.4:6.6) v/v and flow rate 1mL /min. The developed method was thus validated as per ICH guidelines for various parameters whose results advocated the reliability of the method. The results for parameters viz. retention times of Tenofovir Disoproxil fumarate, Emtricitabine and Rilpivirine were 3.09 min, 2.78 min and 3.68 min, linearity range was between 7.5-90, 5-60, and 0.625-7.5µg/mL, respectively. Thus the new RP-HPLC method is optimum, reliable and can be used

for the simultaneous estimation of Tenofovir Disoproxil fumarate, Emtricitabine and Rilpivirine hydrochloride.

Pasha *et al..,* developed a Stability representing Ultra Performance LC method was developed for Assay of multi drug Combination of Rilpivirine, Emtricitabine and Tenofovir alafenamide in bulk active pharmaceutical Ingredients & its tablet formulation, Validation was performed for all parameters .Retention times Of Reference Standard Emtricitabine, Tenofovir Alafenamide and Rilpivirine was found to be 0.965, 1.528, 2.186 respectively, with the flow rate of 0.3 mille liters per minute by Injecting Volume of 2 micro liters by maintaining Run time of 3 minutes. Developed method was subjected to forced Degradation studies under specified conditions, which meets the required criteria. Degradation product At 1.975 Rt was collected under various stress condition, cleavage of imidazole ring of Tenofovir Alafenamide confirmed with Proton NMR, ESI –MASS in + MODE.

4. AIM AND OBJECTIVE

AIM:

As per the literature review, there are no analytical methods reported for the estimation of Cabotegravir, Rilpivirine in combined pharmaceutical dosage form by HPLC. Various publications are available regarding the UV simultaneous estimation, LC-MS method for Cabotegravir and Rilpivirine, either alone or in combination with other drugs in pharmaceutical dosage form.

Hence, there is a need for suitable HPLC method for routine analysis of Cabotegravir and Rilpivirine in the combined formulation. The work was an attempt to develop simple, rapid, cost effective and sensitive analytical method for the simultaneous estimation of Cabotegravir and Rilpivirine in the combined formulation in accordance with ICH Q2B guidelines and to extend the method for routine analysis.

OBJECTIVE AND PLAN:

Present work is to develop and validate a new simple, rapid, and sensitive method for the simultaneous estimation of Cabotegravir and Rilpivirine in the pharmaceutical dosage form by following 3 method

1. Selection
2. System suitability parameters
3. Validation parameters
    1. **Selection:** Steps involved are,
        - Selection of suitable wavelength
        - Selection of stationary phase
        - Selection of mobile phase
        - Selection of detector

2. **System suitability parameters:** steps involved are
    - ✓ Capacity factor
    - ✓ Retention time
    - ✓ Tailing factor
    - ✓ Resolution
    - ✓ % Relative standard deviation

3. **Validation parameters:** steps involved are
    - ❖ Linearity
    - ❖ Precision
    - ❖ Method precision
    - ❖ Accuracy
    - ❖ Range
    - ❖ Robustness
    - ❖ Ruggedness
    - ❖ Limit of detection
    - ❖ Limit of quantification
    - ❖ Specificity

5. MATERIALS AND METHODS

Materials:

- Cabotegravir and Rilpivirine pure drugs (API) received from spectrum labs.
- Combination Cabotegravir and Rilpivirine injections, received from local market.
- Distilled water, Acetonitrile, Phosphate buffer, Methanol, Potassium dihydrogen ortho phosphate buffer, Ortho-phosphoric acid. All the above chemicals and solvents are from Rankem

Instruments:

- Electronics Balance-Denver
- p^H meter -BVK enterprises, India
- Ultrasonicator-BVK enterprises
- WATERS HPLC 2695 SYSTEM equipped with quaternary pumps, Photo Diode Array detector and Auto sampler integrated with Empower 2 Software.
- UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2 mm and 10mm and matched quartz cells integrated with UV win 6 Software was used for measuring absorbance's of Cabotegravir and Rilpivirine solutions.

Methods:

Diluent: Based up on the solubility of the drugs, diluent was selected, Acetonitrile and Water taken in the ratio of 50:50

Preparation of Standard stock solutions: Accurately weighed 100 mg of Cabotegravir, 150 mg of Rilpivirine and transferred to 50 mL volumetric flasks and 3/4 th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (2000µg/mLof Cabotegravir and 3000µg/mLRilpivirine)

Preparation of Standard working solutions (100% solution): 1mL from each stock solution was pipetted out and taken into a 10 mL volumetric flask and made up with diluent. (200 µg/mLof Cabotegravir and 300 µg/mLof Rilpivirine)

Preparation of Sample stock solutions: Accurately weighed 100 mg of Cabotegravir, 150 mg of Rilpivirine and transferred to 50 mL volumetric flasks and 3/4 th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (2000 µg/mLof Cabotegravir and 3000 µg/mL Rilpivirine)

Preparation of Sample working solutions (100% solution):): 1mL from each stock solution was pipetted out and taken into a 10mL volumetric flask and made up with diluent. (200 µg/mLof Cabotegravir and 300 µg/mLof Rilpivirine)

Preparation of buffer:

0.1%OPA Buffer: 1mL of ortho phosphoric acid was diluted to 1000mL with HPLC grade water.

Buffer: 0.01N Potassium dihydrogen Ortho phosphate

Accurately weighed 1.36gm of Potassium dihydrogen Ortho phosphate in a 1000 mL of Volumetric flask add about 900mL of milli-Q water added and degas to sonicate and finally make up the volume with water then added 1mL of Triethylamine then PH adjusted to 3.8 with dil. Orthophosphoric acid solution

VALIDATION:

SYSTEM SUITABILITY PARAMETERS:

The system suitability parameters were determined by preparing standard solutions of Cabotegravir (200ppm) and Rilpivirine (300ppm) and the solutions were injected six times and the parameters like peak tailing, resolution and USP plate count were determined. The % RSD for the area of six standard injections results should not be more than 2%.

SPECIFICITY:

Checking of the interference in the optimized method. We should not find interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

PRECISION:

Preparation of Standard stock solutions: Accurately weighed 100 mg of Cabotegravir, 150 mg of Rilpivirine and transferred to 50mL volumetric flasks and 3/4 th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (2000 µg/mLof Cabotegravir and 3000 µg/mL Rilpivirine)

Preparation of Standard working solutions (100% solution): 1mL from each stock solution was pipetted out and taken into a 10 mL volumetric flask and made up with diluent. (200 µg/mLof Cabotegravir and 300 µg/mL of Rilpivirine)

LINEARITY:

25% Standard solution: 0.25 mL each from two standard stock solutions was pipetted out and made up to 10mL. (50 µg/mLof Cabotegravir and 75 µg/mLof Rilpivirine)

50% Standard solution: 0.5 mL each from two standard stock solutions was pipetted out and made up to 10 mL. (100 µg/mLof Cabotegravir and 150 µg/mLof Rilpivirine)

75% Standard solution: 0.75mL each from two standard stock solutions was pipetted out and made up to 10 mL. (150 µg/mLof Cabotegravir and 225 µg/mLof Rilpivirine)

100% Standard solution: 1.0 mL each from two standard stock solutions was pipetted out and made up to 10 mL. (200 µg/mLof Cabotegravir and 300 µg/mL of Rilpivirine)

125% Standard solution: 1.25 mL each from two standard stock solutions was pipetted out and made up to 10 mL. (250 µg/mLof Cabotegravir and 375 µg/mLof Rilpivirine)

150% Standard solution: 1.5 mL each from two standard stock solutions was pipetted out and made up to 10 mL (300 µg/mLof Cabotegravir and 450 µg/mLof Rilpivirine)

ACCURACY:

Preparation of Standard stock solutions: Accurately weighed 100 mg of Cabotegravir, 150 mg of Rilpivirine and transferred to 50 mL volumetric flasks and 3/4 th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (2000 µg/mLof Cabotegravir and 3000 µg/mL Rilpivirine)

Preparation of 50% Spiked Solution: 0.5 mL of sample stock solution was taken into a 10 mL volumetric flask, to that 1.0 mL from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 100% Spiked Solution: 1.0 mL of sample stock solution was taken into a 10 mL volumetric flask, to that 1.0mL from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 150% Spiked Solution: 1.5 mL of sample stock solution was taken into a 10 mL volumetric flask, to that 1.0 mL from each standard stock solution was pipetted out, and made up to the mark with diluent.

Acceptance Criteria:

The % Recovery for each level should be between 98.0 to 102

ROBUSTNESS:

Small deliberate changes in method like Flow rate, mobile phase ratio, and temperature are made but there were no recognized change in the result and are within range as per ICH Guide lines.

Robustness conditions like Flow minus (0.9 mL/min), Flow plus (1.1 mL/min), mobile phase minus, mobile phase plus, temperature minus (25°C) and temperature plus (35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

LOD sample Preparation: 0.25 mL each from two standard stock solutions was pipetted out and transferred to two separate 10mL volumetric flasks and made up with diluents. From the above solutions 0.1 mL each of Cabotegravir, Rilpivirine, solutions respectively were transferred to 10 mL volumetric flasks and made up with the same diluents

LOQ sample Preparation: 0.25 mL each from two standard stock solutions was pipetted out and transferred to two separate 10mL volumetric flask and made up with diluent. From the above solutions 0.3 mL each of Cabotegravir, Rilpivirine, and solutions respectively were transferred to 10mL volumetric flasks and made up with the same diluent.

DEGRADATION STUDIES:

Oxidation:

To 1 mL of stock solution of Cabotegravir and Rilpivirine, 1 mL of 20% hydrogen peroxide (H2O2) was added separately. The solutions were kept for 30 min at 60^0c. For HPLC study, the resultant solution was diluted to obtain 200 µg/mL & 300µg/mL solution and 10 µL were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies:

To 1 mL of stock s solution Cabotegravir and Rilpivirine, 1 mL of 2N Hydrochloric acid was added and refluxed for 30mins at 60^0c .The resultant solution was diluted to obtain 200 µg/mL & 300 µg/mL solution and 10 µL solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies:

To 1 mL of stock solution Cabotegravir and Rilpivirine, 1 mL of 2N sodium hydroxide was added and refluxed for 30mins at 60^0c. The resultant solution was diluted to obtain 200 µg/mL & 300 µg/mL solution and 10 µL were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies:

The standard drug solution was placed in oven at 105°C for 1 h to study dry heat degradation. For HPLC study, the resultant solution was diluted to 200 µg/mL & 300 µg/mL solution and10µL were injected into the system and the chromatograms were recorded to assess the stability of the sample**.**

Photo Stability studies:

The photochemical stability of the drug was also studied by exposing the 2000 µg/mL Cabotegravir & 3000 µg/mL Rilpivirine µg/mL solution to UV Light by keeping the beaker in UV Chamber for 1days or 200 Watt hours/m^2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 200 µg/mL & 300 µg/mL solutions and 10 µL were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies:

Stress testing under neutral conditions was studied by refluxing the drug in water for 1hrs at a temperature of 60º. For HPLC study, the resultant solution was diluted to 200µg/mL & 300 µg/mL solution and 10 µL were injected into the system and the chromatograms were recorded to assess the stability of the sample**.**

6. RESULTS AND DISCUSSION

Optimized wavelength selected was 260nm.

Method development: Method development was done by changing various, mobile phase ratios, buffers etc.

Trial 1:

Chromatographic conditions:

Mobile phase	**:**	Acetonitrile and Water taken in the ratio 50:50
Flow rate	**:**	1 mL/min
Column	**:**	Kromasil C18 C18 (4.6 x 150mm, 2.7µm)
Detector wave length	**:**	260 nm
Column temperature	**:**	30°C
Injection volume	**:**	10µL
Run time	**:**	5 min
Diluent	**:**	Water and Acetonitrile in the ratio 50:50

Result:

In this trail both peaks were eluted but broad peak shape was observed for both drug peaks. So, further trial is carried out.

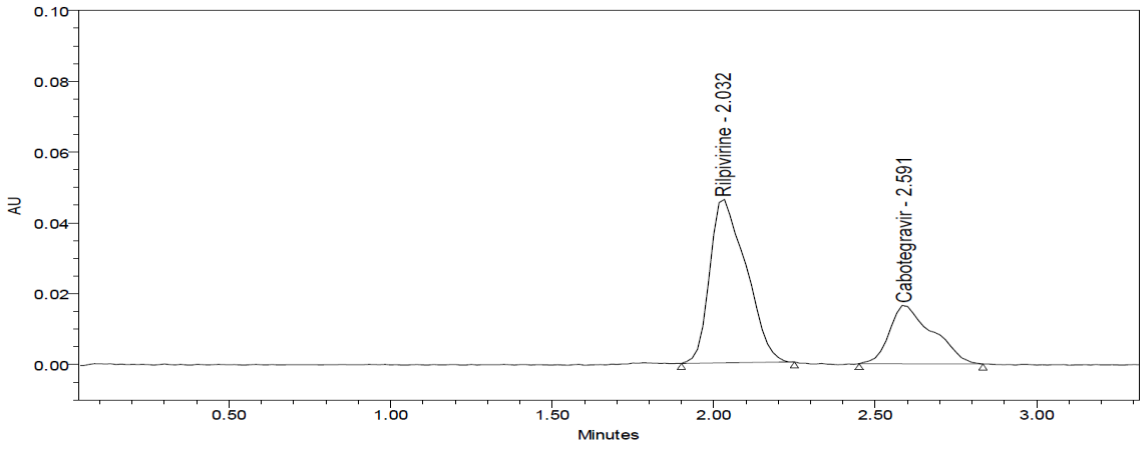


Fig 6.1 Trial chromatogram 1

Trial 2:

Chromatographic conditions:

Mobile phase	:	Acetonitrile: 0.01N OPA (50:50)
Flow rate	:	1 mL/min
Column	:	Kromasil C18 (4.6 x 150mm, 2.7µm)
Detector wave length	:	260 nm
Column temperature	:	30°C
Injection volume	:	10 µL
Run time	:	10 min
Diluent	:	Water and Acetonitrile in the ratio (50:50)

Result:

In this trail by changing mobile phase both peaks were eluted but Less USP resolution (<2) is observed between both drug peaks. So, further trial is carried out.

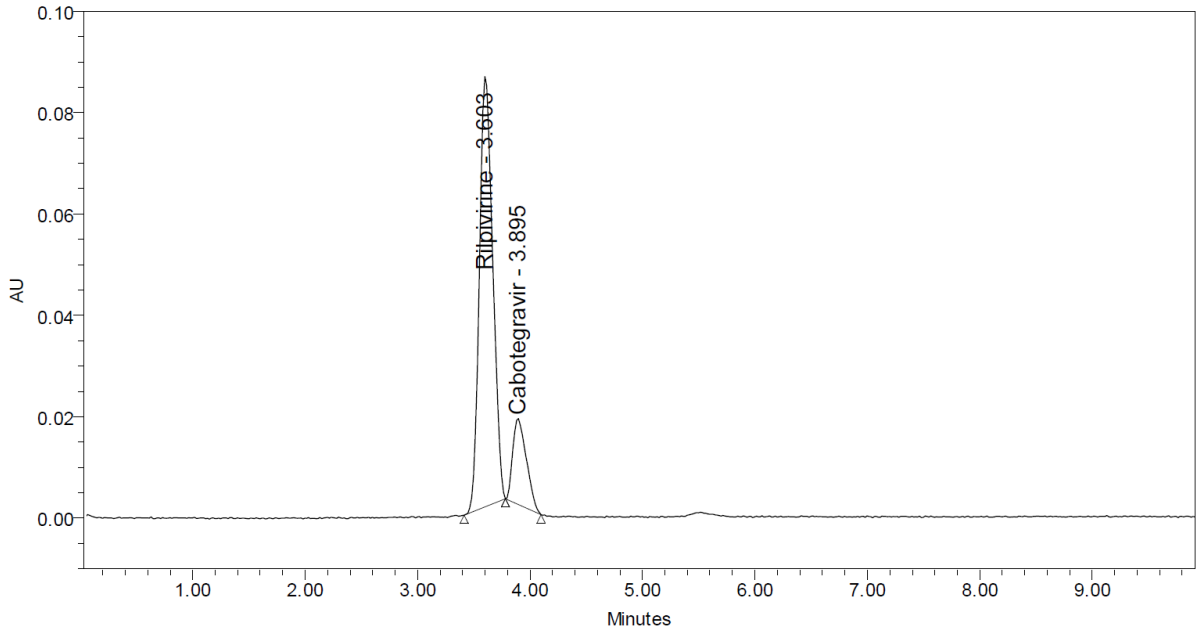


Fig 6.2 Trial chromatogram 2

Trial 3:

Chromatographic conditions:

Mobile phase	**:**	Acetonitrile: 0.1% OPA (60:40)
Flow rate	**:**	1 mL/min
Column	**:**	Kromasil C18 (4.6 x 150mm, 5µm)
Detector wave length	**:**	260 nm
Column temperature	**:**	30°C
Injection volume	**:**	10 µL
Run time	**:**	10 min
Diluent	**:**	Water and Acetonitrile in the ratio 50:50

Result:

In this trail by changing mobile phase both peaks were eluted but Peak tailing is occurred for Rilpivirine peak and Retention time was in void range. So, further trial is carried out.

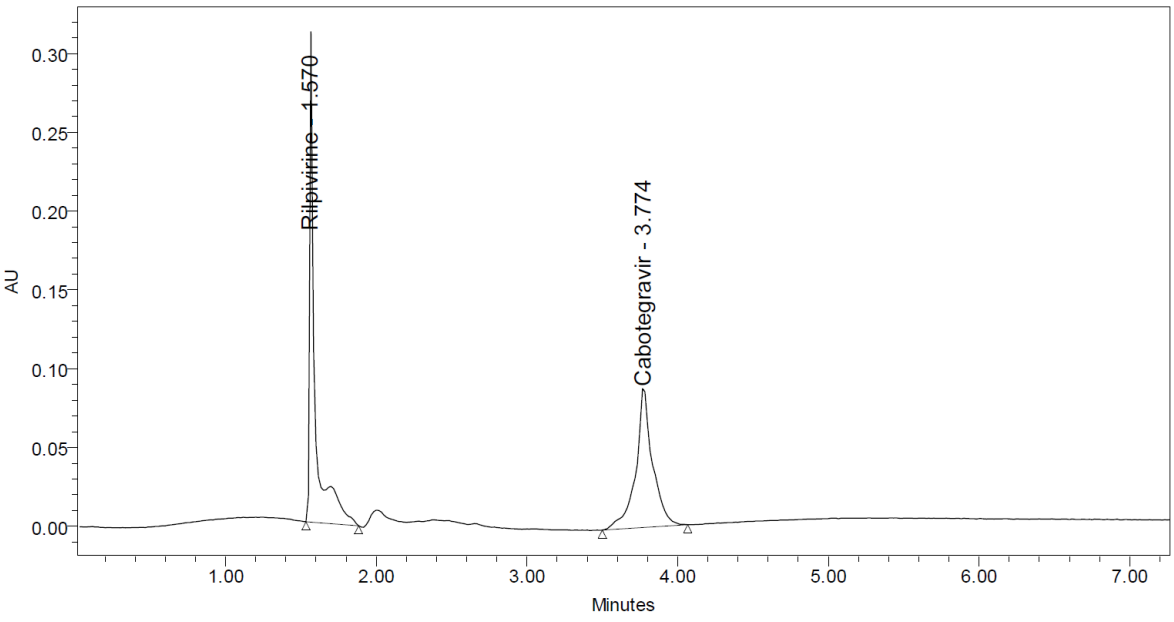


Fig 6.3 Trial chromatogram 3

Trial 4:

Chromatographic conditions:

Mobile phase	**:**	Acetonitrile: 0.1% OPA (50:50)
Flow rate	**:**	1 mL/min
Column	**:**	Agilent C18 (4.6 x 150mm, 5µm)
Detector wave length	**:**	260nm
Column temperature	**:**	30°C
Injection volume	**:**	10µL
Run time	**:**	10 min
Diluent	**:**	Water and Acetonitrile in the ratio 50:50

Result:

In this trail by changing column both peaks were eluted but Rilpivirine peak was eluted in voided range (<2mins) and peak splitting is observed for Cabotegravir peak. So, further trial is carried out.

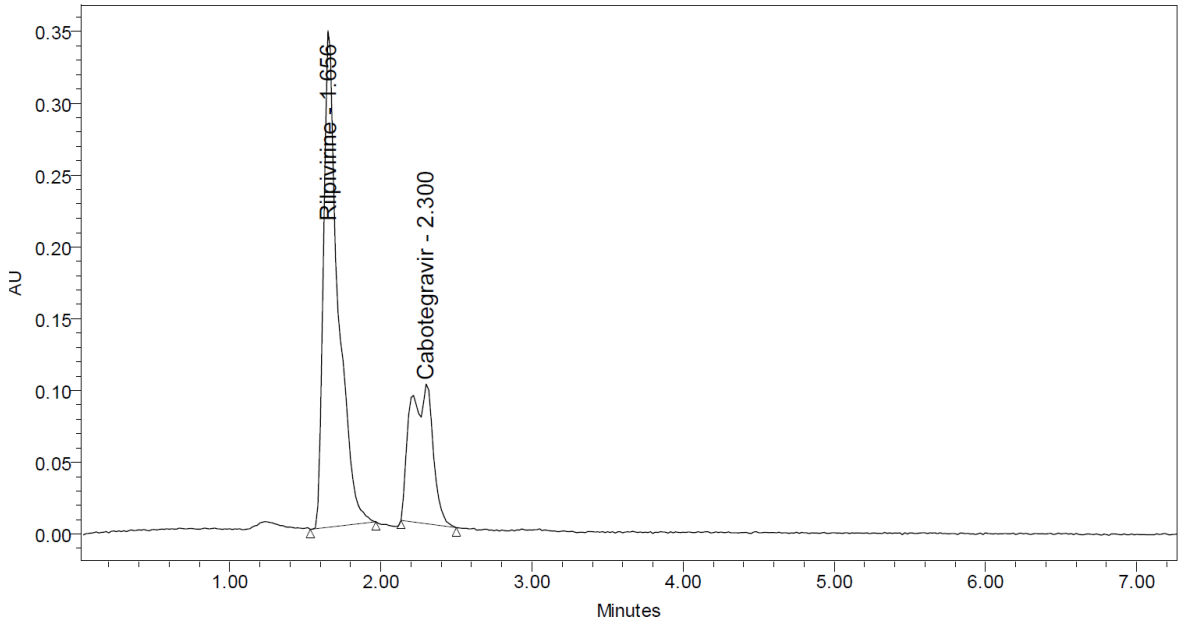


Fig. No. 6.4 Trial chromatogram

Optimized method:

Chromatographic conditions:

Mobile phase	**:**	**0.**1% OPA : Acetonitrile(60:40)
Flow rate	**:**	**1** ml/min
Column	**:**	Denali C18 (4.6 x 150mm, 5µm)
Detector wave length	**:**	260nm
Column temperature	**:**	30°C
Injection volume	**:**	10µL
Run time	**:**	10 min
Diluent	**:**	Water and Acetonitrile in the ratio 50:50

Result:

Both peaks have good resolution, tailing factor, theoretical plate count and resolution.

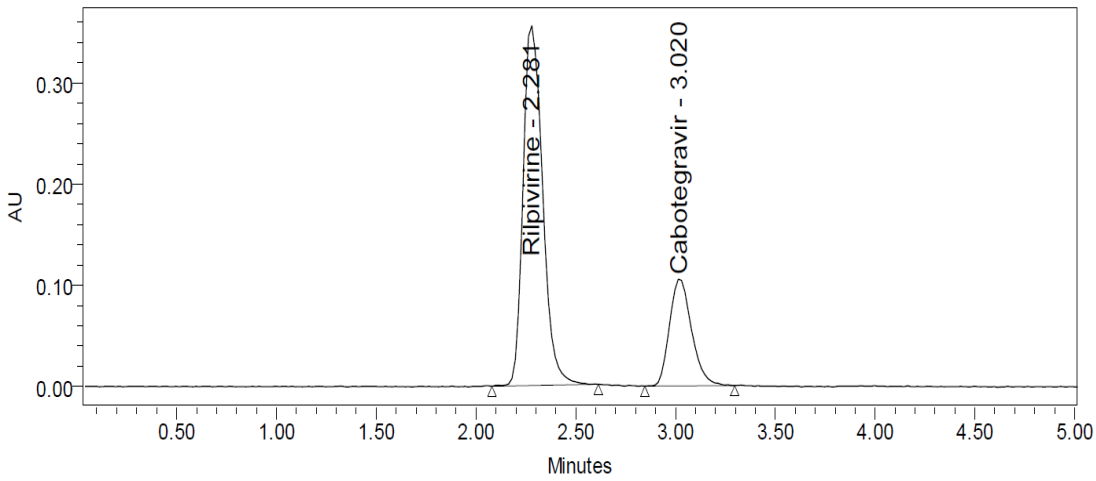


Fig 6.5 Optimized Chromatogram

Observation:

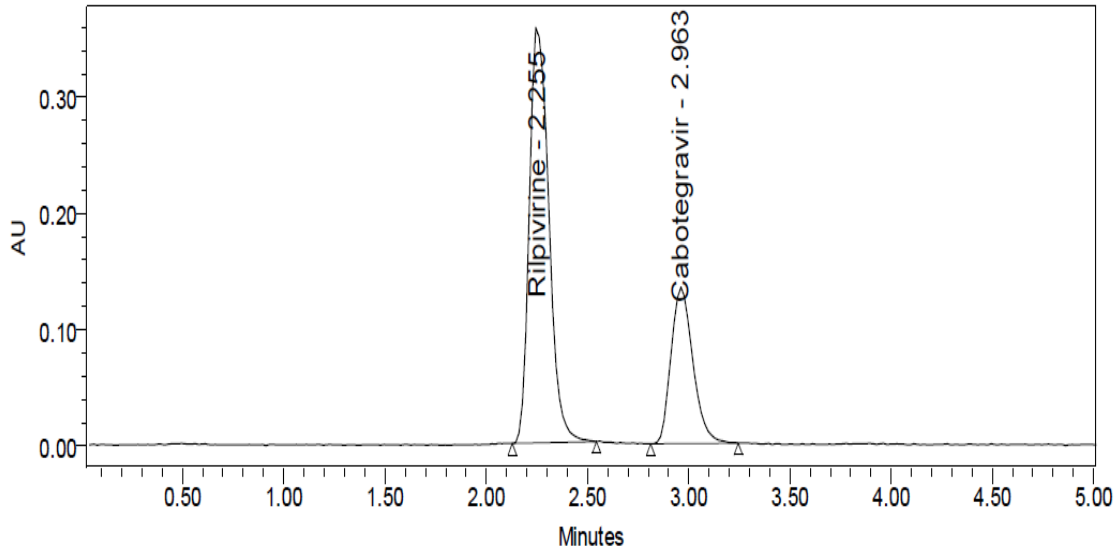
Rilpivirine and Cabotegravir were eluted at 2.260 min and 2.974 min respectively with good resolution. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated.

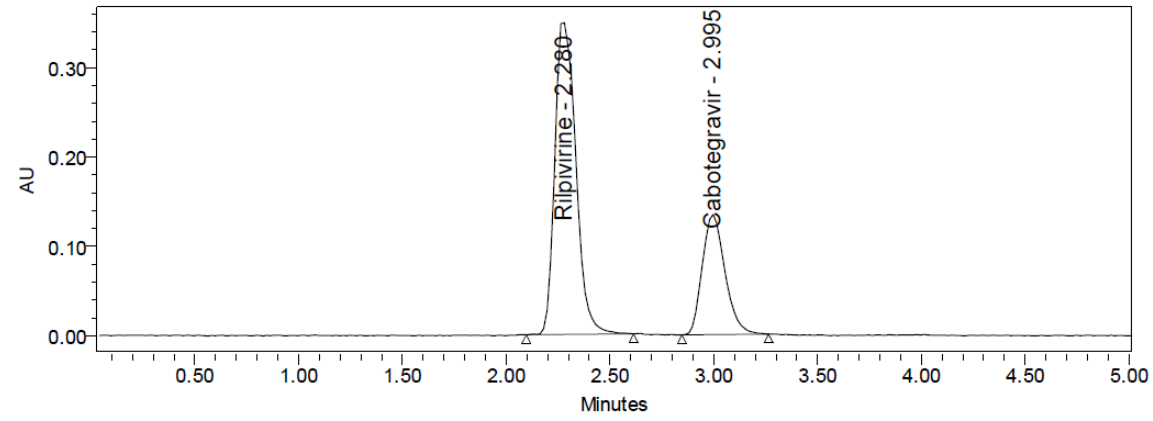
SYSTEM SUITABILITY:

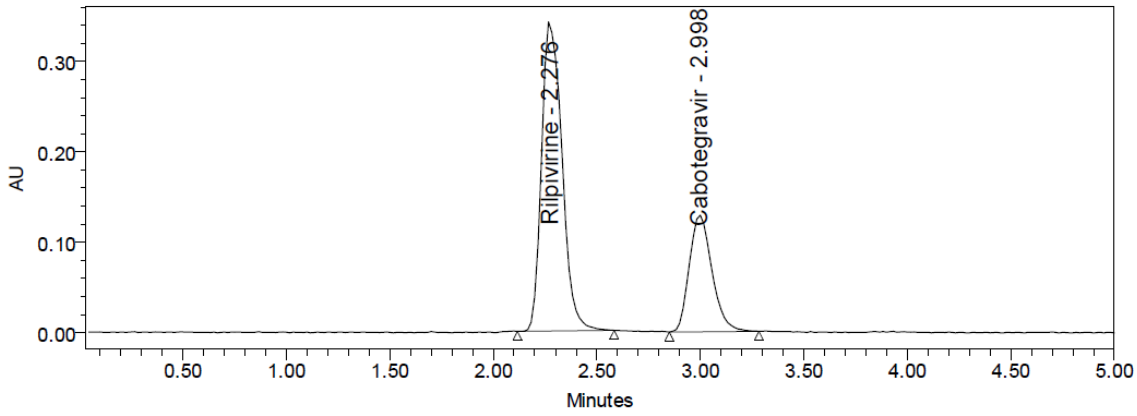
All the system suitability parameters were within the range and satisfactory as per ICH guidelines

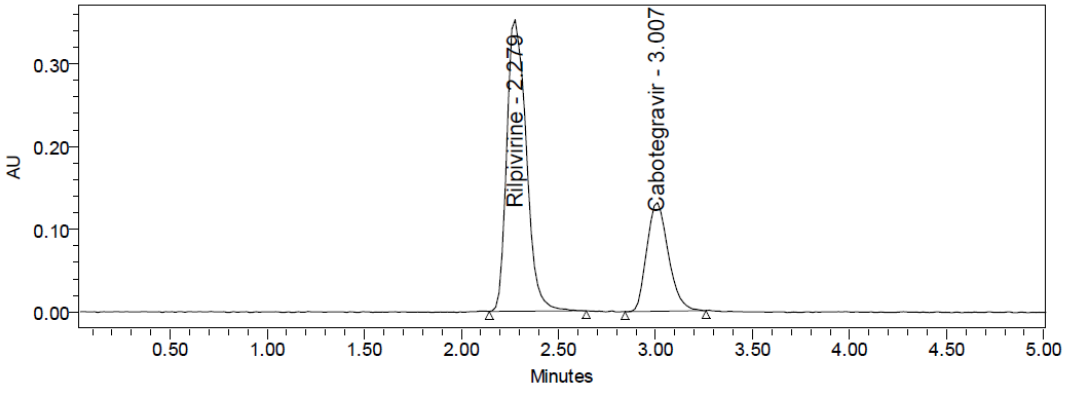
Table: 6.1 System suitability parameters for Rilpivirine and Cabotegravir

S no	Rilpivirine			Cabotegravir			
Inj	RT(min)	USP Plate Count	Tailing	RT(min)	USP Plate Count	Tailing	RS
1	2.255	2691	1.27	2.963	3438	1.25	3.8
2	2.274	2650	1.27	2.995	3462	1.23	3.8
3	2.276	2619	1.26	2.998	3488	1.23	3.8
4	2.279	2733	1.26	3.003	3419	1.23	3.8
5	2.279	2631	1.26	3.006	3579	1.22	3.8
6	2.280	2637	1.26	3.007	3454	1.21	3.8




Rilpivirine - 2.280
Cabotegravir - 2.995
AU
Minutes
0.00
0.10
0.20
0.30
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00


Rilpivirine - 2.276
Cabotegravir - 2.998
AU
Minutes
0.00
0.10
0.20
0.30
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00


Rilpivirine - 2.279
Cabotegravir - 3.007
AU
Minutes
0.00
0.10
0.20
0.30
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00

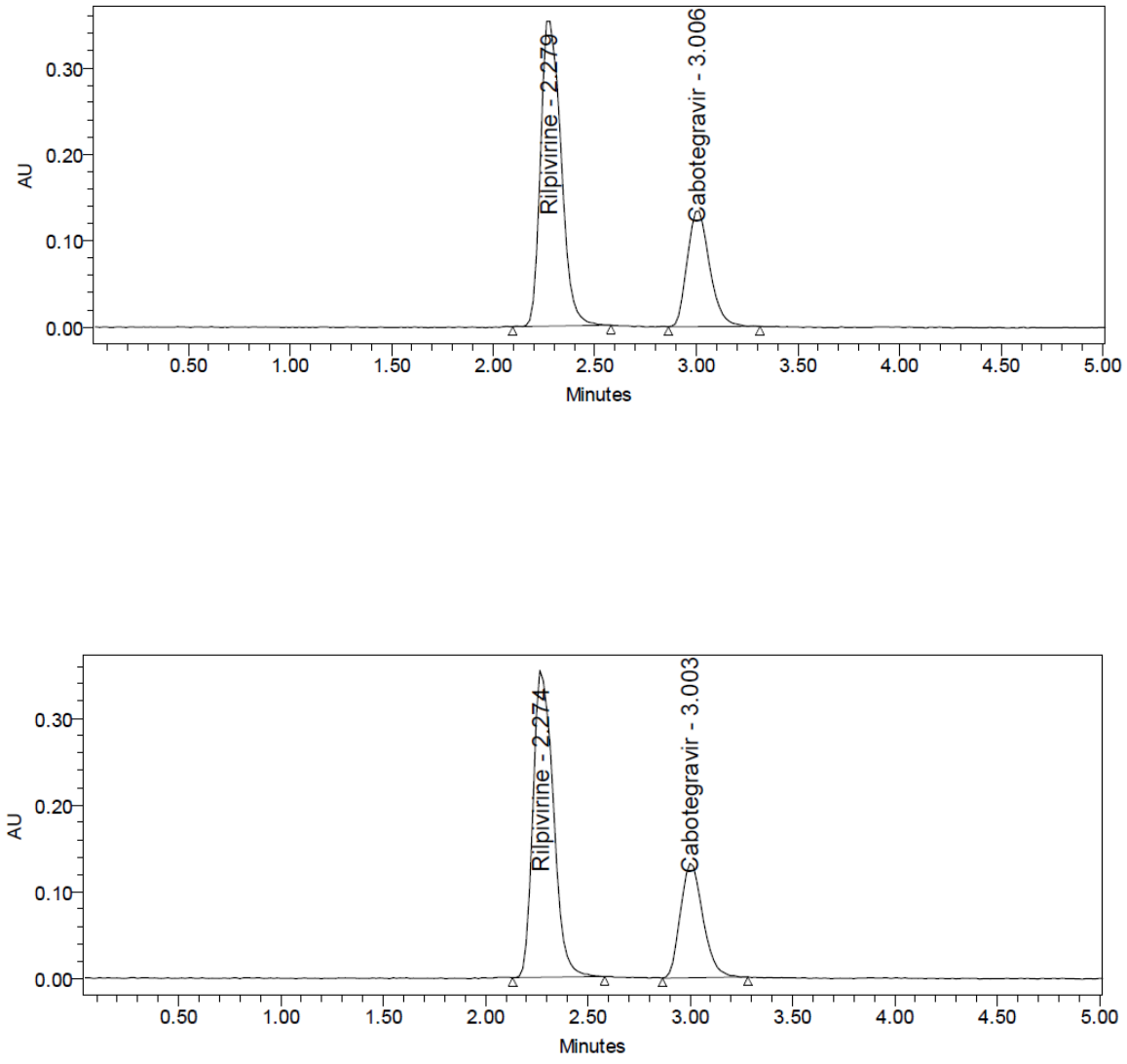


Fig 6.6 System suitability Chromatogram

Discussion:

According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits.

VALIDATION:

Specificity:

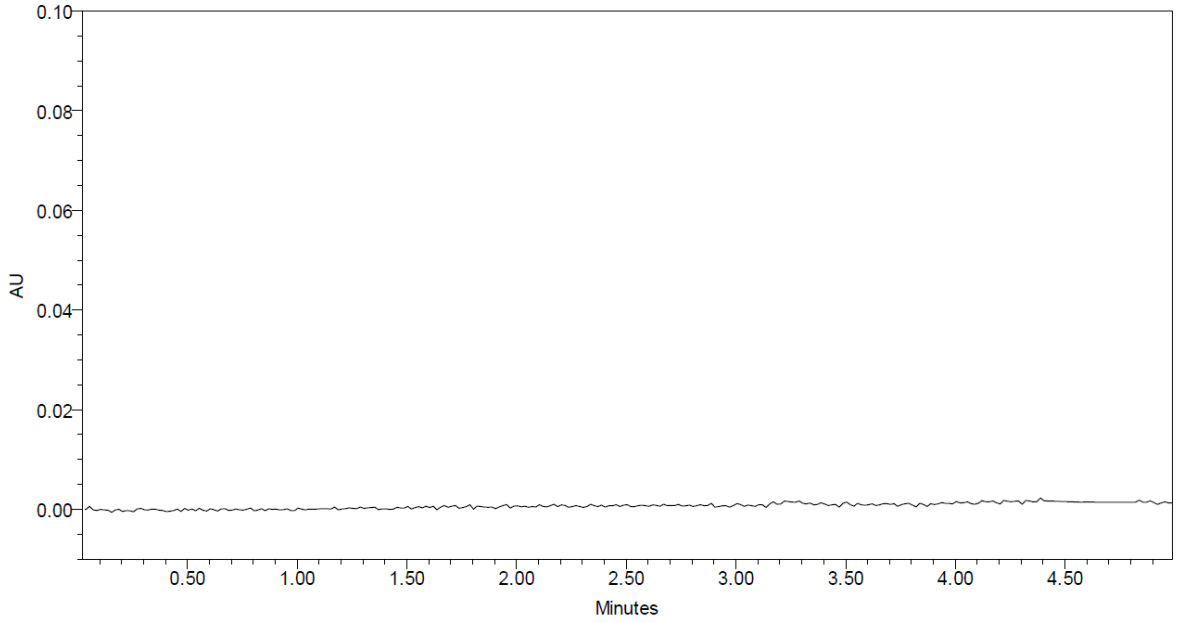


Figure No. 6.7. Chromatogram of blank.

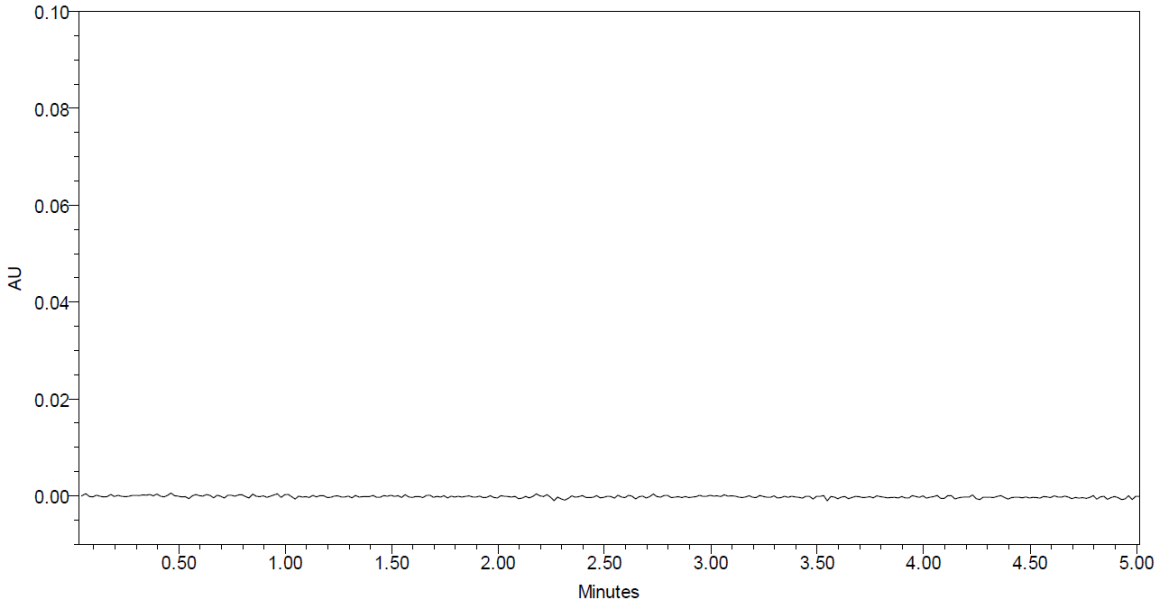


Figure No. 6.8 Chromatogram of placebo

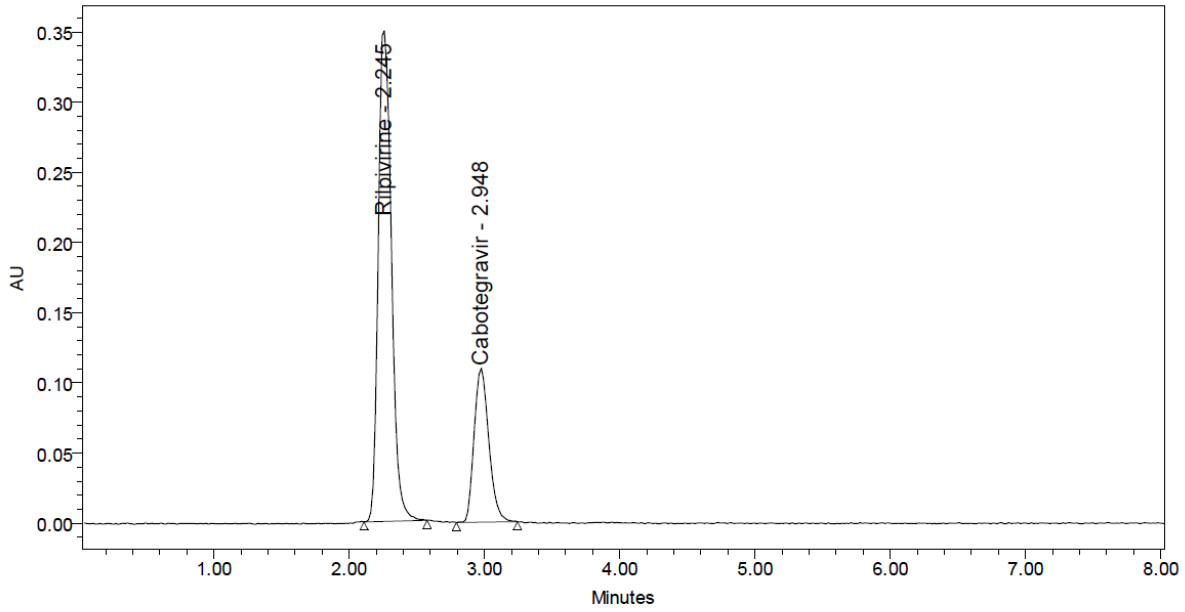


Fig 6.9 Typical Chromatogram

Discussion:

Retention times of Rilpivirine and Cabotegravir were 2.245 min and 2.948 min respectively. We did not found and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

LINEARITY:

Table 6.2 Linearity table for Cabotegravir and Rilpivirine

Cabotegravir		Rilpivirine	
Conc (µg/mL)	**Peak area**	**Conc (µg/mL)**	**Peak area**
0	0	0	0
50	204596	75	535285
100	425302	150	1116064
150	623343	225	1587753
200	827172	300	2133880
250	1034525	375	2647492
300	1225622	450	3123149

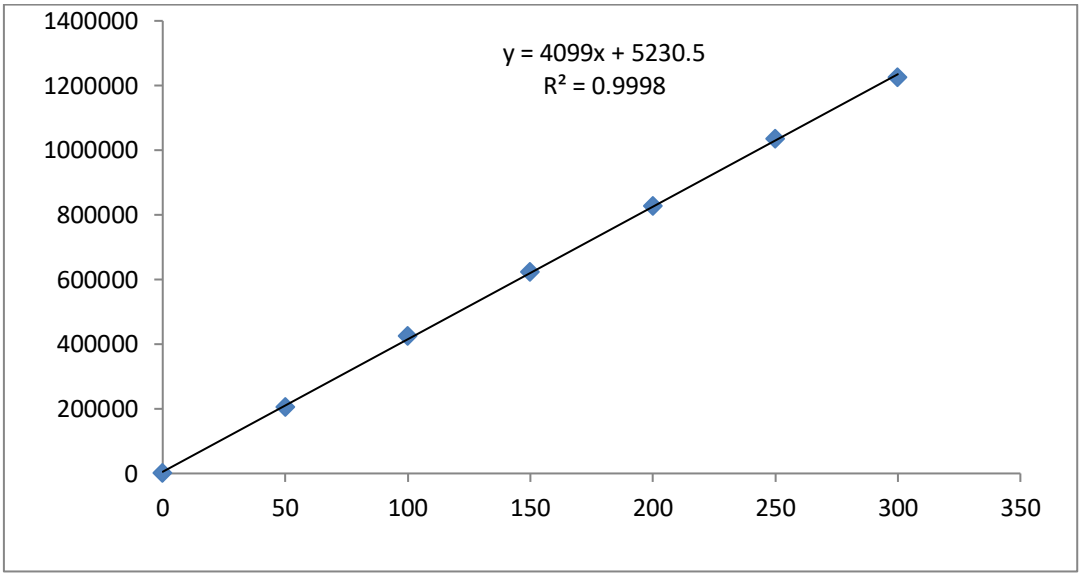


Fig No. 6.10 Calibration curve of Cabotegravir

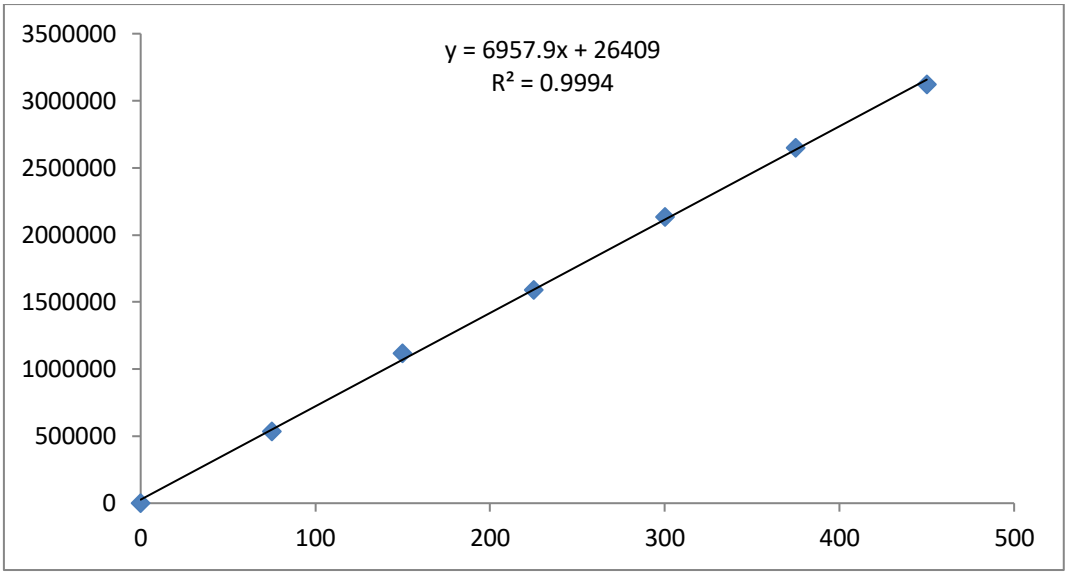


Fig No. 6.11 Calibration curve of Rilpivirine

Discussion:

Six linear concentrations of Cabotegravir (50-300 µg/mL) and Rilpivirine (75-450 µg/mL) were injected in a duplicate manner. Average areas were mentioned above and linearity equations obtained for Cabotegravir was y = 4099x + 5230 and of Rilpivirine was y = 6957x + 26409. Correlation coefficient obtained was 0.999 for the two drugs.

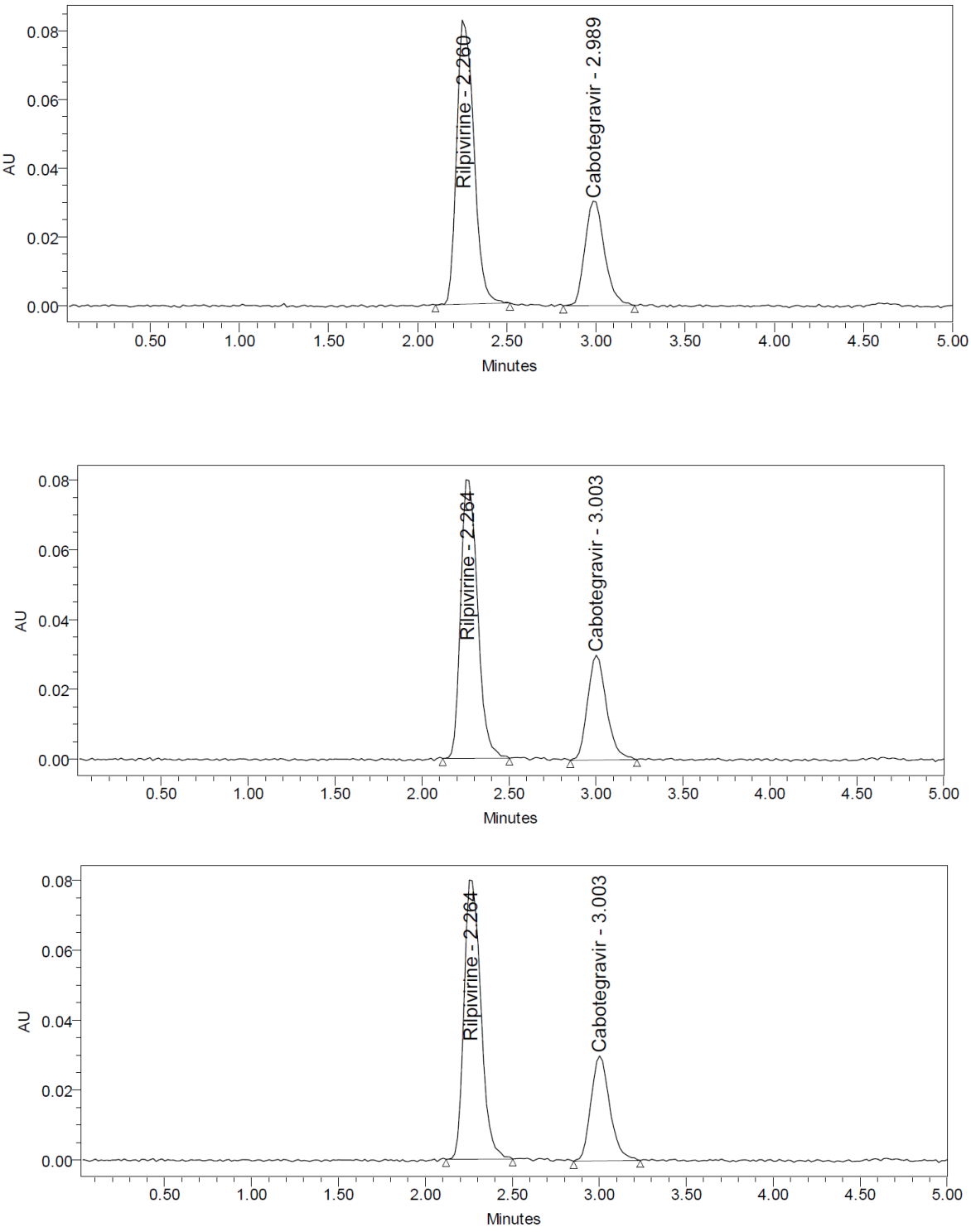


Fig. No. 6.12 Linearity 25% Chromatogram of Cabotegravir and Rilpivirine

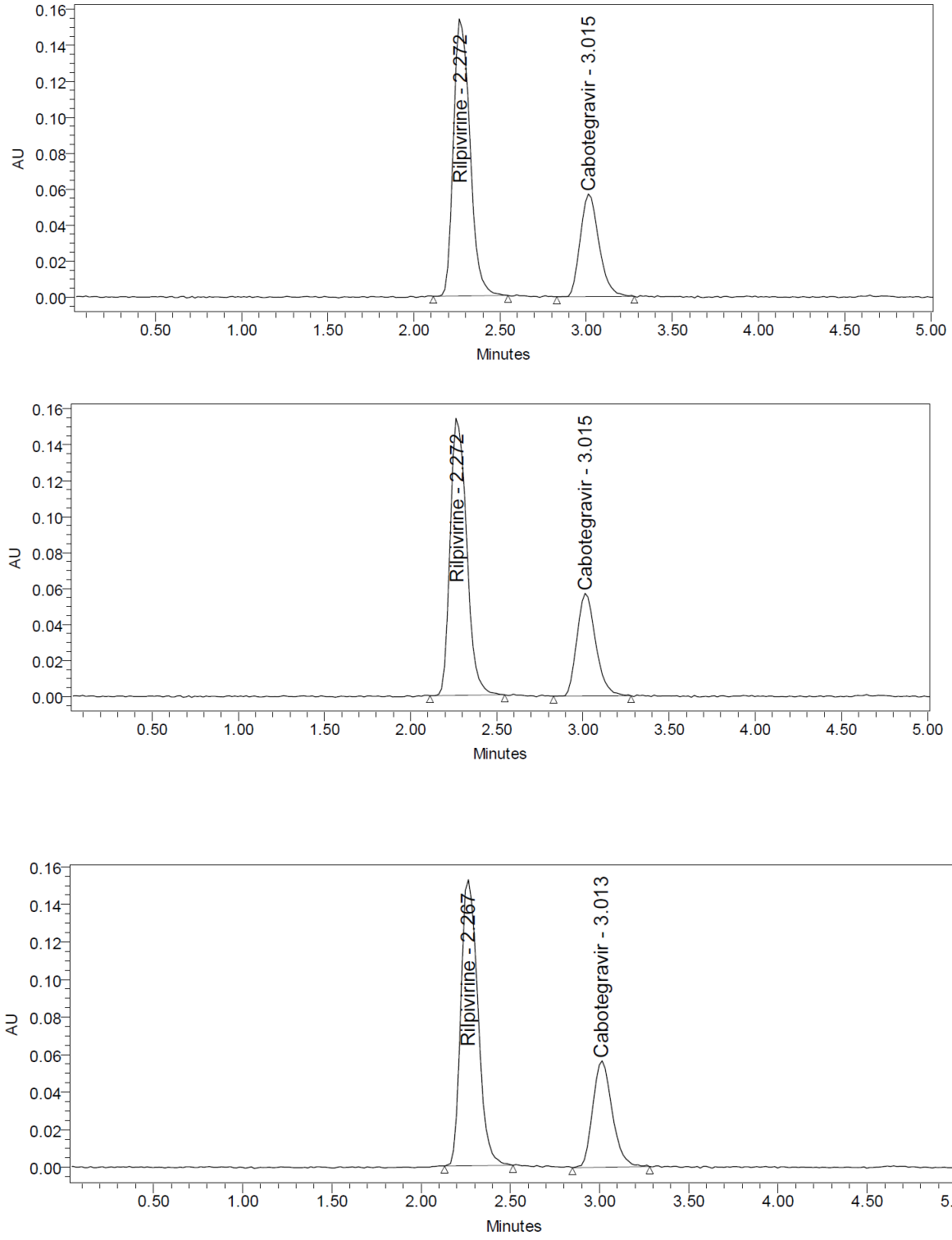


Fig No. 6.13 Linearity 50% Chromatogram of Cabotegravir and Rilpivirine

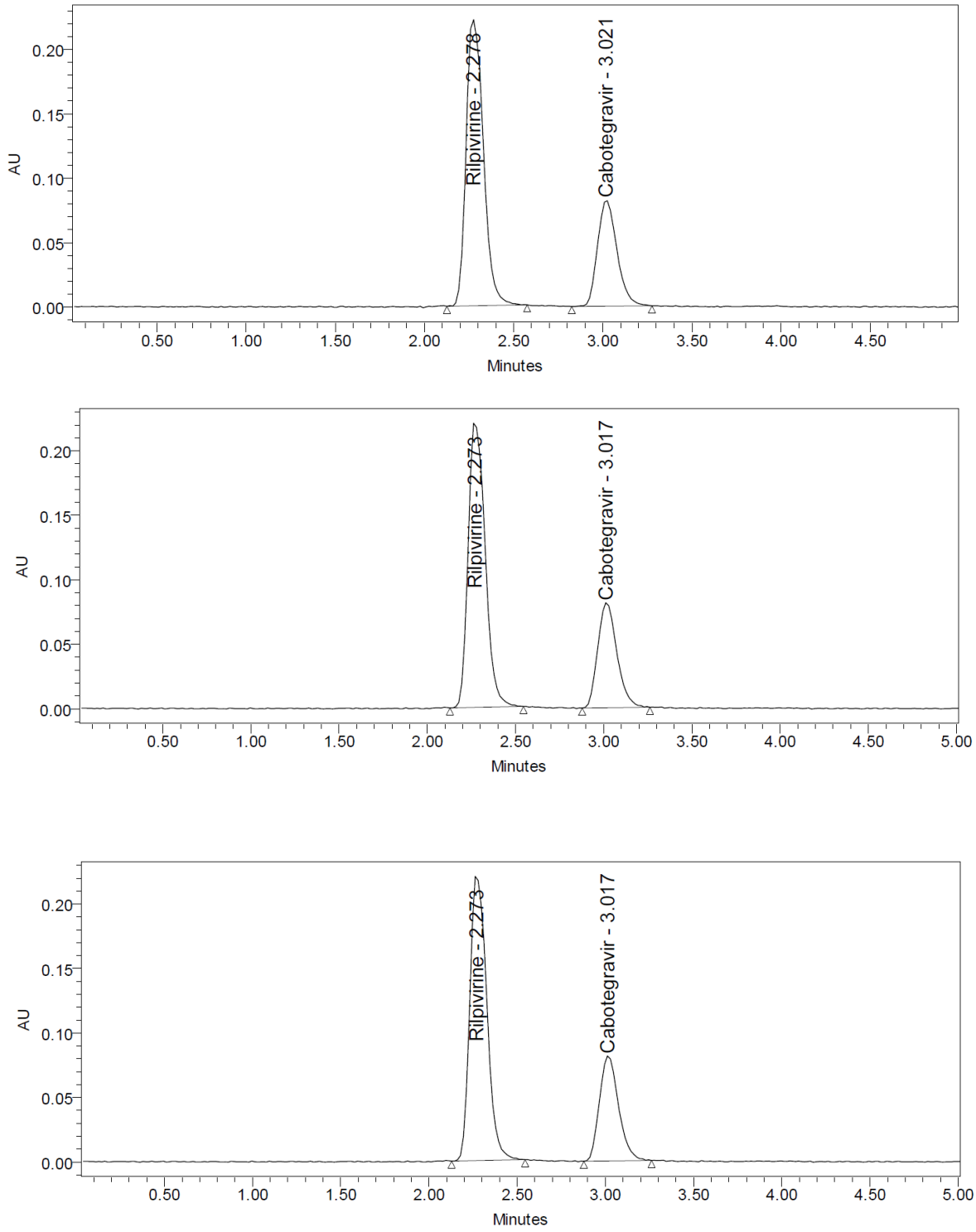


Fig No. 6.14 Linearity 75% Chromatogram of Cabotegravir and Rilpivirine

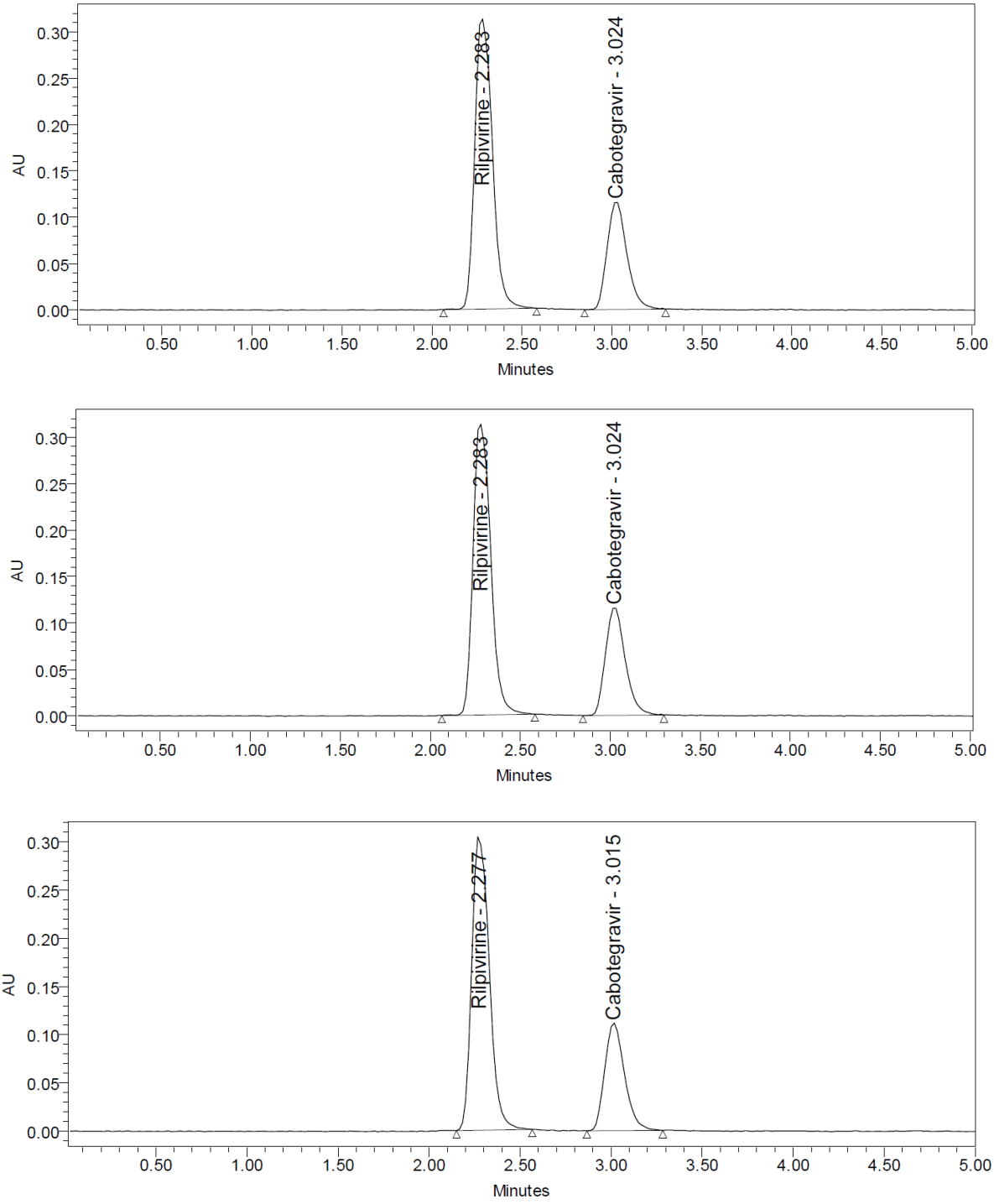


Fig No. 6.15 Linearity 100% Chromatogram of Cabotegravir and Rilpivirine

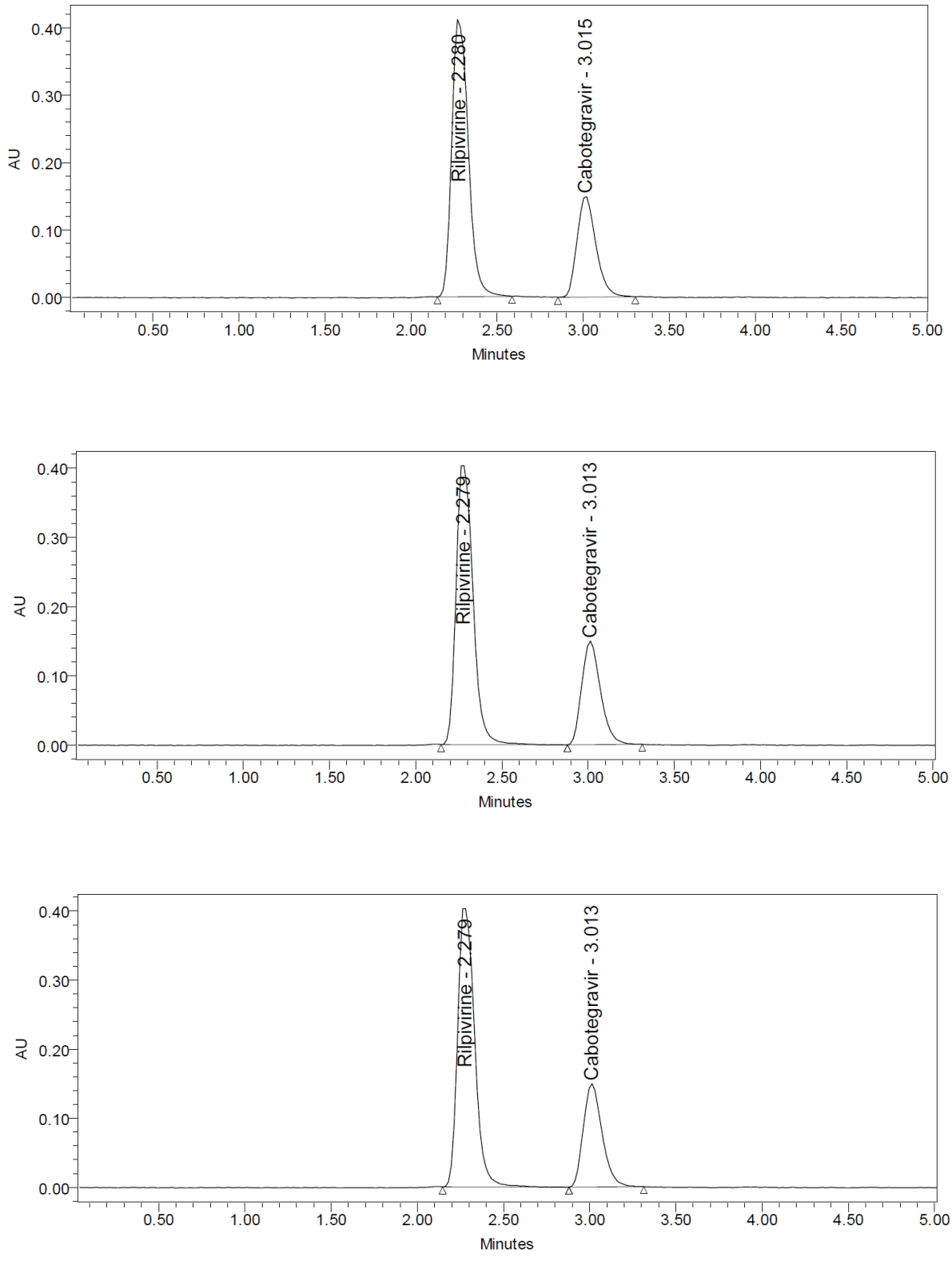


Fig No. 6.16 Linearity 125% Chromatogram of Cabotegravir and Rilpivirine

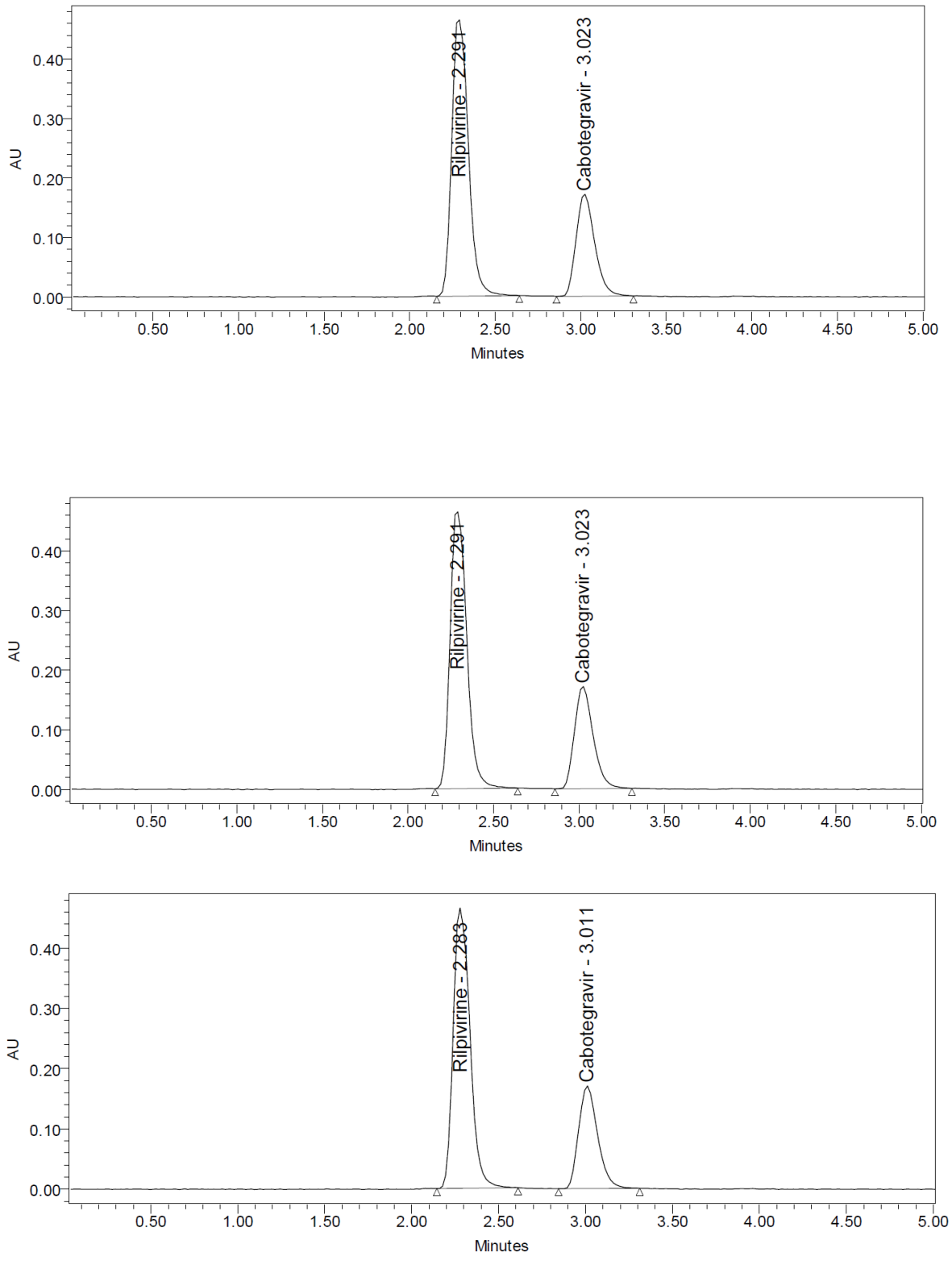


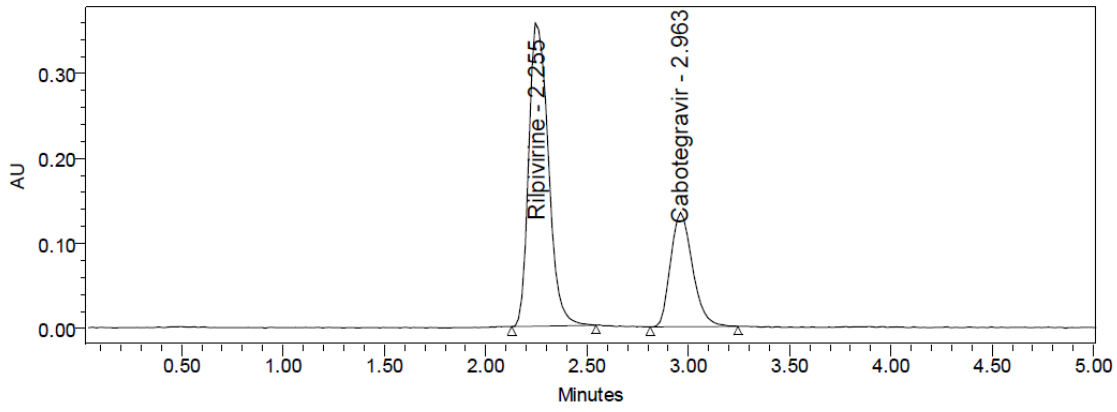
Fig No. 6.17 Linearity 150% Chromatogram of Cabotegravir and Rilpivirine

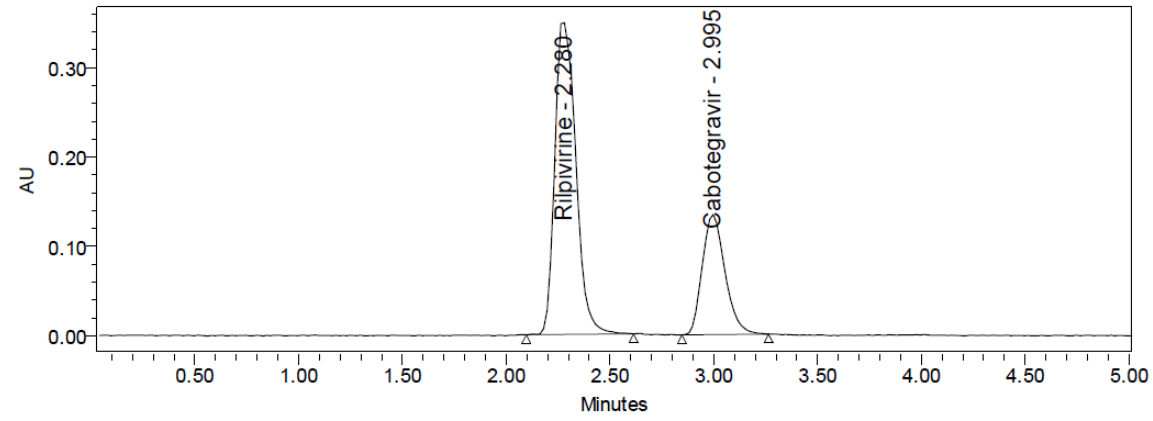
PRECISION:

System Precision:

Table 6.3 System precision table of Cabotegravir and Rilpivirine

S. No	Area of Cabotegravir	Area of Rilpivirine
1.	821182	2122901
2.	823203	2137480
3.	837264	2141383
4.	821751	2106471
5.	835180	2121350
6.	829485	2138947
Mean	828011	2128089
S.D	7044.5	13583.0
%RSD	0.9	0.6




Rilpivirine - 2.280
Cabotegravir - 2.995
AU
Minutes
0.00
0.10
0.20
0.30
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
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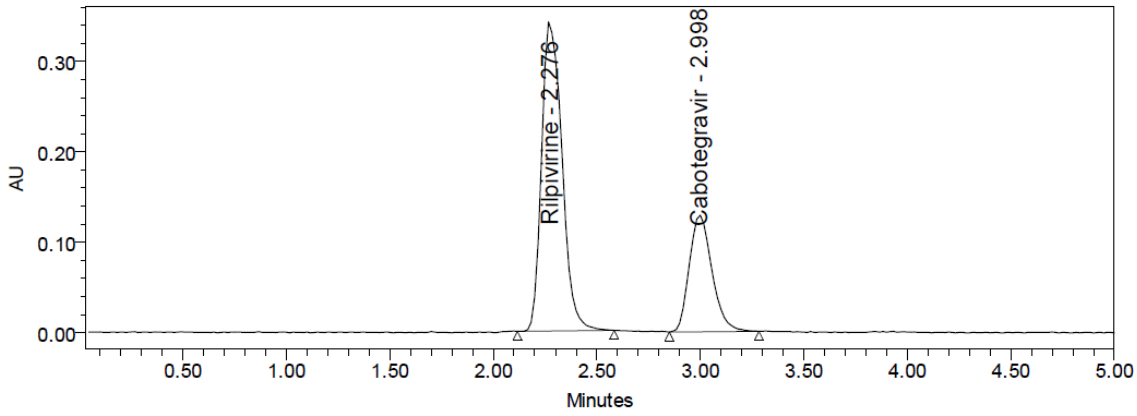
5.00


Rilpivirine - 2.276
Cabotegravir - 2.998
AU
Minutes
0.00
0.10
0.20
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0.50
1.00
1.50
2.00
2.50
3.00
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4.00
4.50
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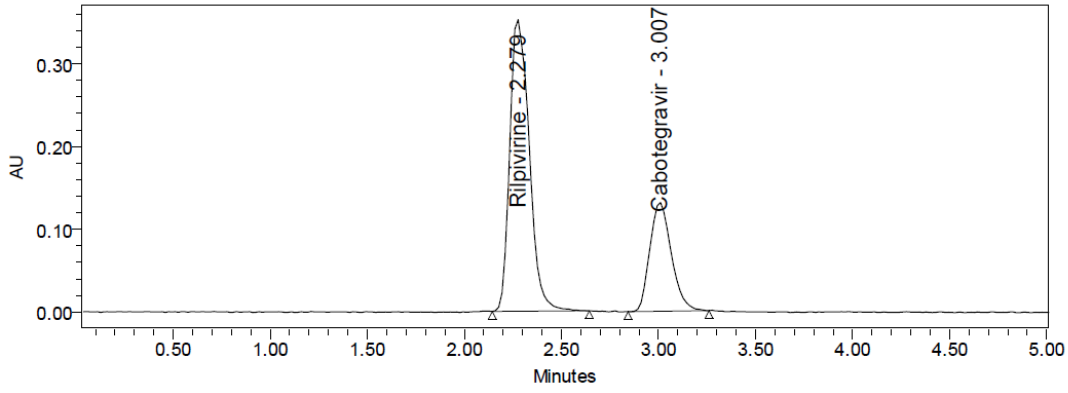
Rilpivirine - 2.279
Cabotegravir - 3.007
AU
Minutes
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0.10
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1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00

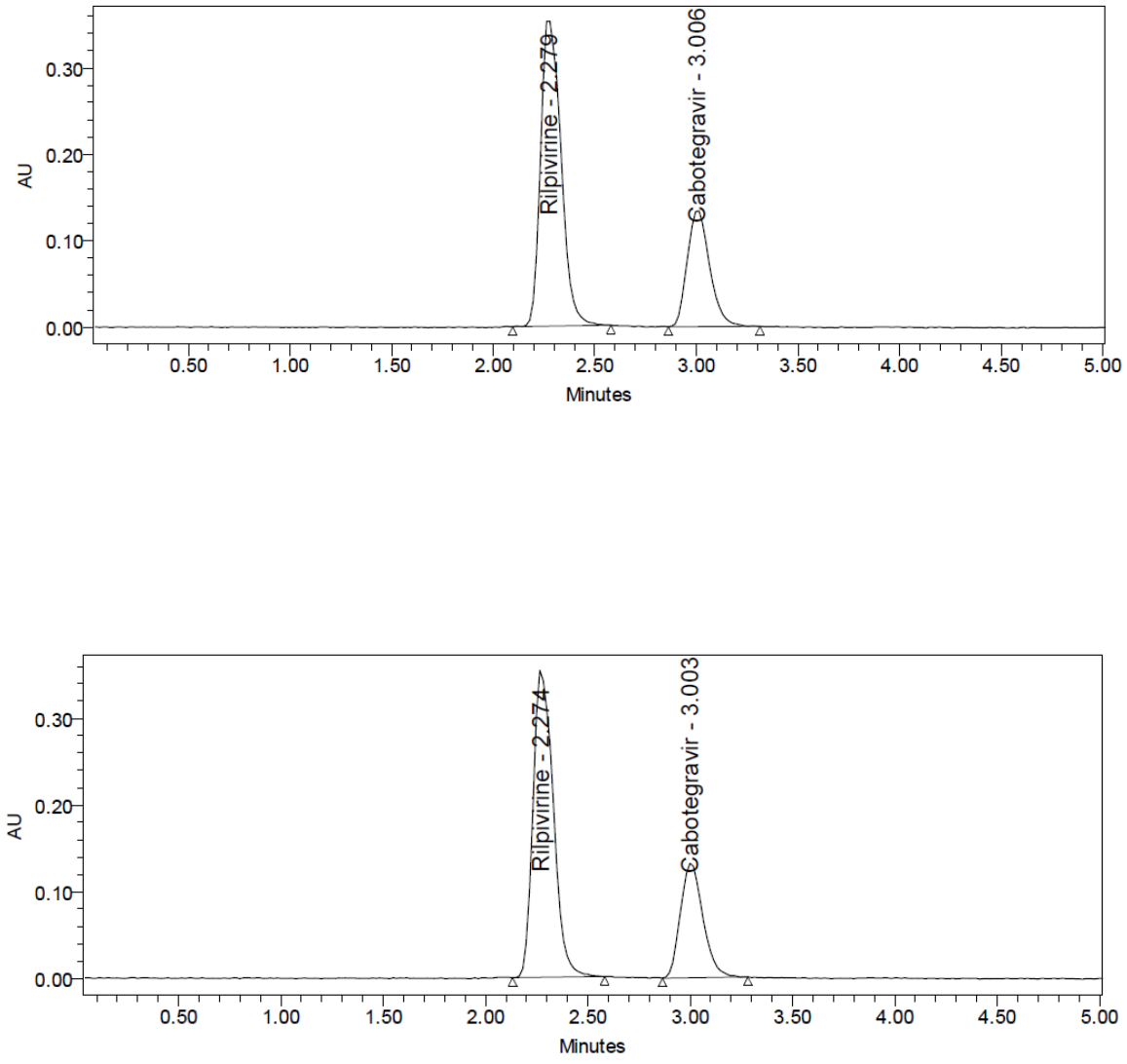


Fig 6.18 System precision chromatogram

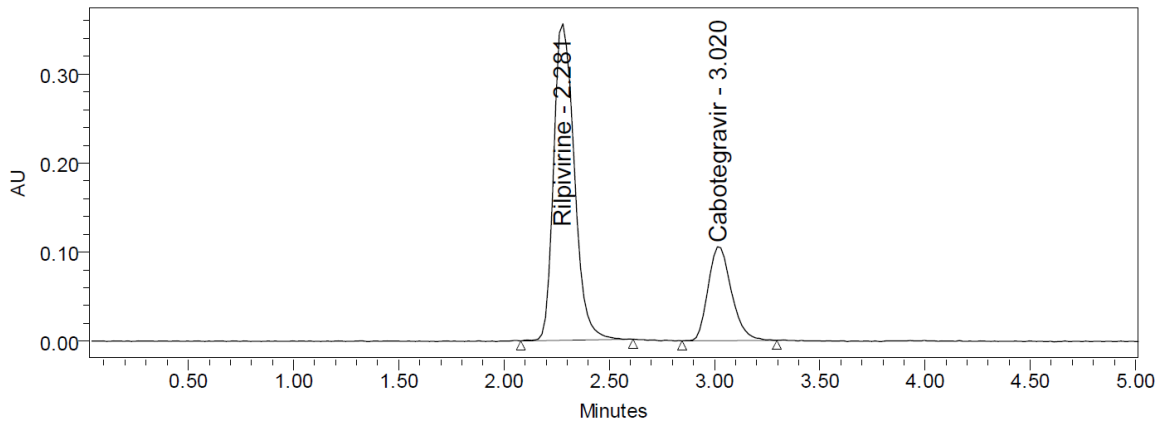
Discussion:

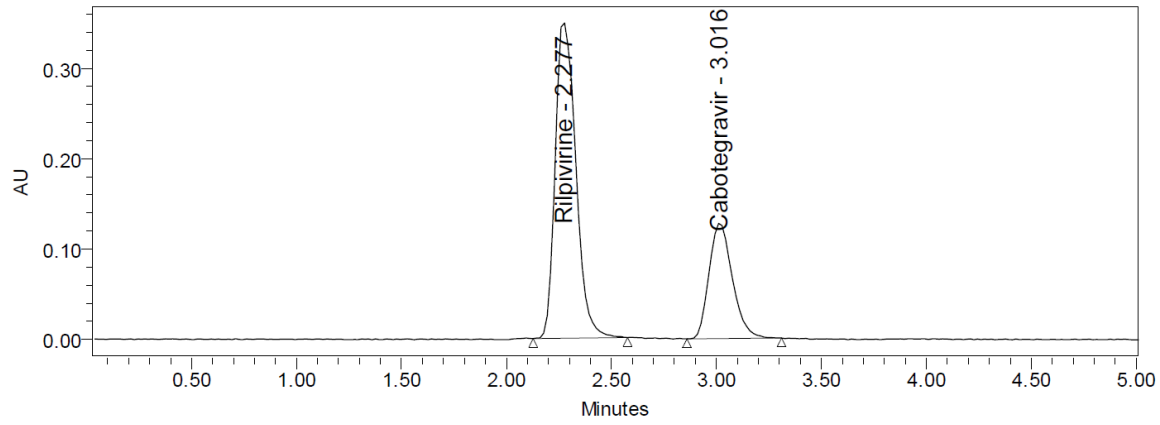
From a single volumetric flask of working standard solution six injections were given and the obtained areas were mentioned above. Average area, standard deviation and % RSD were calculated for two drugs. % RSD obtained as 0.9% and 0.6% respectively for Cabotegravir and Rilpivirine .As the limit of Precision was less than “2” the system precision was passed in this method.

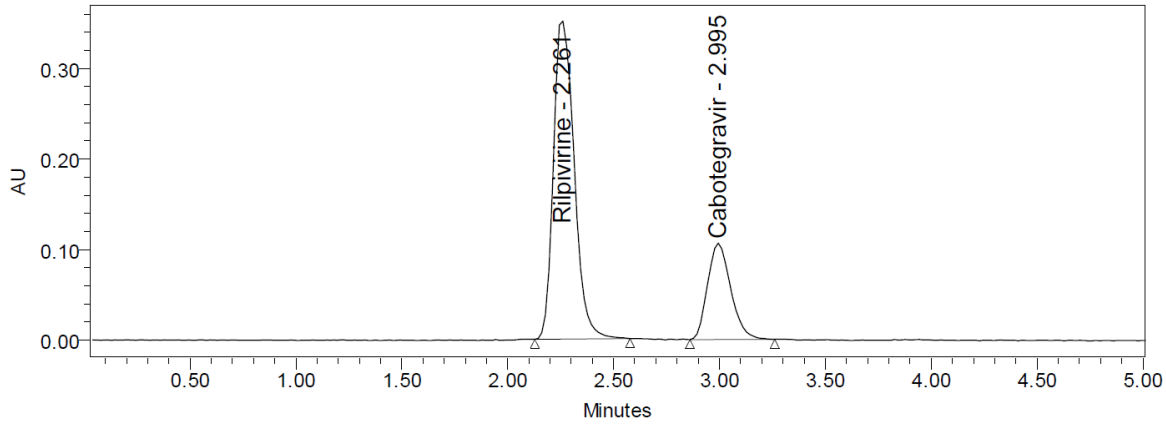
REPEATABILITY:

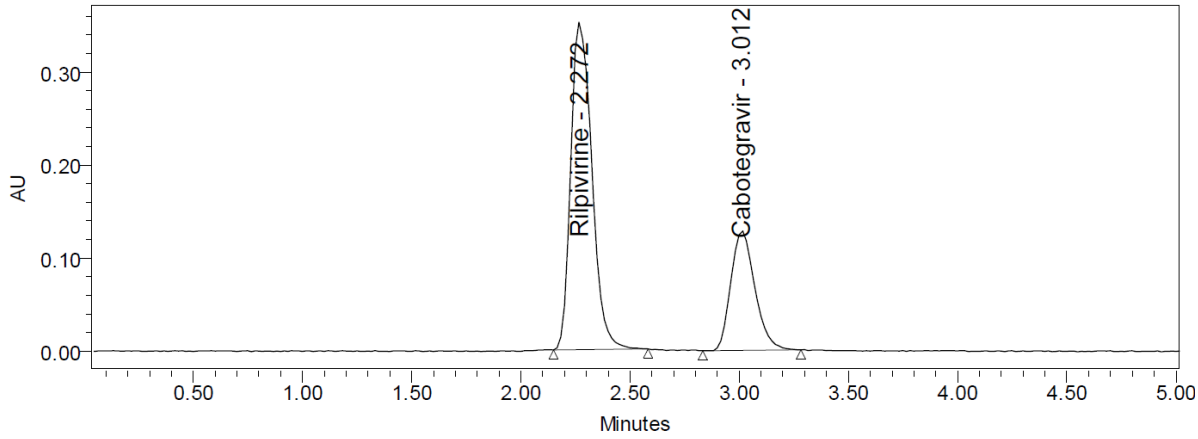
Table 6.4 Repeatability table of Cabotegravir and Rilpivirine

S. No	Area of Cabotegravir	Area of Rilpivirine
1.	832177	2129808
2.	830082	2140536
3.	827638	2142313
4.	833713	2131561
5.	831500	2145571
6.	839076	2157859
Mean	832364	2141275
S.D	3878.0	10207.5
%RSD	0.5	0.5




Rilpivirine - 2.277
Cabotegravir - 3.016
AU
Minutes
0.00
0.10
0.20
0.30
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00


Rilpivirine - 2.261
Cabotegravir - 2.995
AU
Minutes
0.00
0.10
0.20
0.30
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00


Rilpivirine - 2.272
Cabotegravir - 3.012
AU
Minutes
0.00
0.10
0.20
0.30
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00

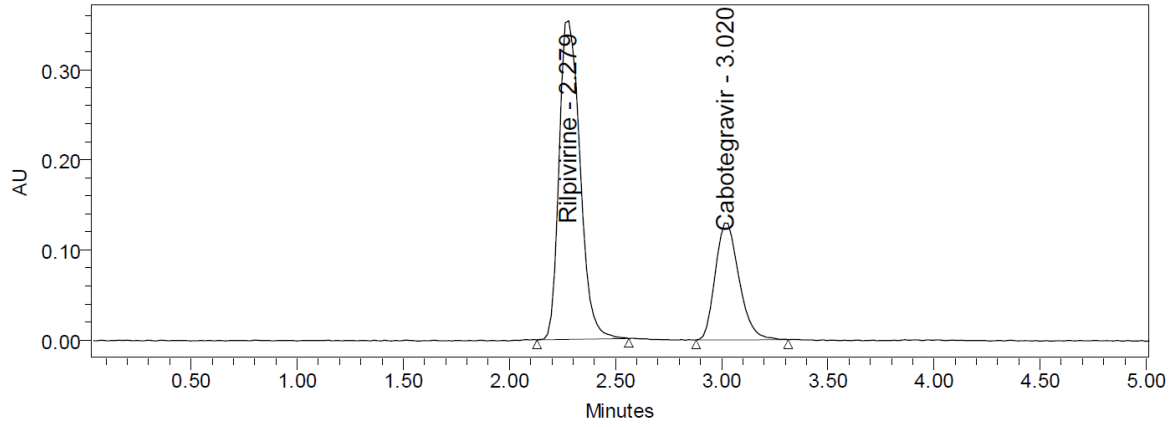


Fig No. 6.19 Repeatability chromatogram

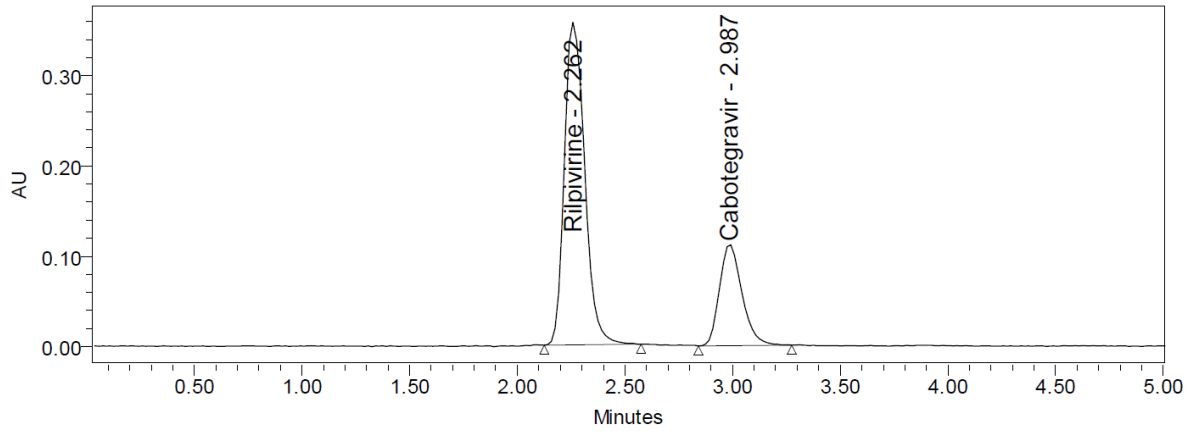
Discussion:

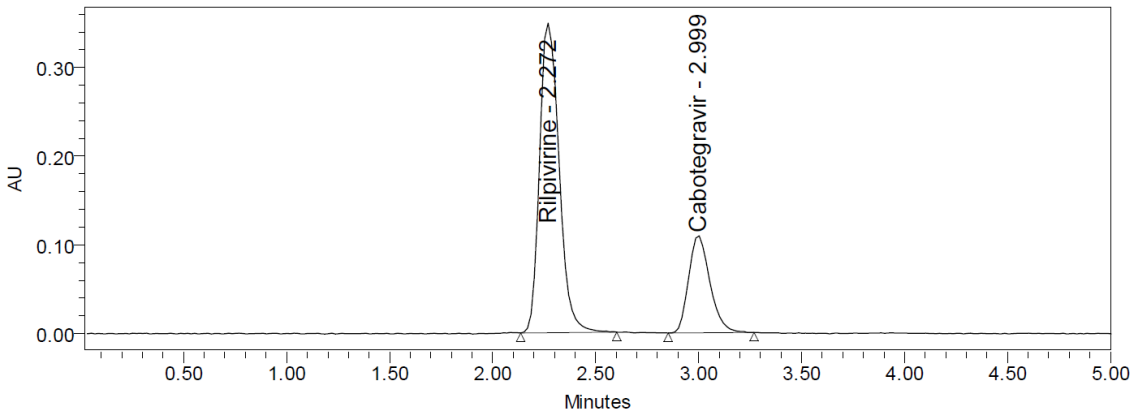
Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated for two drugs and obtained as 0.5% and 0.5% respectively for Cabotegravir and Rilpivirine. As the limit of Precision was less than "2" the system precision was passed in this method.

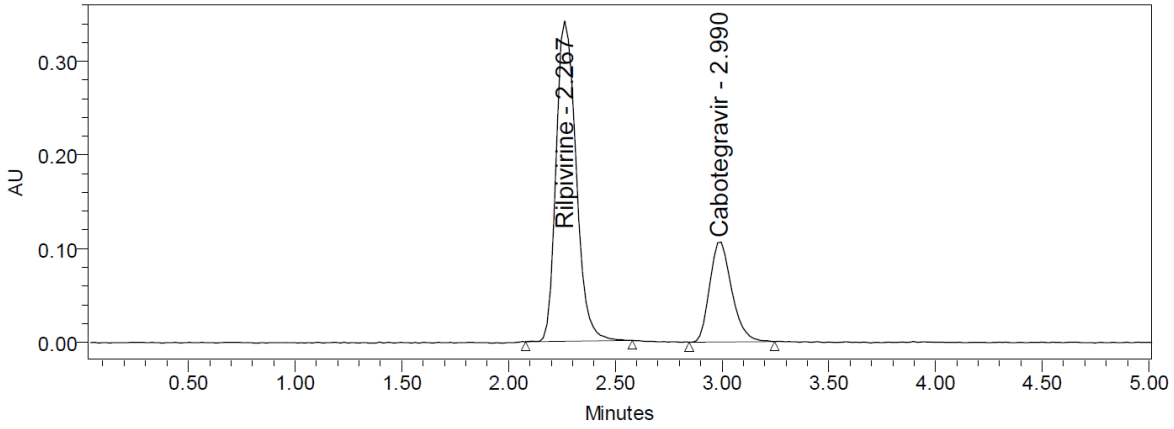
INTERMEDIATE PRECISION (DAY- DAY PRECISION):

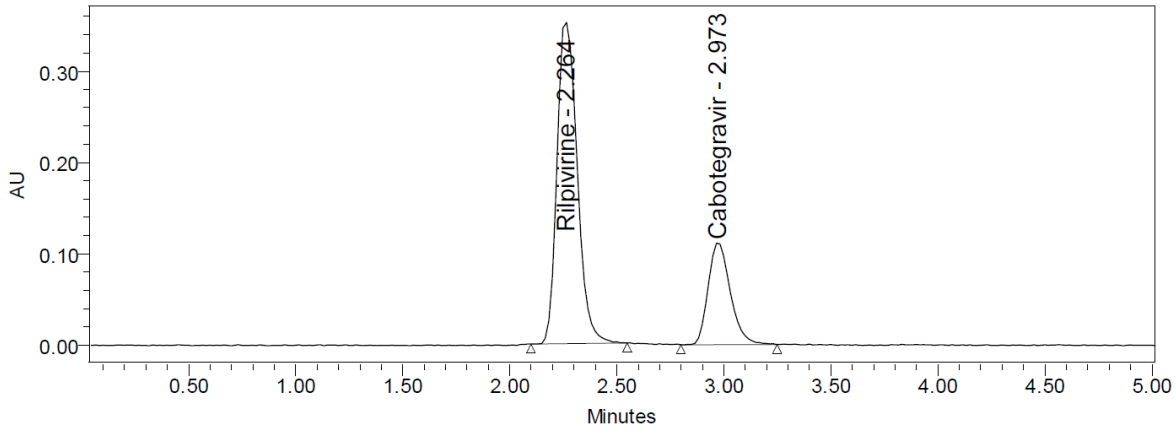
Table 6.5 Intermediate precision table of Cabotegravir and Rilpivirine

S. No	Area of Cabotegravir	Area of Rilpivirine
1.	829805	2144913
2.	823401	2102555
3.	828907	2148670
4.	831824	2137523
5.	831576	2116976
6.	825468	2108588
Mean	828497	2126538
S.D	3393.3	19682.3
%RSD	0.4	0.9




Rilpivirine - 2.272
Cabotegravir - 2.999
AU
Minutes
0.30
0.20
0.10
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00


Rilpivirine - 2.267
Cabotegravir - 2.990
AU
Minutes
0.30
0.20
0.10
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00


Rilpivirine - 2.264
Cabotegravir - 2.973
AU
Minutes
0.30
0.20
0.10
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00

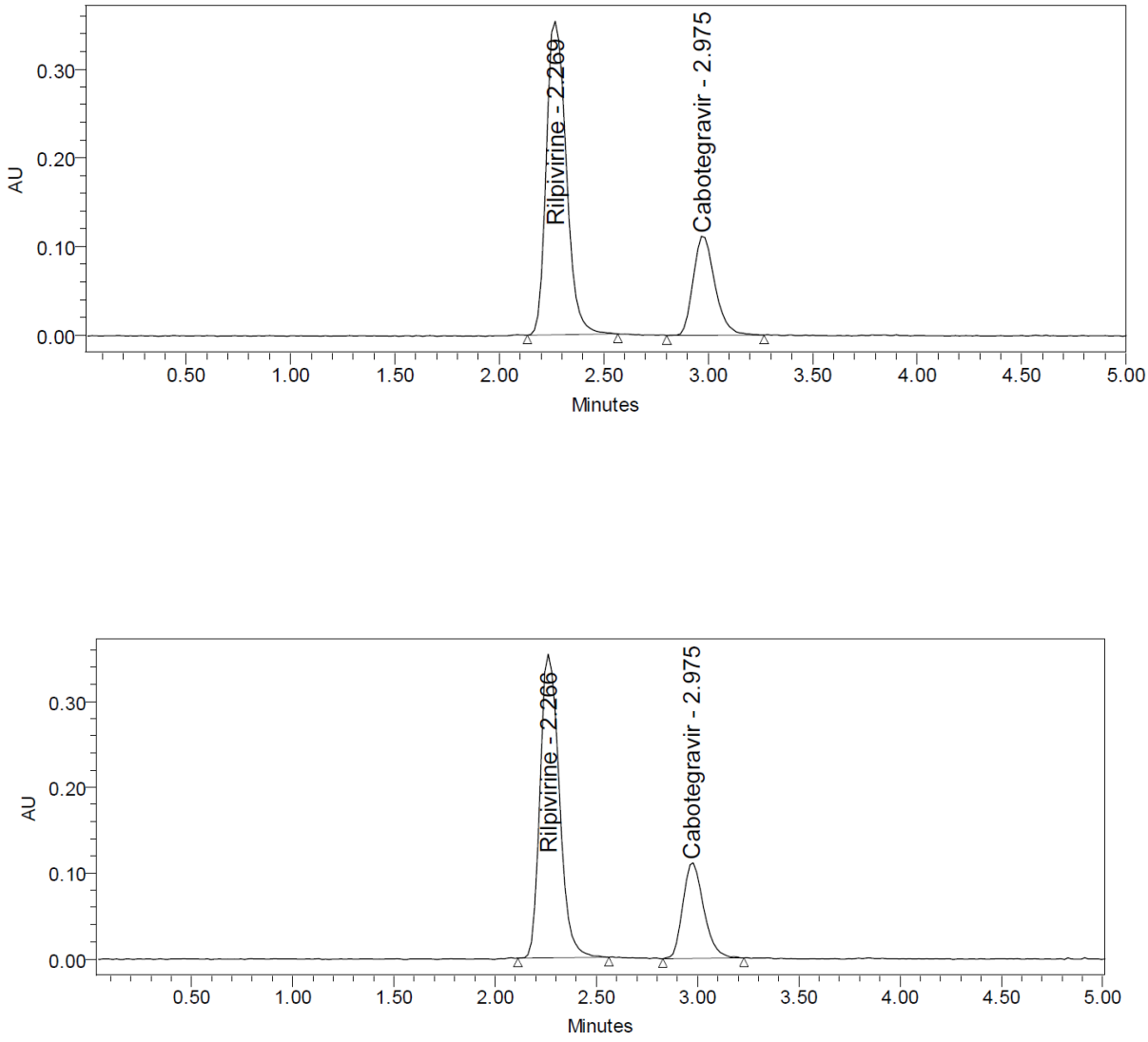


Fig: 6.20 Inter Day precision Chromatogram

Discussion:

Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given on the next day of the sample preparation and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated for two drugs and obtained as 0.4% and 0.9% respectively for Cabotegravir and Rilpivirine. As the limit of Precision was less than "2" the system precision was passed in this method.

ACCURACY:

Table 6.6 Accuracy table of Cabotegravir

% Level	**Amount Spiked (µg/mL)**	**Amount recovered (µg/mL)**	**% Recovery**	Mean %Recovery
50%	100	100.51	100.51	100.60%
	100	100.72	100.72	
	100	100.41	100.41	
100%	200	199.87	99.94	
	200	202.60	101.30	
	200	200.51	100.26	
150%	300	300.01	100.00	
	300	302.09	100.70	
	300	304.68	101.56	

Table 6.7 Accuracy table of Rilpivirine

% Level	**Amount Spiked (µg/mL)**	**Amount recovered (µg/mL)**	**% Recovery**	Mean %Recovery
50%	150	151.08	100.72	100.54%
	150	150.60	100.40	
	150	152.30	101.53	
100%	300	302.14	100.71	
	300	300.31	100.10	
	300	301.98	100.66	
150%	450	450.70	100.15	
	450	451.58	100.35	
	450	450.80	100.18	

Discussion:

Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 100.60% and 100.54% for Cabotegravir and Rilpivirine respectively.

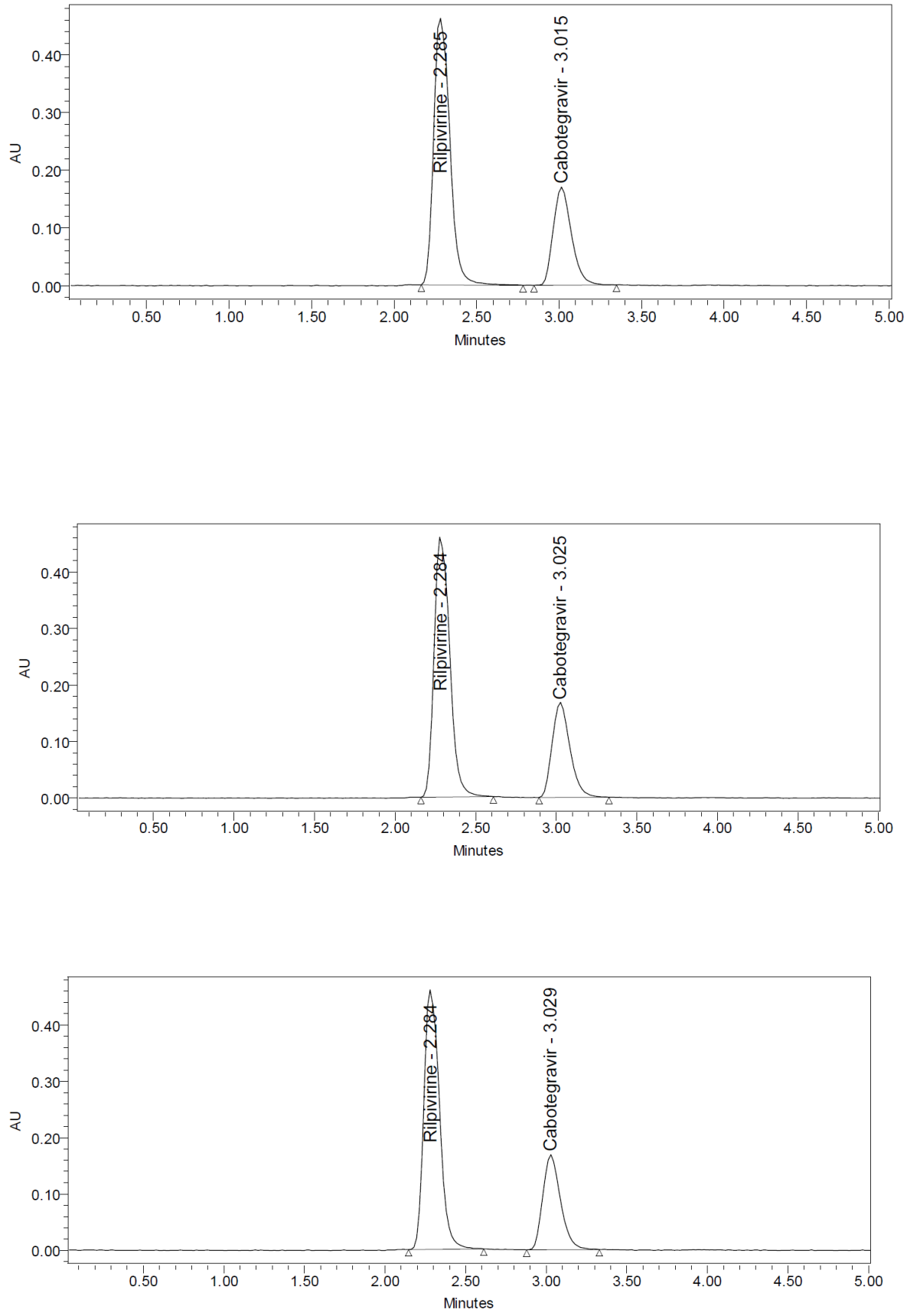
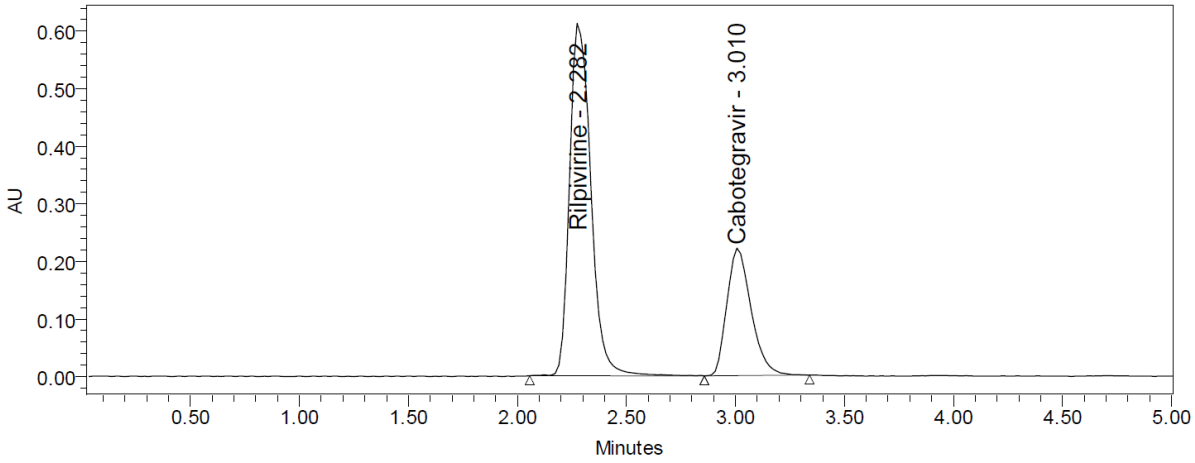
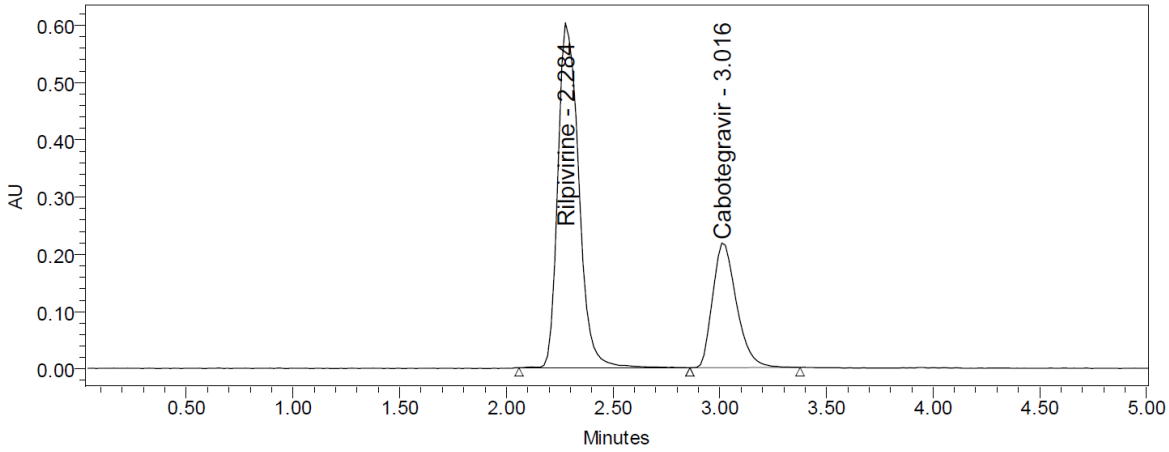


Fig No. 6.21 Accuracy 50% Chromatogram of Cabotegravir and Rilpivirine





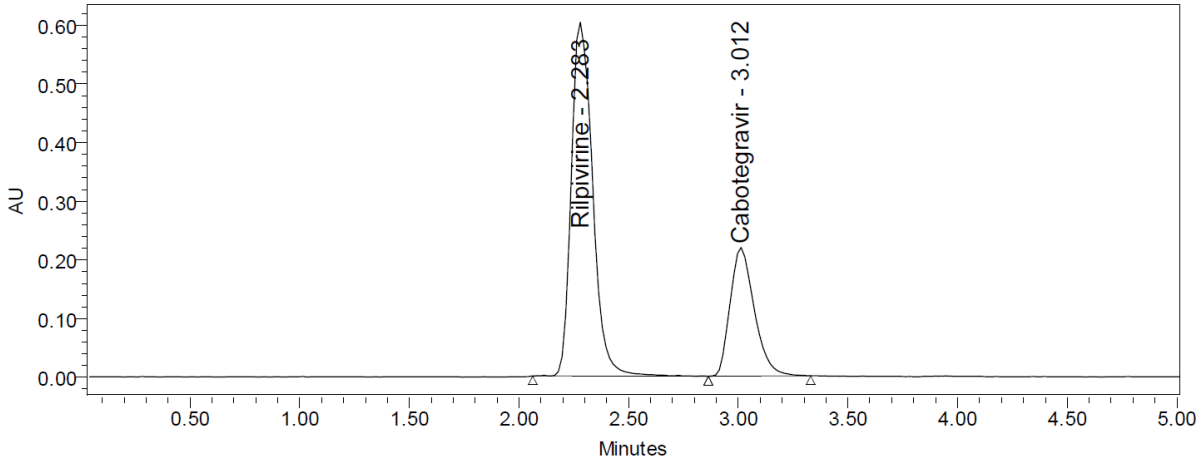


Fig No. 6.22 Accuracy 100% Chromatogram of Cabotegravir and Rilpivirine

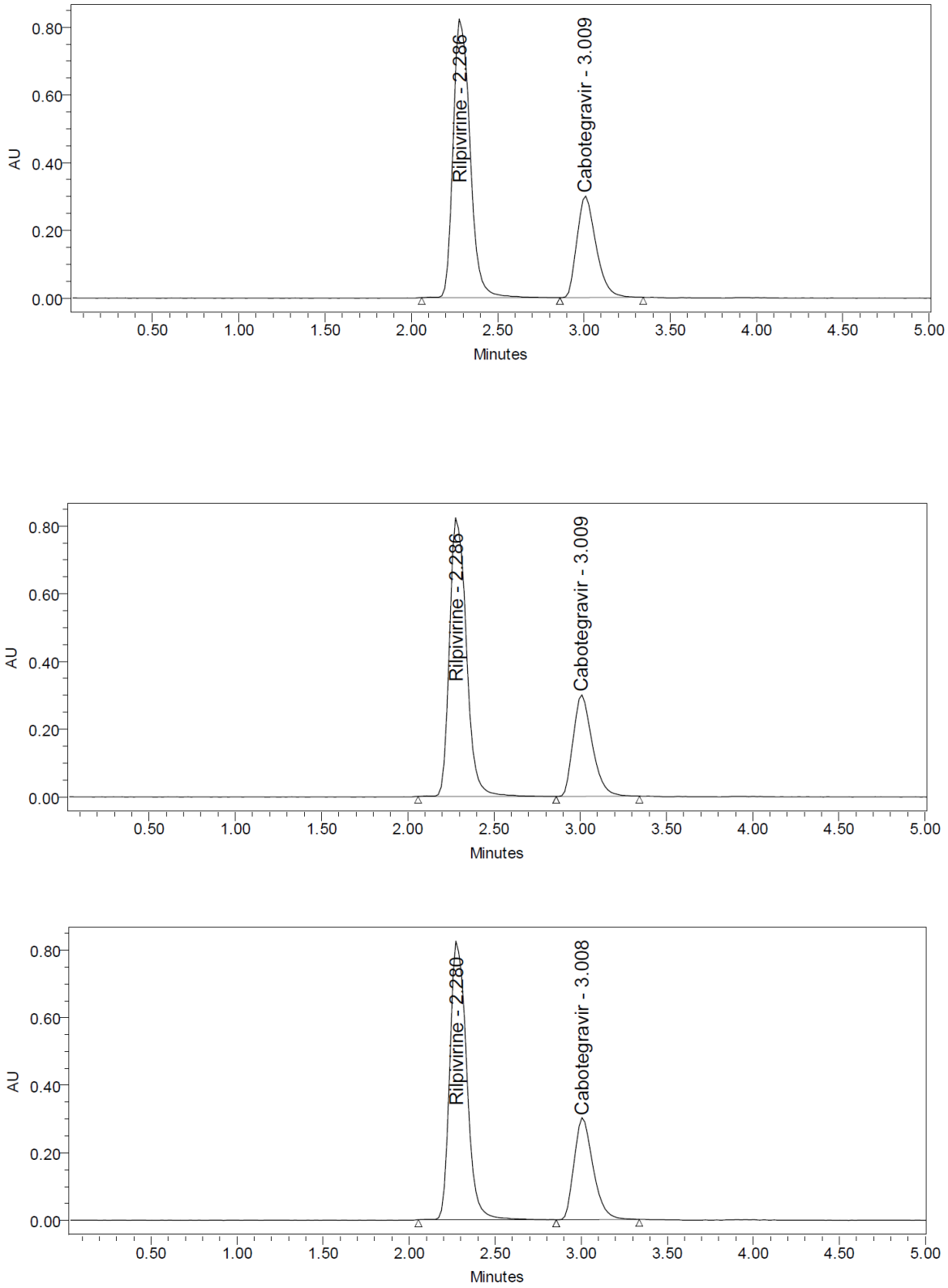


Fig No. 6.23 Accuracy 150% Chromatogram of Cabotegravir and Rilpivirine

SENSITIVITY:

Table 6.8 Sensitivity table of Cabotegravir and Rilpivirine

Molecule	LOD	LOQ
Cabotegravir	0.91	2.74
Rilpivirine	3.88	11.77

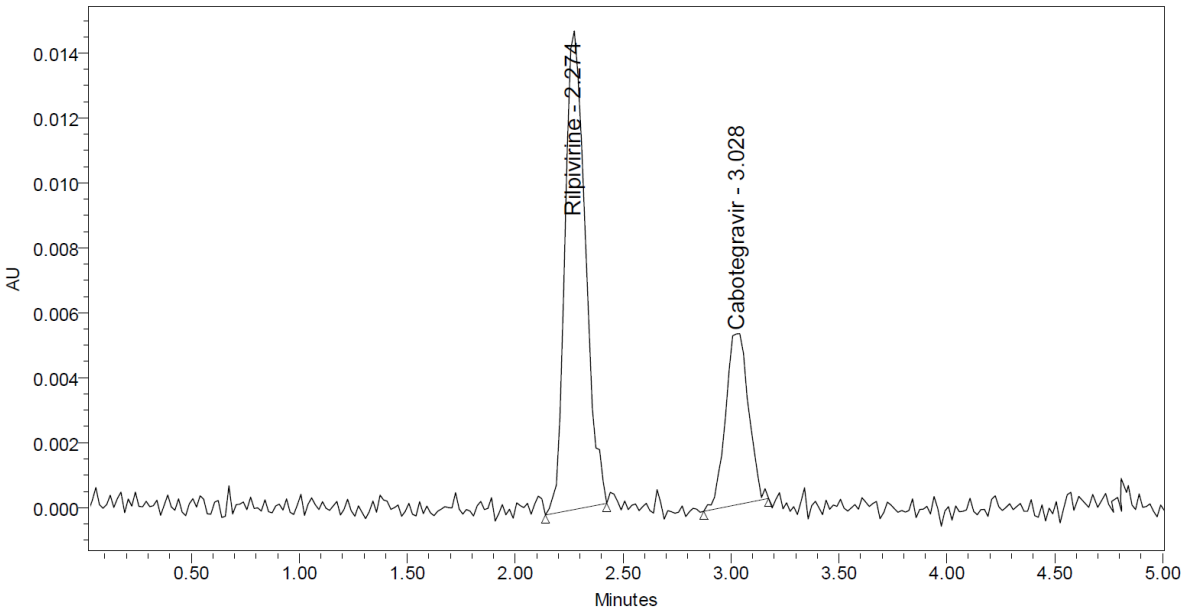


Fig. No. 6.24 LOD Chromatogram of Standard

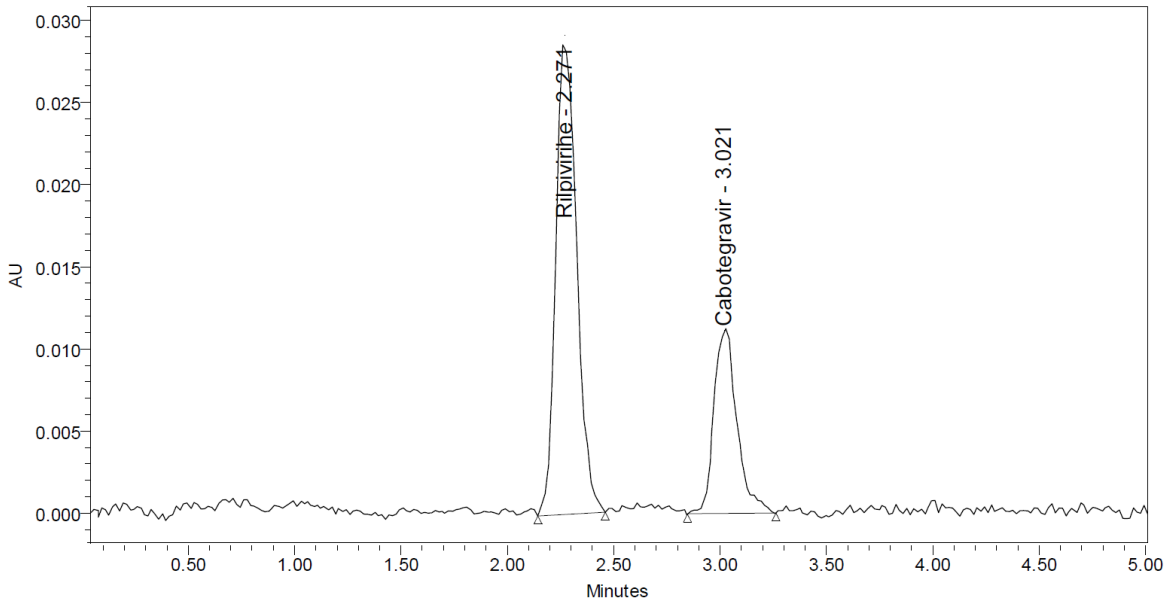


Fig.No. 6.25 LOQ Chromatogram of of Standard

ROBUSTNESS:

Table 6.9 Robustness data for Cabotegravir and Rilpivirine.

S.no	Condition	%RSD of Cabotegravir	%RSD of Rilpivirine
1	Flow rate (-) 0.9ml/min	0.8	0.8
2	Flow rate (+) 1.1ml/min	0.1	1.5
3	Mobile phase (-) 55B:45A	0.7	0.8
4	Mobile phase (+) 45B:55A	0.2	0.8
5	Temperature (-) 25°C	0.5	0.5
6	Temperature (+) 35°C	0.6	1.2

Discussion:

Robustness conditions like Flow minus (0.9ml/min), Flow plus (1.1ml/min), mobile phase minus (65B:35A), mobile phase plus (55B:45A), temperature minus (25°C) and temperature plus(35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

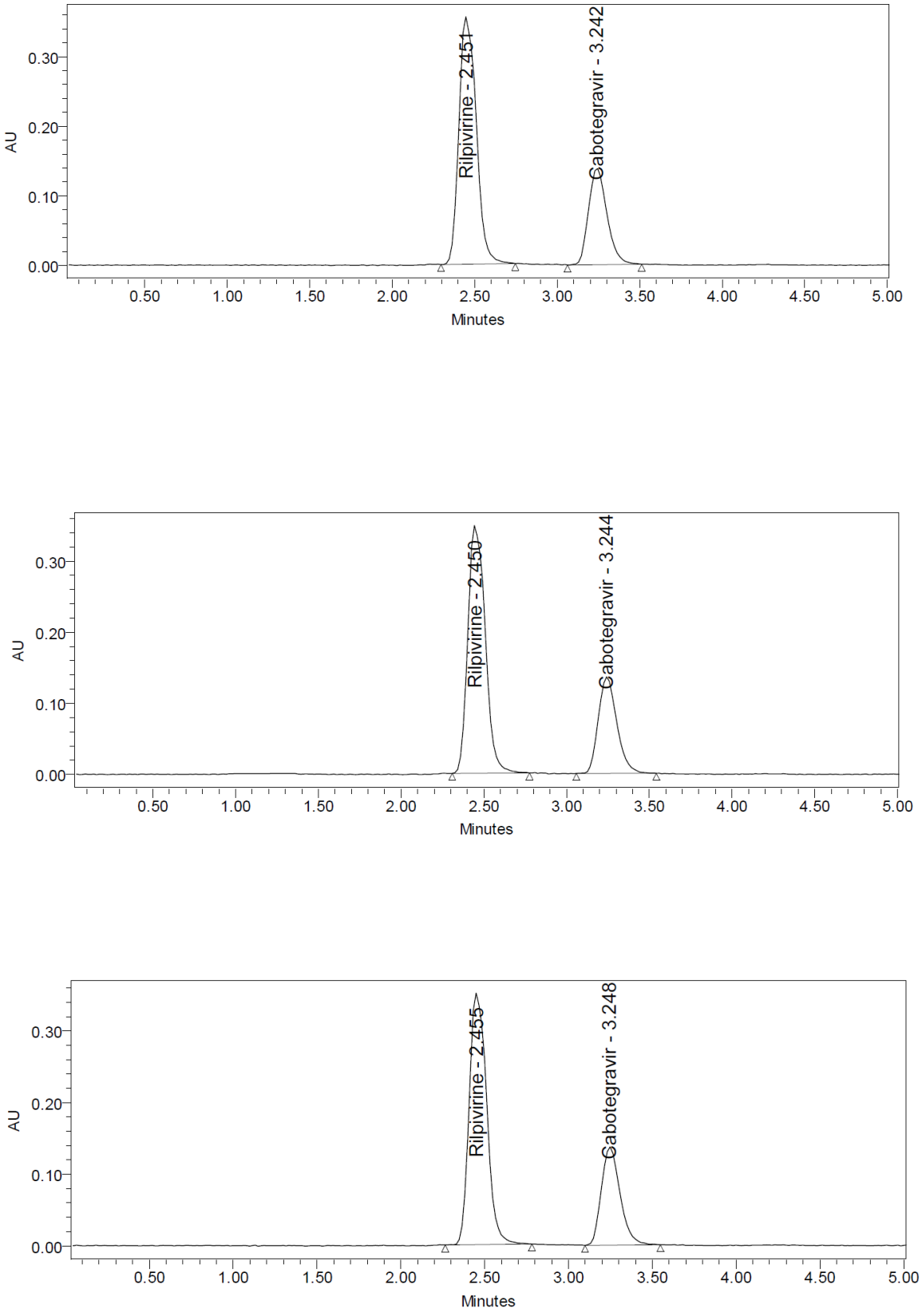


Fig No. 6.26 Flow minus Chromatogram of Cabotegravir and Rilpivirine.

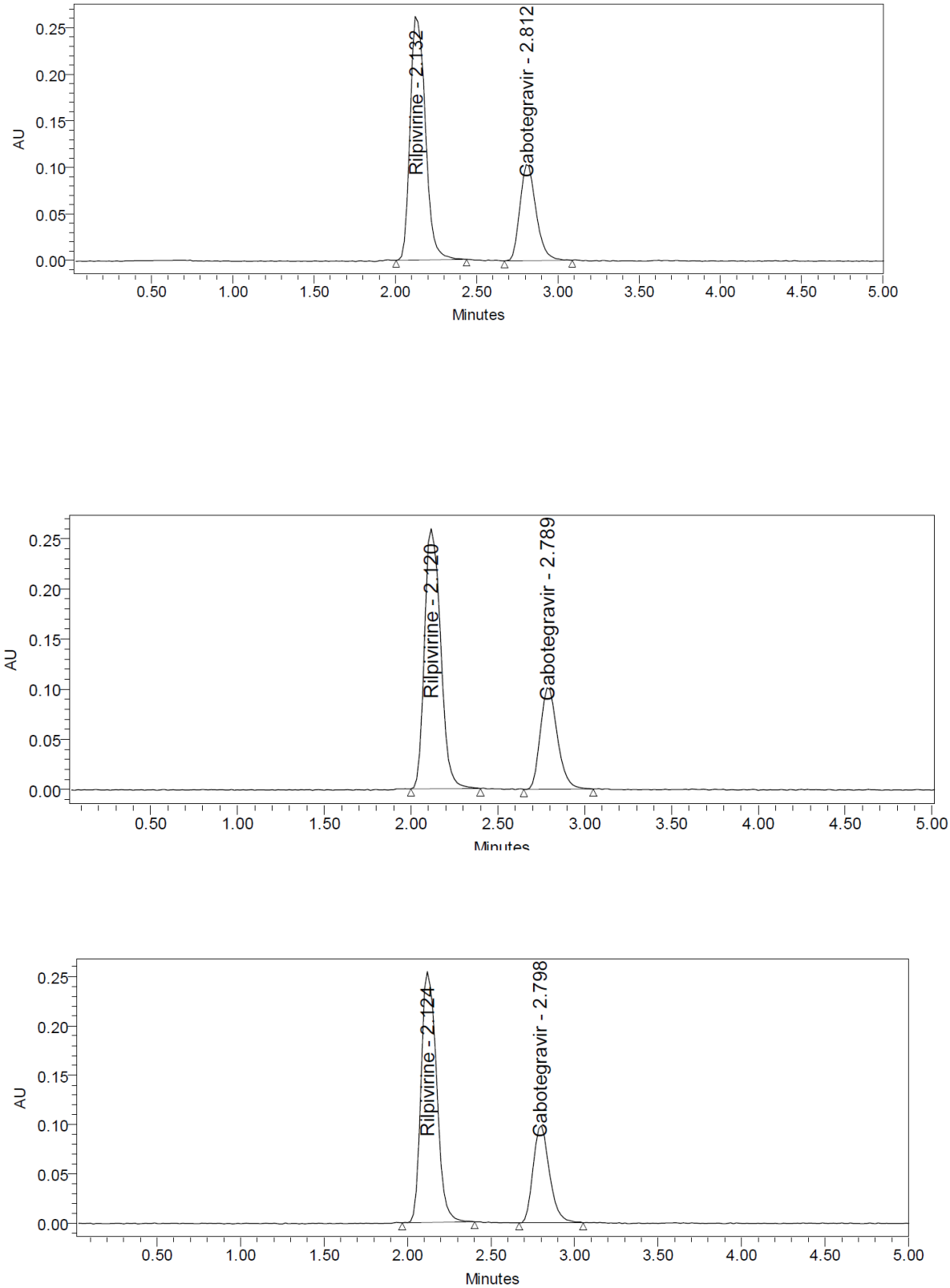


Fig No. 6.27 Flow plus Chromatogram of Cabotegravir and Rilpivirine.

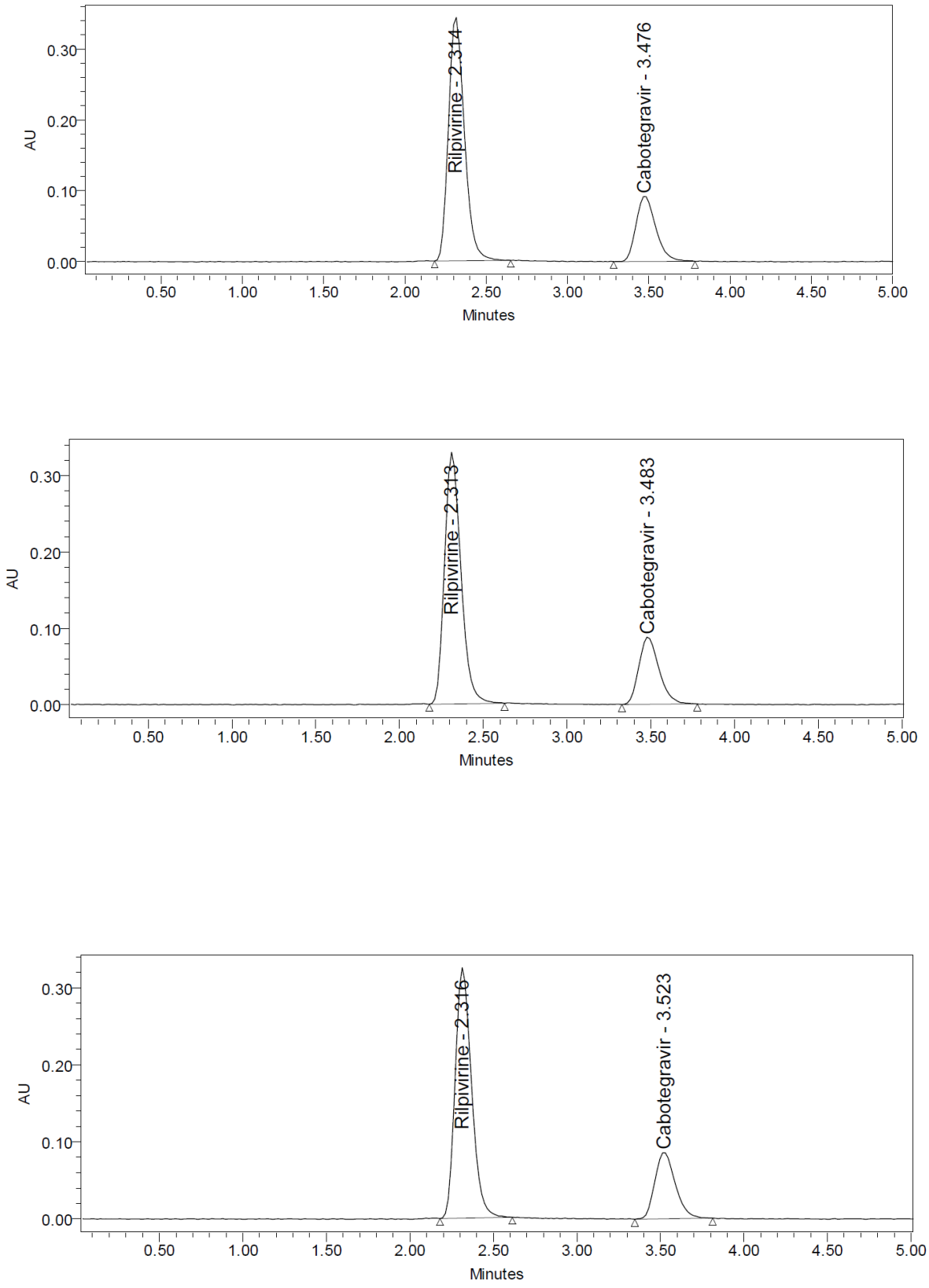


Fig No. 6.28 Mobile phase minus Chromatogram of Cabotegravir and Rilpivirine.

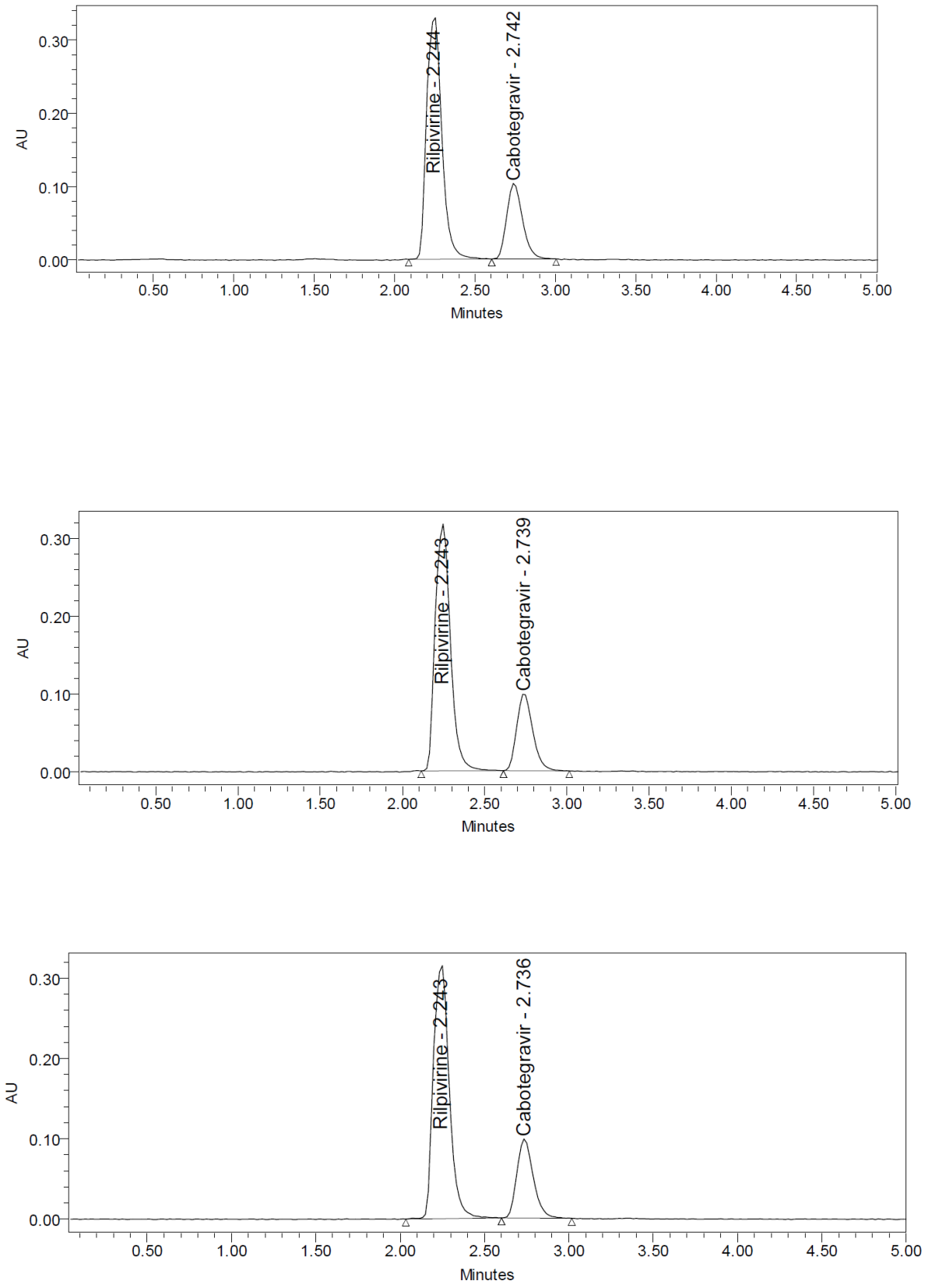


Fig No. 6.29 Mobile phase Plus Chromatogram of Cabotegravir and Rilpivirine.

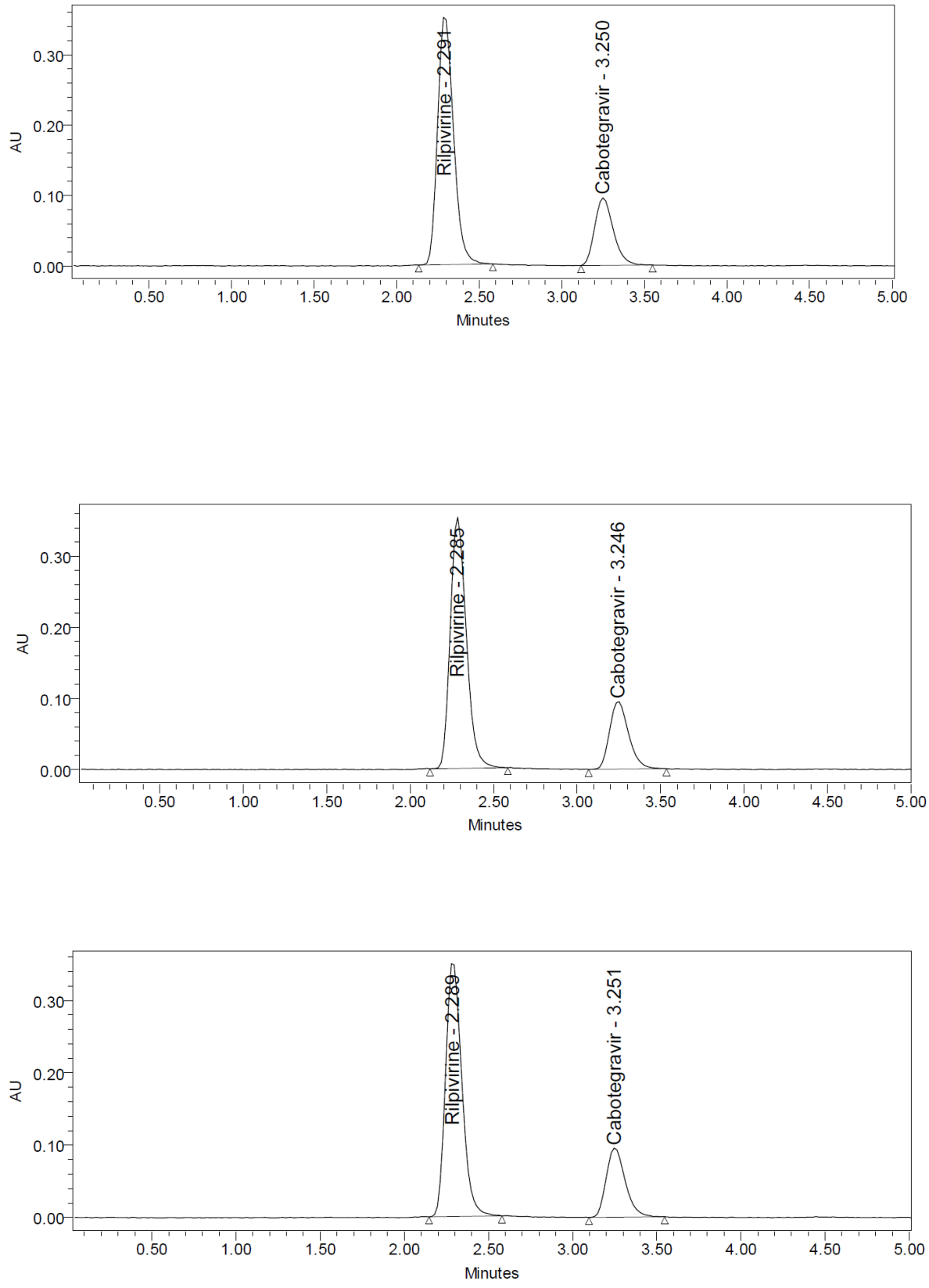


Fig No. 6.30 Temperature minus Chromatogram of Cabotegravir and Rilpivirine .

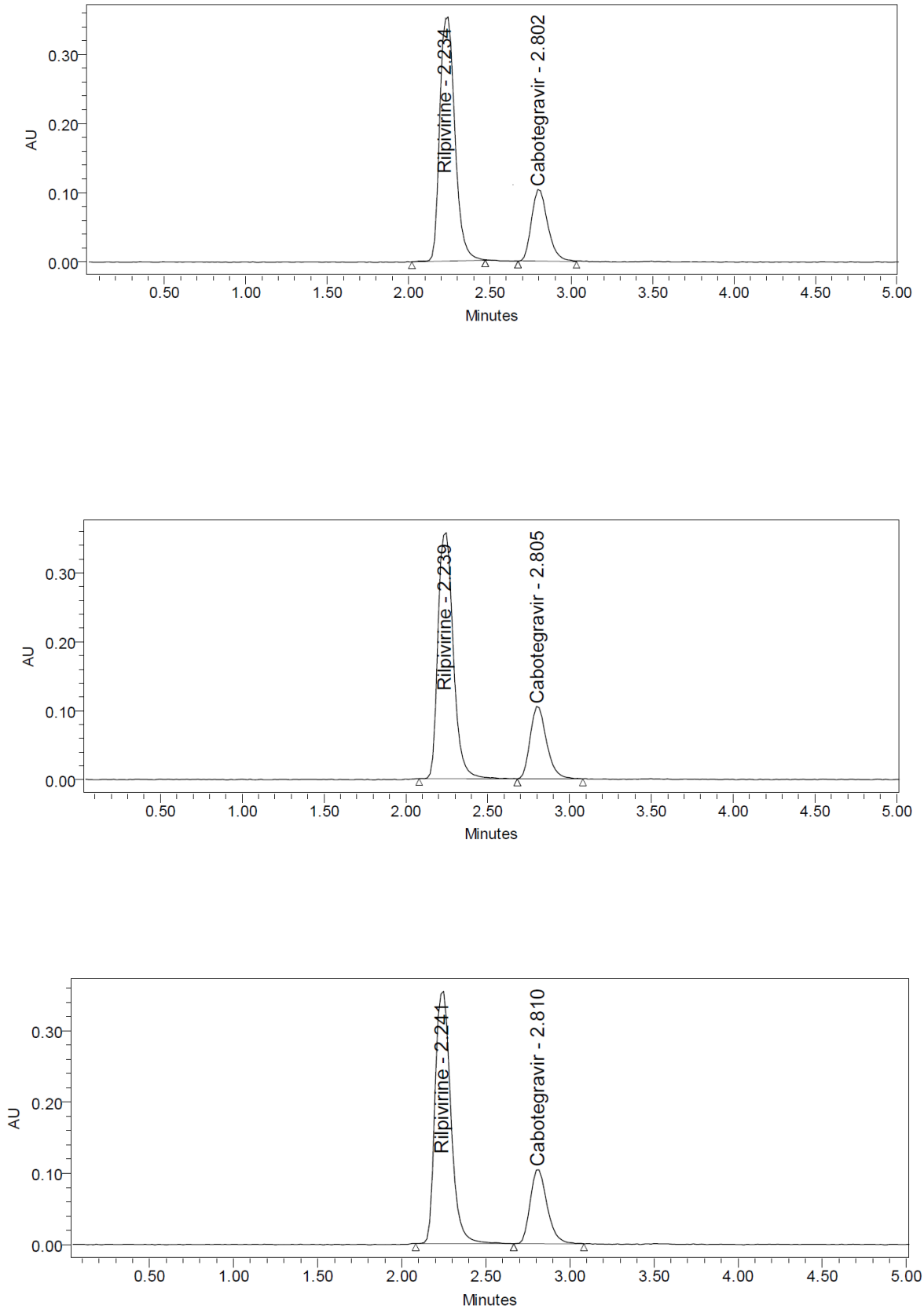


Fig No. 6.31 Temperature plus Chromatogram of Cabotegravir and Rilpivirine

Assay: (Cabenuva)

Bearing the label claims Cabotegravir 400mg, Rilpivirine 600mg/2ml. Assay was performed with the above formulation. Average % Assay for Cabotegravir and Rilpivirine obtained was 100.32 % and 100.42% respectively

Table 6.10 Assay Data of Cabotegravir

S.no	Standard Area	Sample area	% Assay
1	821182	832177	100.30
2	823203	830082	100.05
3	837264	827638	99.76
4	821751	833713	100.49
5	835180	831500	100.22
6	829485	839076	101.13
Avg	828011	832364	100.32
Stdev	7044.5	3878.0	0.47
%RSD	0.9	0.5	0.5

.**Table 6.11 Assay Data of Rilpivirine**

S.no	Standard Area	Sample area	% Assay
1	2122901	2129808	99.88
2	2137480	2140536	100.38
3	2141383	2142313	100.47
4	2106471	2131561	99.96
5	2121350	2145571	100.62
6	2138947	2157859	101.20
Avg	2128089	2141275	100.42
Stdev	13583.0	10207.5	0.48
%RSD	0.6	0.5	0.5

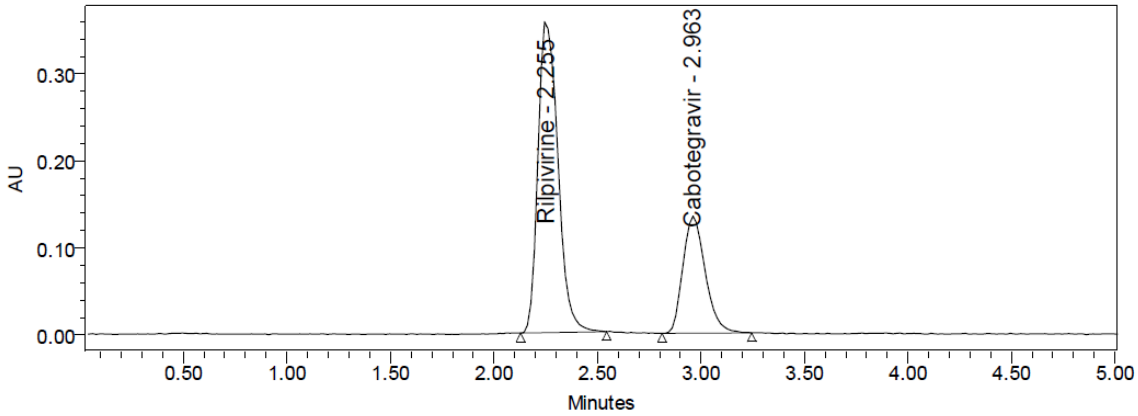


Fig 6.32 Chromatogram of working standard solution

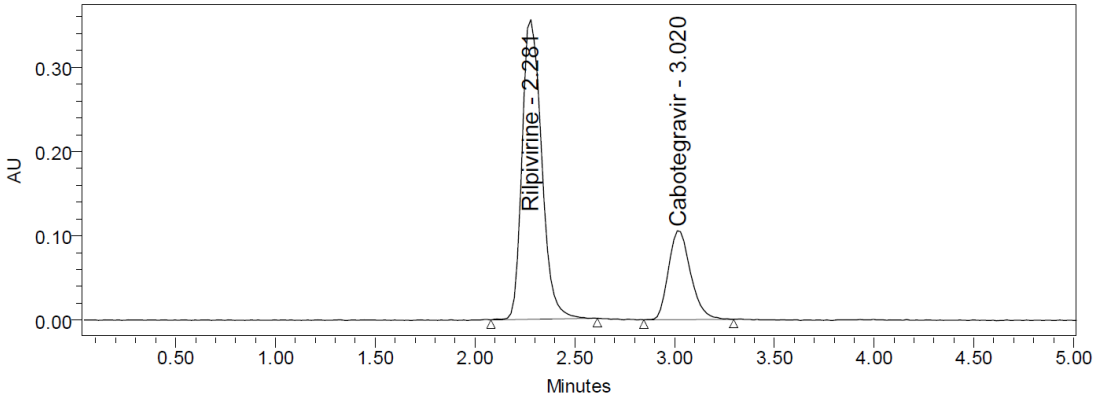


Fig No. 6.33 Chromatogram of working sample solution

DEGRADATION

DegradationStudies:

Degradation studies were performed with the formulation and the degraded samples were injected. Assay of the injected samples was calculated and all the samples passed the limits of degradation.

Table 6.12 Degradation Data of Cabotegravir

S.NO	Degradation Condition	% Drug Ungraded	% Drug Degraded
1	Acid	94.31	5.69
2	Alkali	95.70	4.30
3	Oxidation	95.51	4.49
4	Thermal	97.61	2.39
5	UV	98.80	1.20
6	Water	97.85	2.15

Table 6.13 Degradation Data of Rilpivirine

S.NO	Degradation Condition	% Drug Ungraded	% Drug Degraded
1	Acid	93.75	6.25
2	Alkali	95.58	4.42
3	Oxidation	95.87	4.13
4	Thermal	98.18	1.82
5	UV	99.18	0.82
6	Water	99.33	0.67

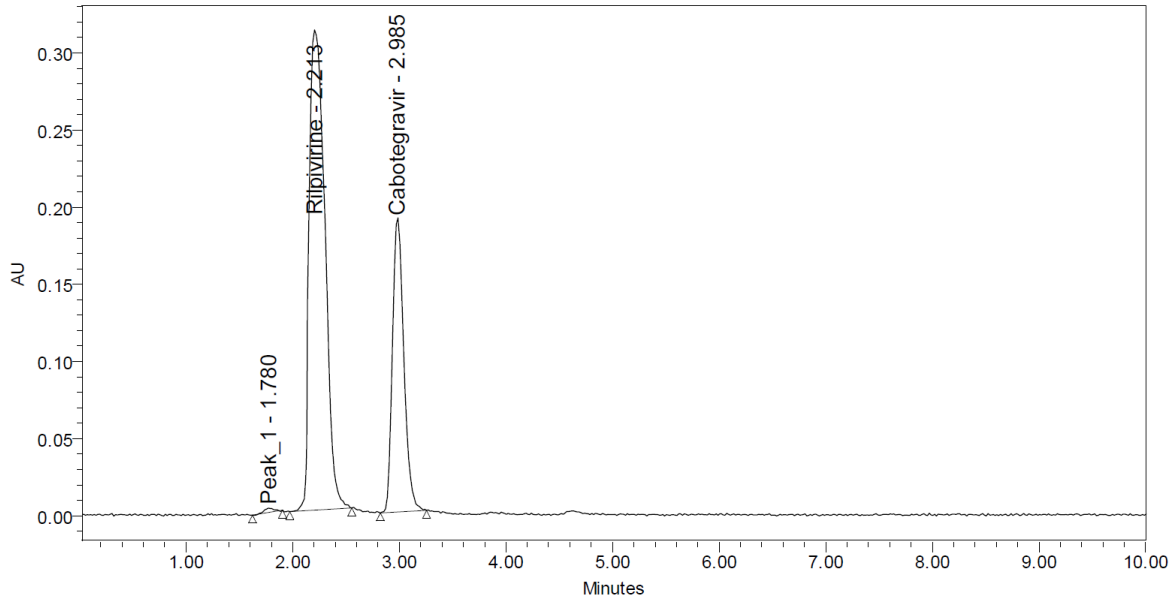


Fig. No. 6.34 Acid chromatogram of Cabotegravir and Rilpivirine

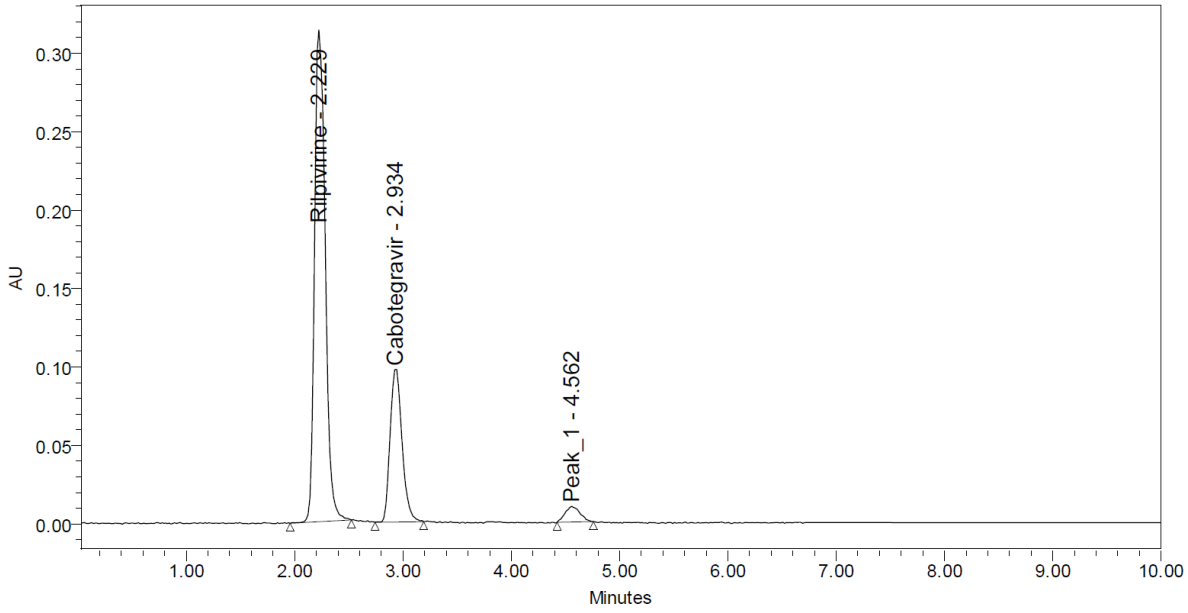


Fig. No. 6.35 Base chromatogram of Cabotegravir and Rilpivirine

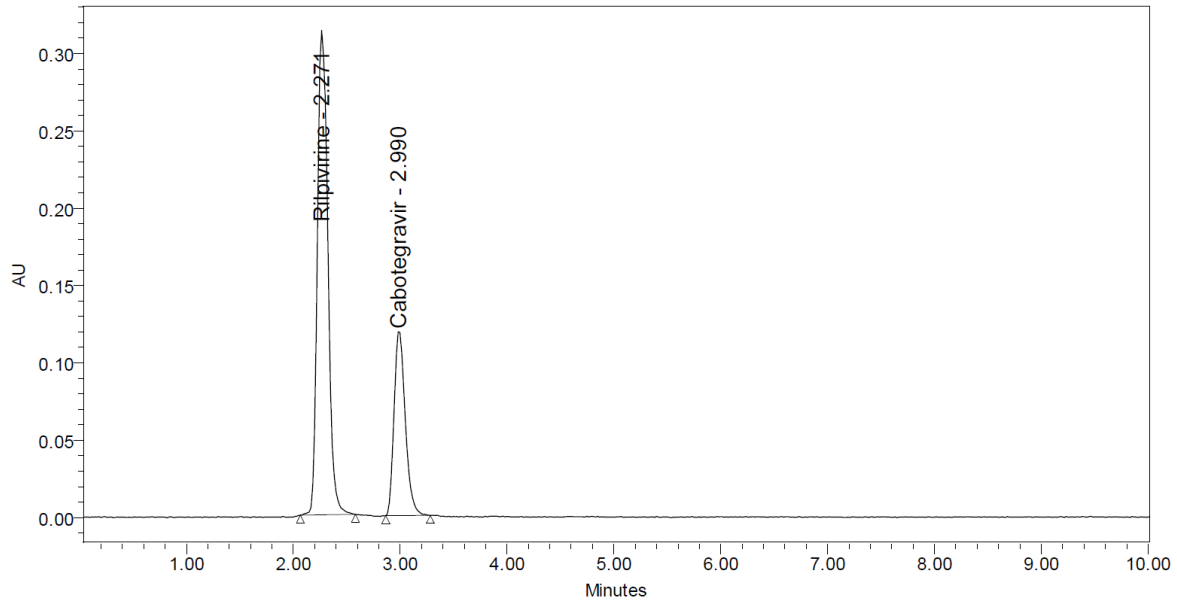


Fig. No. 6.36 Peroxide chromatogram of Cabotegravir and Rilpivirine

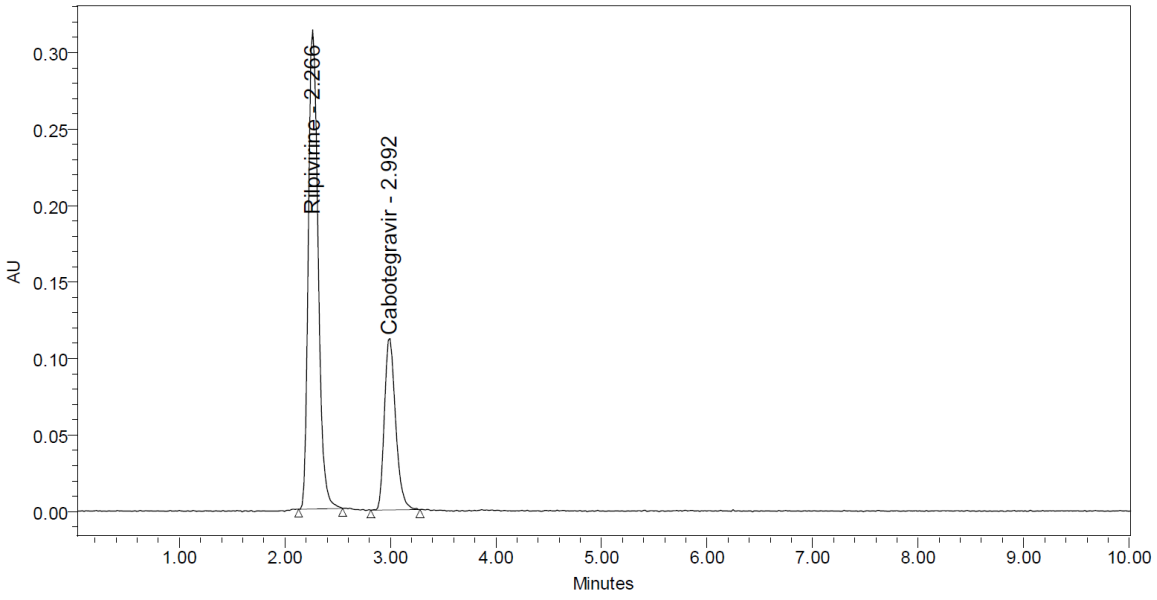


Fig. No. 6.37 Thermal chromatogram of Cabotegravir and Rilpivirine

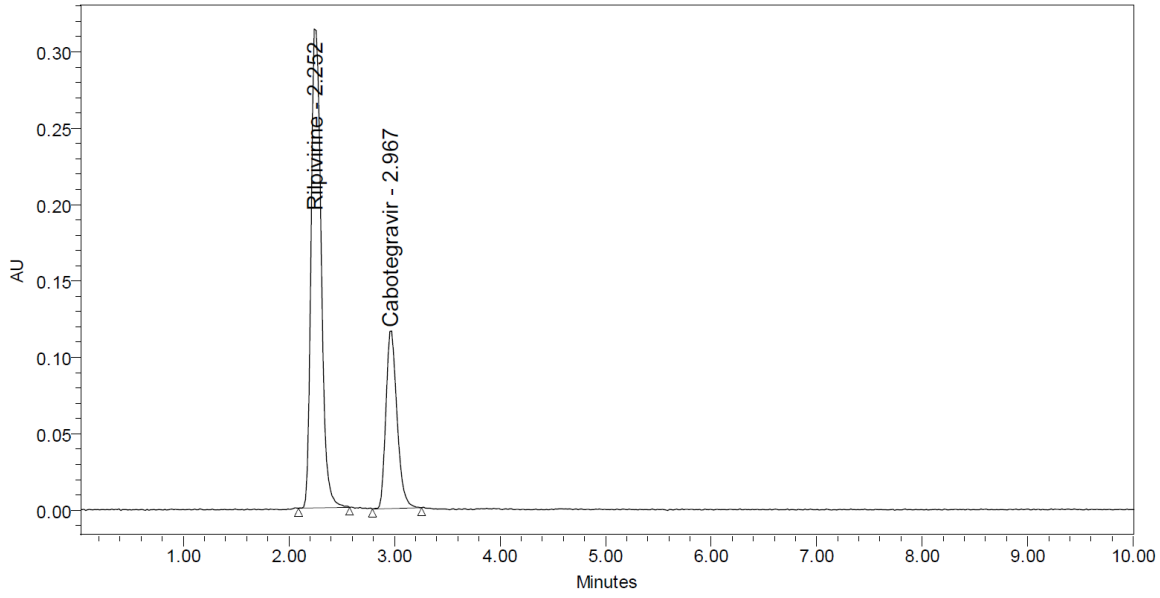


Fig.No. 6.38 UV chromatogram of Cabotegravir and Rilpivirine

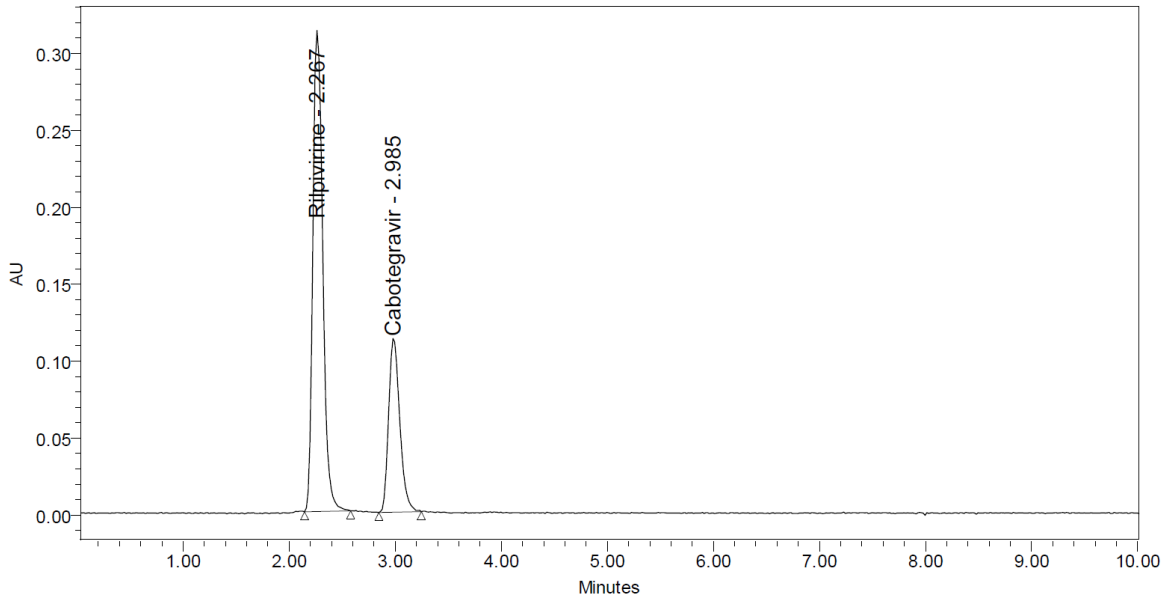


Fig.No. 6.39 Water chromatogram of Cabotegravir and Rilpivirine

Discussion: Regarding the pH adjustment in mobile phase for the acid and base degradation studies have movement in retention time of drugs. But due to neutralized acid sample with 2N Base solution and base sample with 2N Acid solution there will be no change in retention time**.**

7. SUMMARY AND CONCLUSION

7.1 Summary Table

Parameters		Cabotegravir	Rilpivirine	LIMIT
Linearity Range (µg/ml)		50-300 µg/ml	75-450µg/ml	R< 1
Regression coefficient		0.999	0.999	
Slope(m)		4099	6957	
Intercept(c)		5230	26409	
Regression equation (Y=mx+c)		y = 4099.x + 5230	y = 6957.x + 26409	
Assay(% mean assay)		100.32%	100.42%	90-110%
Specificity		Specific	Specific	No interference of any peak
System precision %RSD		0.9	0.6	NMT 2.0%
Method precision %RSD		0.5	0.5	NMT 2.0%
Accuracy %recovery		100.60%	100.54%	98-102%
LOD		0.91	2.74	NMT 3
LOQ		3.88	11.77	NMT 10
Robustness	**FM**	0.8	0.8	%RSD NMT 2.0
	FP	0.1	1.5	
	MM	0.7	0.8	
	MP	0.2	0.8	
	TM	0.5	0.5	
	TP	0.6	1.2	

Conclusion

A simple, Accurate, precise method was developed for the simultaneous estimation of the Cabotegravir and Rilpivirine. Retention time of Cabotegravir and Rilpivirine were found to be 3.020 min and 2.281 min. %RSD of the Cabotegravir and Rilpivirine were and found to be 0.9 and 0.6 respectively. %Recovery was obtained as 100.19% and 100.16% for Cabotegravir and Rilpivirine respectively. LOD, LOQ values obtained from regression equations of Cabotegravir and Rilpivirine were 0.91, 2.74 and 3.88, 11.72 respectively. %Assay was obtained as 100.32% and 100.42% for Cabotegravir and Rilpivirine respectively. Regression equation of Cabotegravir is y =4099.x + 5230, y = 6957.x + 26409 of Rilpivirine. Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

8. BIBLIOGRAPHY

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