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(RESEARCH ARTICLE)

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Fast and efficient related substance determination of Rilpivirine and Dolutegravir in combined HIV therapy with RP-HPLC

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Abstract

The abstract discusses the development and validation of a Related Substance RP-HPLC method for the simultaneous estimation of Rilpivirine and Dolutegravir in a combined pharmaceutical dosage form, following their FDA approval for HIV treatment. The developed method utilized a simple and economical approach, employing an LC-20 AT C18 column with a Water:Methanol (70:30) mobile phase at a flow rate of 1 ml/min, and detection at 258 nm. Retention times for Rilpivirine and Dolutegravir were determined to be 3.82 min and 7.51 min, respectively, while Rilpivirine and Dolutegravir impurity retention times were found to be 4.32 min and 7.12 min, respectively. The method demonstrated excellent linearity for Rilpivirine impurity (5.0-15.0 µg/ml) and Dolutegravir impurity (7.5-22.5 µg/ml). Limits of detection (LOD) were 0.079µg/ml and 0.145µg/ml for Rilpivirine and Dolutegravir impurities, while limits of quantification (LOQ) were 0.241µg/mL and 0.441µg/mL, respectively. The proposed method was successfully applied for the simultaneous estimation of both drugs and their related impurities in a commercial combined dosage form.

Keywords: Rilpivirine; Dolutegravir; Related Substance RP-HPLC Method; ICH Q2 (R1) guidelines

1. Introduction

The advent of combination therapies has revolutionized the treatment landscape for human immunodeficiency virus (HIV), offering enhanced efficacy and reduced side effects. Among the notable combinations, the use of Rilpivirine and Dolutegravir has gained significant attention and earned approval from the Food and Drug Administration (FDA) for HIV treatment. Rilpivirine, a non-nucleoside reverse transcriptase inhibitor (NNRTI), and Dolutegravir, an integrase strand transfer inhibitor (INSTI), exhibit synergistic effects when used together, making them a potent therapeutic option.

In the realm of pharmaceutical analysis, ensuring the quality, purity, and consistency of these combined formulations is paramount. A critical aspect of this quality control is the determination of related substances, which are impurities or degradation products that may impact the safety and efficacy of the drug. While individual analytical methods exist for Rilpivirine and Dolutegravir, there is a notable gap in the literature regarding a fast and efficient Related Substance determination for the simultaneous analysis of both components in combined HIV therapy [1].

The aim of this study is to address the existing gap in analytical methods for estimating Rilpivirine and Dolutegravir in their individual and combined dosage forms. While numerous methods exist for individual estimation, there is a lack of related impurities methods for the simultaneous estimation of these drugs in combined pharmaceutical dosage forms using RP-HPLC. The objective is to develop a specific, sensitive, and selective related impurities [2].RP-HPLC method that is simple, accurate, precise, and rapid for the simultaneous estimation of Rilpivirine and Dolutegravir in combined pharmaceutical dosage forms. Given the importance of these antiretroviral medications in managing HIV, the study aims to contribute to analytical method development and validation, ensuring the identity, purity, potency, and performance

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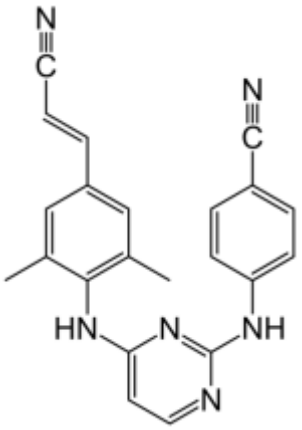
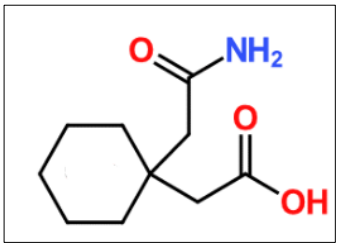
of drug products. The ultimate goal is to apply the newly developed and validated analytical method to estimate Rilpivirine and Dolutegravir in pharmaceutical.

Table 1 Description of Rilpivirine

Name	Rilpivirine
Official in	Not Official in any Pharmacopoeia
Description	Rilpivirine is a non-nucleoside reverse transcriptase inhibitor (NNRTI), used to treat HIV-1 infections in persons who have not received treatment.
Structure	
Chemical Formula	C22H18N6
Mol. Weight	366.42g/mol
IUPAC Name	4-[[4-[[4-[(1E)-2-Cyanoethenyl]-2,6-dimethylphenyl]amino]-2-pyrimidinyl]amino]benzonitrile
Categories	Anti-HIV Agents
Solubility	with a solubility of approximately in water
PHARMACOLOGY	
Classes	Non-nucleoside reverse transcriptase inhibitor (NNRTI)
Mechanism of Action	Rilpivirine is a Inhibits the reverse transcriptase enzyme, preventing the conversion of viral RNA into DNA in the early stages of HIV replication.
PROPERTIES	
State	Solid.
CAS NO.	500287-72-9
Melting point	241-243 °C
pKa	5.6
Log P	4.3

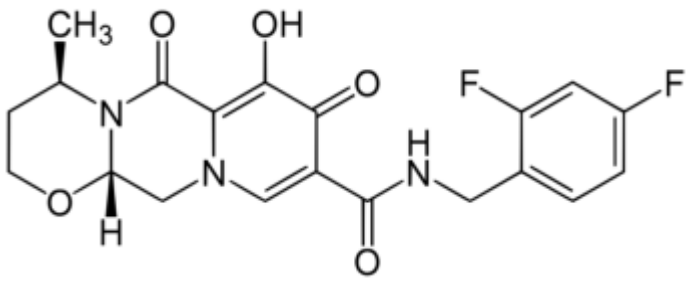
1.1. Related Impurities Of Rilpivirine

Table 2 Name and Structure of Impurities of Rilpivirine

Name	Structure	Chemical name
Rilpivirine related impurity		1,1-Cyclohexanediacetic Acid Monoamide

1.2. Drug Profile of Dolutegravir

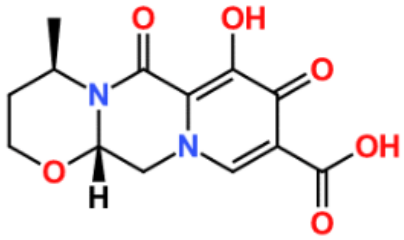
Table 3 Description of Dolutegravir

Name	Dolutegravir
Official in	Not Official in any Pharmacopoeia
Description	Dolutegravir an HIV-1 integrase inhibitor prevents the viral genome from being integrated into the host cell by blocking the strand transfer step (INSTI).
Structure	
Chemical Formula	$C_{20}H_{19}F_2N_3O_5$
Mol. Weight	419.4 g/mol
IUPAC Name	(4R,12aS)-N-(2,4-difluorobenzyl)-7-hydroxy-4-methyl-6,8-dioxo-3,4,6,8,12,12a-hexahydro-2H-pyrido[1',2':4,5]pyrazino[2,1-b] [1,3]oxazine-9-carboxamide
Categories	Integrase inhibitors
Solubility	is soluble in methanol
PHARMACOLOGY	
Classes	HIV-1 integrase inhibitor
Mechanism of Action	Dolutegravir is antiviral medication for HIV-1. Through binding to the active site and obstructing the strand transfer stage of retroviral DNA integration in the host cell, it inhibits HIV integrase. The HIV replication cycle's strand transfer stage is crucial because it stops the virus from replicating. The mean EC50 value of dolutegravir in peripheral blood mononuclear cells (PBMCs) and MT-4 cells ranges from 0.5 nM (0.21 ng/mL) to 2.1 nM (0.85 ng/mL).
PROPERTIES	
State	Solid.
CAS NO.	1051375-16-6

Melting point	190-193^{0}C
pK_a	8.2
Log P	7.3

1.3. Related Impurities of Dolutegravir

Table 4 Name and Structure of Impurities of Dolutegravir

Name	Structure	Chemical name
Dolutegravir impurity		(4R,12As)-7-Hydroxy-4-methyl-6,8-dioxo-3,4,6,8,12,12a-hexahydro-2h-pyrido[1',2':4,5]pyrazino[2,1-b][1,3]oxazine-9-carboxylic Acid

1.4. Combination Product

Table 5 Combination Brand Available in Market

Brand Name	Content	Marketed By	Dosage Form	Dose
JULUCA	Dolutegravir + Rilpivirine	GSK Pharmaceuticals Pvt Ltd	Tablet	Dolutegravir (50mg) and Rilpivirine Sodium (25mg)

Figure 1 Marketed Formulation of Dolutegravir and Rilpivirine

2. Materials and Methods:

In present research work, an attempt was made for development and validation of Related Substance method for simultaneous estimation of Rilpivirine and Dolutegravir in pharmaceutical dosage form by RP-HPLC.

2.1. Instruments

Table 6 List of Instruments

Instruments Name	Manufacturer
HPLC	Shimadzu LC-20 AT
UV Visible spectrophotometer	Systronic 119
Electronic balance	Shimadzu ATX-240
Sonicator	Frontline Ultrasonic Cleaner
Hot air oven	Thermolab Mumbai, India
pH meter	Analab Scientific Pvt Ltd

2.2. Apparatus

Table 7 List of Apparatus

Components	Description
Volumetric flasks	Borosilicate glass
Pipettes	Borosilicate glass
Measuring cylinder	Borosilicate glass
Beaker	Borosilicate glass
Whatman Filter	Filter Paper No.42

2.3. Reagents

Table 8 List of Reagents

Chemicals	Grade	Manufacturer
Acetonitrile	HPLC	Merck, Rankem
Potassium Dihydrogen Phosphate	AR	Merck, Rankem
Water	HPLC	HPLC Grade
Orthophosphoric Acid	AR	Merck, Rankem
Methanol	HPLC	Merck, Rankem
Rilpivirine and its Related Impurities	Dial pharmaceutical	
Dolutegravir and its Related Impurities	Avantika medix Pvt.	

2.4. Method development

2.4.1. Selection and Detection of Wavelength

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is the one that gives good response for the drugs that are to be detected. At 258 nm both drug give good response. So 258 nm was selected as detection wavelength for estimation of Rilpivirine and Dolutegravir in tablet dosage form by RP-HPLC. [1-20]

2.4.2. Selection of Chromatographic Condition

Proper selection of the HPLC method depends upon the nature of the sample (ionic or neutral molecules), its molecular weight, pK_a and solubility. RP-HPLC was selected for the initial separation based on literature survey and its simplicity and suitability. To optimize the chromatographic conditions the effect of chromatographic variables such as mobile phase, pH, flow rate and solvent ratio were studied. Finally the chromatographic condition was chosen that give the best resolution, symmetry and capacity factor for estimation of both drugs and its related impurities. [1-20]

2.4.3. Selection of Column

For RP-HPLC method, various columns are available and pure drugs chromatogram was developed in different mobile phase, different columns (e.g. C_8, C_{18}, phenyl etc) with different dimensions. The retention time and tailing factor was calculated for each drugs and its related impurities and for each chromatogram. Sharp peak and good resolution was found in C_{18}. Finally BDS Hypersil C_{18} (250mm X 4.6mm, 5µm) column was chosen for method development. [1-20]

2.4.4. Procedure for Solution Preparation

Preparation of Standard Stock Solution

- Standard Stock Solution of Rilpivirine (100 ppm)

Take 10 mg of Rilpivirine an into a 100ml volumetric flask and dissolve with methanol upto the mark to get 100 µg/ml of Rilpivirine Standard Stock Solution.

- Standard Stock Solution of Rilpivirine Impurity (100 ppm)

Take 10 mg of Rilpivirine into a 100ml volumetric flask and dissolve with methanol upto the mark to get 100 µg/ml of Rilpivirine Impurity Standard Stock Solution.

- Standard Stock Solution of Dolutegravir (150 ppm)

Take 15 mg of Dolutegravir into a 100ml volumetric flask and dissolve with methanol upto the mark to get 150 µg/ml of Dolutegravir Standard Stock Solution.

- Standard Stock Solution of Dolutegravir Impurity (150 ppm)

Take 15 mg of Dolutegravir into a 100ml volumetric flask and dissolve with methanol upto the mark to get 150 µg/ml of Dolutegravir Impurity Standard Stock Solution.

Preparation of Working Standard Solution

- Working Standard Solution of Rilpivirine (10 ppm)

From above Rilpivirine Standard Stock solution 1 ml was taken in to 10 ml volumetric flask and was made up to the mark with the mobile phase to get 10 µg/ml of Rilpivirine Working Standard Solution.

- Working Standard Solution of Rilpivirine Impurity (10 ppm)

From above Rilpivirine Impurity Standard Stock solution 1 ml was taken in to 10 ml volumetric flask and was made up to the mark with the mobile phase to get 10 µg/ml of Rilpivirine Impurity Working Standard Solution.

- Working Standard Solution of Dolutegravir (15 ppm)

From above Dolutegravir Standard Stock solution 1 ml was taken in to 10 ml volumetric flask and was made up to the mark with the mobile phase to get 15 µg/ml of Dolutegravir Working Standard Solution.

- Working Standard Solution of Dolutegravir Impurity (15 ppm)

From above Dolutegravir Impurity Standard Stock solution 1 ml was taken in to 10 ml volumetric flask and was made up to the mark with the mobile phase to get 15 µg/ml of Dolutegravir Impurity Working Standard Solution.

Preparation of Mobile Phase

Prepare 0.05M Potassium Dihydrogen Phosphate by dissolving 6.8 gm of Potassium Dihydrogen Phosphate in 1000 ml water; adjust pH 5 with 0.1N NaOH. This solution was sonicated for 5 min for degassing and filtered through 0.45µ Millipore filter. Prepare the different ratio of Buffer (pH 5.0): Acetonitrile.

Preparation of Test Solution

The average weight of 10 tablets was determined and was ground in a mortar. Test solution was prepared by dissolving tablet powder equivalent to 200 mg of Rilpivirine or 300 mg of Dolutegravir was transferred to 100ml volumetric flask. Then 60 ml mobile phase was added and sonicated for 15 mins to ensure complete solubilization of drug. Further dilute 5ml of above solution and make up with 100 ml of mobile phase, After sonication, volume was made up to the mark with mobile phase. Filter the solution with 0.45 micron membrane filter and the final filtrate is collected as test solution.

2.4.5. Chromatographic Separation

Standard solutions of Rilpivirine and Dolutegravir along with its related impurities were injected in column with 20 µl micro-syringe. The chromatogram was run for appropriate minutes with mobile phase. The detection was carried out at wavelength 258 nm. The chromatogram was stopped after separation achieved completely. Data related to peak like area, height, retention time, resolution etc. were recorded using software. [1-21]

2.4.6. Chromatographic Conditions

Table 9 Chromatographic Conditions of HPLC

Components	**Description**
Column	C18 (25 cm × 0.46 cm) Hypersil BDS
Mobile Phase	Water : Methanol (70:30 v/v)
Flow Rate	1.0 ml/min
Detection Wavelength	258 nm
Run time	10 min
Injection volume	20.0 µl

2.5. Validation of RP-HPLC method

2.5.1. System Suitability Test

It is an integral part of chromatographic method. These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as a whole. System suitability testing provides assurance that the method will provide accurate and precise data for its intended use.

Acceptance criteria

- Theoretical Plates for the analyte peak should not be less than 2000.
- Tailing factor for the analyte peak should not be more than 2.0.

2.5.2. Linearity and Range

The linearity for Rilpivirine and Dolutegravir were assessed by analysis of combined standard solution in range of 5.0-15.0 µg/ml and 7.5-22.5 µg/ml respectively, 0.5,0.75,1,1.25 and 1.5 ml solutions were pipette out from the Stock solution of Rilpivirine and Dolutegravir and transfer to 100 ml volumetric flask and make up with mobile phase to obtain 5,7.5,10,12.5 and 15µg/ml and 7.5,11.25,15,18.75 and 22.5µg/ml for Rilpivirine and Dolutegravir respectively. In term of slope, intercept and correlation co-efficient value. The graph of peak area obtained verses respective concentration was plotted.

Acceptance criteria: Value of r2 should be nearer to 1 or equal to 1.

2.5.3. Precision

- Repeatability

Standard solution containing Rilpivirine (10 µg/ml) and Dolutegravir (5 µg/ml) was injected six times and areas of peaks were measured and % R.S.D. was calculated.

Acceptance criteria: % RSD of Area should not be more than 5.0%

- Intraday Precision

Standard solution containing (0.3,5.0,7.5µg/ml) of Rilpivirine and (0.15,10.0,15.0µg/ml) of Dolutegravir were analyzed three times on the same day and % R.S.D was calculated.

Acceptance criteria: % RSD of Area should not be more than 5.0%

- Interday Precision

Standard solution containing (0.3,5.0,7.5µg/ml) of Rilpivirine (0.15,10.0,15.0)µg /ml) of Dolutegravir were analyzed three times on the different day and % R.S.D was calculated.

Acceptance criteria: % RSD of Area should not be more than 5.0%

2.5.4. Accuracy

- For Rilpivirine

10 µg/ml drug solutions was taken in three different flask label A, B and C. Spiked 80%, 100%, 120% of standard solution in it and diluted up to 10ml. The area of each solution peak was measured at 258 nm. The amount of Rilpivirine was calculated at each level and % recoveries were computed.

- For Dolutegravir

15 µg/ml drug solutions was taken in three different flask label A, B and C. Spiked 80%, 100%, 120% of standard solution in it and diluted up to 10ml. The area of each solution peak was measured at 258 nm. The amount of Dolutegravir was calculated at each level and % recoveries were computed.

Acceptance criteria: % Recovery (individual) at each level should be between 98.00% and 102.00%

2.5.5. Limit of Detection and Limit of Quantitation

The LOD was estimated from the set of 3 calibration curves used to determination method linearity. The LOD may be calculated as,

$$LOD = 3.3 \times (SD/Slope)$$

Where,
SD = Standard deviation of Y-intercepts of 3 calibration curves.
Slope = Mean slope of the 3 calibration curves.

The LOQ was estimated from the set of 3 calibration curves used to determine method

linearity. The LOQ may be calculated as,

$$LOQ = 10 \times (SD/Slope)$$

Where,
SD = Standard deviation of Y-intercepts of 3 calibration curves.
Slope = Mean slope of the 3 calibration curves.

2.5.6. Robustness

Following parameters were changed one by one and their effect was observed on system suitability for standard preparation.

- Flow rate of mobile phase was changed (± 0.2 ml/min) 0.8 ml/min and 1.2 ml/min.
- pH of Mobile phase was changed (± 0.2) 5.2 and 4.8
- Ratio of Mobile phase was changed (±2) Water: Methanol (68:32) and Water: Methanol (72:28).

Acceptance criteria

- Number of theoretical plates for the analyte peak should not be less than 2000.
- Asymmetry value for the analyte peak should not be more than 2.0
- % RSD for the analyte peak should not be more than 5.0%

2.5.7. Calculation of Known Impurities of Rilpivirine and Dolutegravir

Analyzed test solution for three times and calculate % of each known impurities in comparison with standard preparations of Rilpivirine and Dolutegravir.

The amount of known related impurities presents in the formulation of Rilpivirine and Dolutegravir is calculated by using the formula given below.

For each known impurities of Rilpivirine and Dolutegravir:

$$\% \text{ of each known impurities} = (Cu/Cs) \text{ X } (Ru/Rs) \text{ X } 100$$

Where,
Cu= Concentration of each impurity in standard preparation
Cs= Concentration of each impurity in test preparation
Ru= Area of each impurity in test preparation
Rs= Area of each impurity in standard preparation

3. Result

The mobile phase Water:Methanol (70:30 v/v). was selected because it was found to ideally resolve the peaks with retention time (RT) 3.82 and 7.51 min for Rilpivirine and Dolutegravir and the retention time of Rilpivirine impurity and Dolutegravir impurity were found to be 4.32 min and 7.12 min respectively respectively and the same is shown in below figure 2, 3.

Final Chromatographic Condition for Rilpivirine and Dolutegravir

- **Stationary Phase :** BDS Hypersil C18 (250 mm×4.6 mm, 5 µm particle size)
- **Mobile Phase :** Water : Methanol (70:30 v/v).
- **Flow Rate :** 1 ml/min
- **Detection Wavelength :** 258 nm
- **Run Time :** 10 min
- **Injection Volume :** 20 µl

Observed values for System Suitability Test

- Retention Time (Rt): Retention Time was observed depicted in below table 10
- Column efficiency (N): Number of plates observed for Rilpivirine and Dolutegravir was observed depicted in below table with caption 10
- Symmetry factor (S): Tailing factor observed for Rilpivirine and Dolutegravir was observed depicted in below table 10

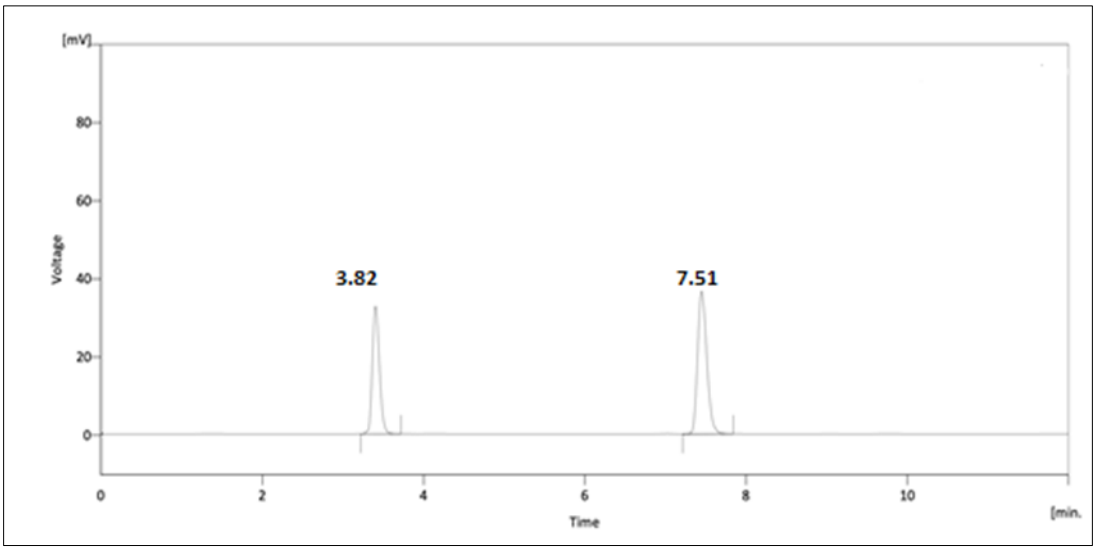


Figure 2 Chromatogram of Rilpivirine and Dolutegravir in water: Methanol (70:30 v/v)

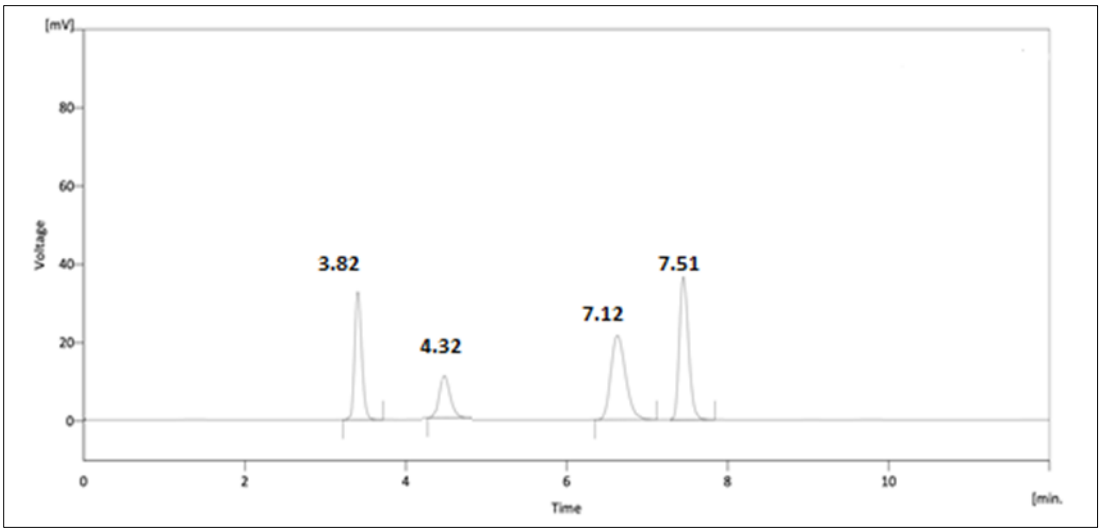


Figure 3 Chromatogram of Rilpivirine and Dolutegravir along with its Related Impurities in water: Methanol (70:30 v/v). Final

Table 10 Results for System Suitability Test

Parameters	Rilpivirine	Dolutegravir	Rilpivirine Impurity	Dolutegravir Impurity
Retention Time	3.82	7.51	4.32	7.12
Theoretical plates per column	8125	10254	8034	11352
Tailing factor	1.18	2.13	1.25	1.82

3.1. Method validation

3.1.1. System Suitability Parameters

System suitability tests are used to verify that the resolution and repeatability of the system were adequate for the analysis intended. The parameters used in this test were the chromatographic peak, retention time, resolution, theoretical plate number and tailing factor. Results are shown in table no 11

Table 11 System Suitability Parameters

Parameters	Rilpivirine	Dolutegravir	Rilpivirine Impurity	Dolutegravir Impurity
Retention Time	3.82	7.51	4.32	7.12
Theoretical plates per column	8125	10254	8034	11352
Tailing factor	1.18	2.13	1.25	1.82

3.1.2. Specificity

The Chromatograms of Rilpivirine and Dolutegravir Impurity standards and Rilpivirine and Dolutegravir sample show no interference with the Chromatogram of Rilpivirine and Dolutegravir Blank, so the Developed method is Specific. Figure 4,5, 6.

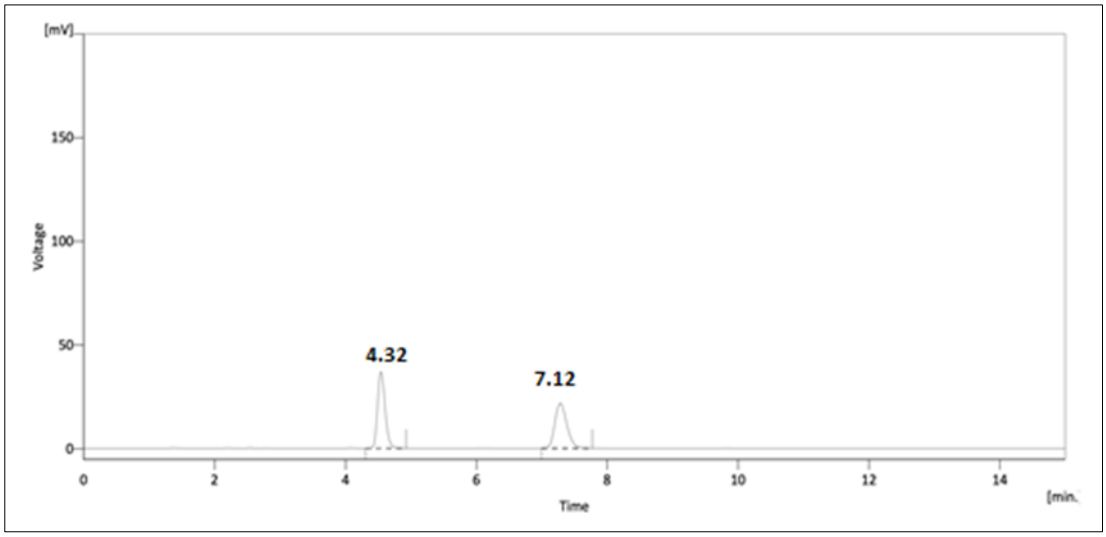


Figure 4 Chromatogram of Rilpivirine Impurity and Dolutegravir Impurity Standard

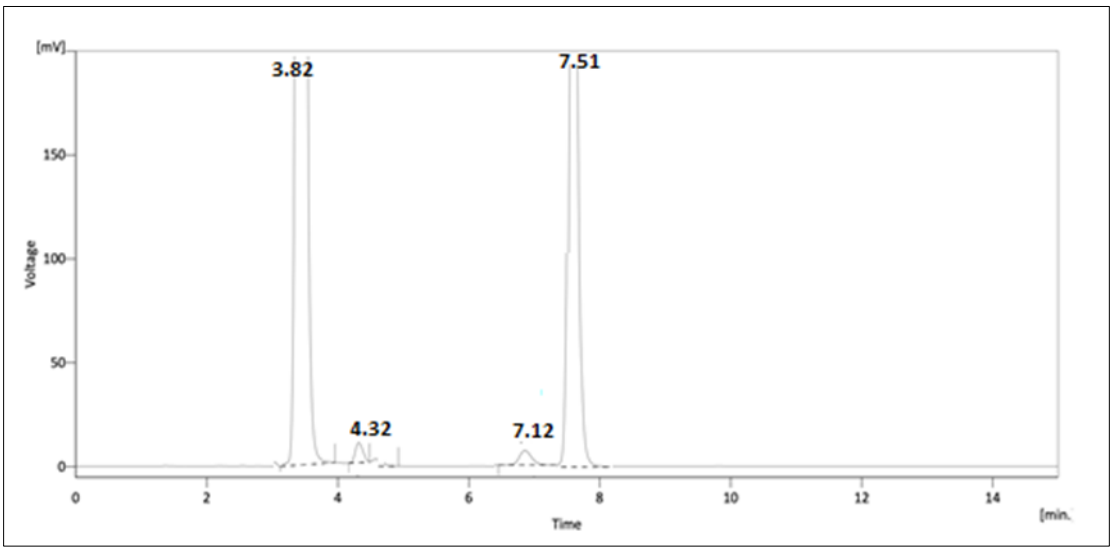


Figure 5 Chromatogram of Rilpivirine and Dolutegravir Sample

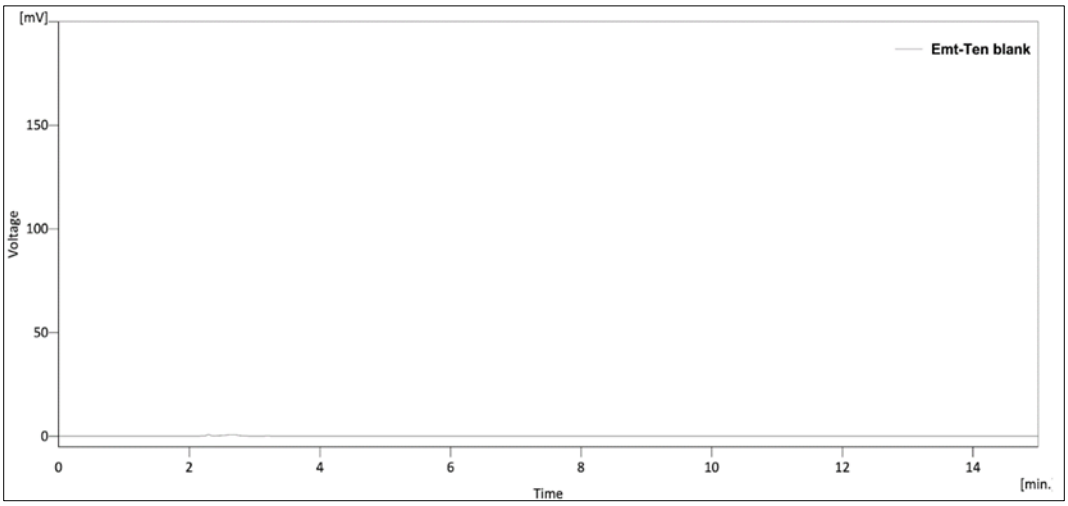


Figure 6 Chromatogram of Rilpivirine and Dolutegravir Blank

3.1.3. Linearity and Range

The linearity for Rilpivirine Impurity and Dolutegravir Impurity were assessed by analysis of combined standard solution in range of 5.0-15.0µg/ml and 7.5-22.5µg/ml respectively. Correlation co-efficient for calibration curve Rilpivirine Impurity and Dolutegravir Impurity was found to be 0.999 respectively. Figures are shown in 7, 8. Linear responses are shown in table 12, 13.

The regression line equation for Rilpivirine and Dolutegravir are as following:

For Rilpivirine Impurity: y = 29.763x – 1.68 and

For Dolutegravir Impurity: y = 18.62x – 2.25

Table 12 Linearity Data for Rilpivirine Impurity

Sr. No	Concentration (µg/ml)	Area
1	5	148.21
2	7.5	225.32
3	10	285.75
4	12.5	375.14
5	15	445.34

Table 13 Linearity Data for Dolutegravir Impurity

Sr. No	Concentration (µg/ml)	Area
1	7.5	135.62
2	11.25	210.58
3	15	277.45
4	18.75	343.16
5	22.5	418.46

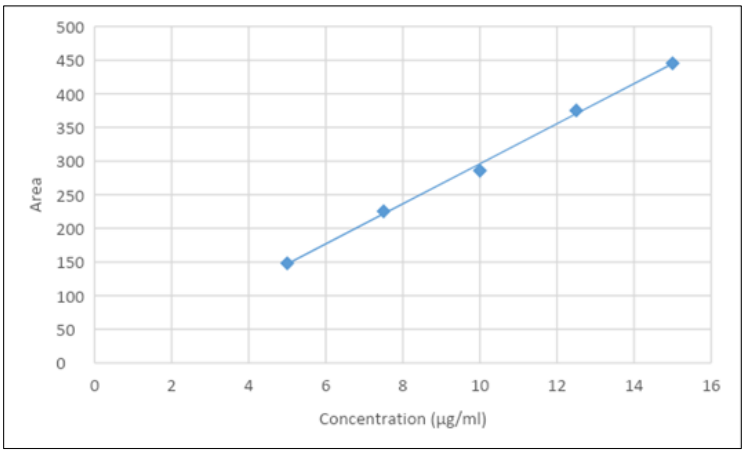


Figure 7 Calibration Curve of Rilpivirine Impurity

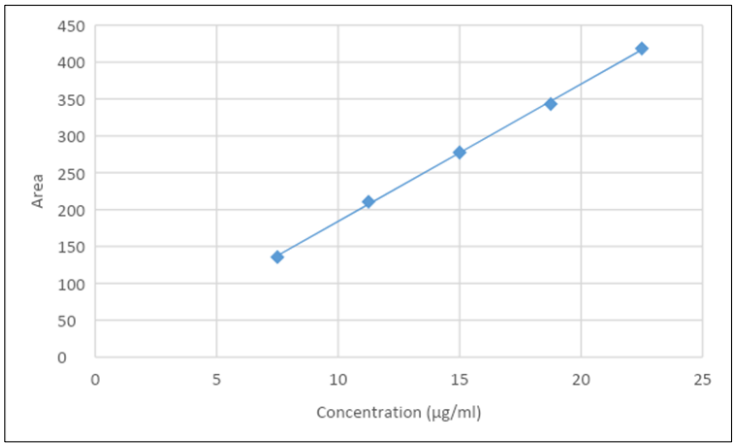


Figure 8 Calibration Curve of Dolutegravir Impurity

3.1.4. Precision

Repeatability

The data for repeatability of peak area measurement for Rilpivirine and Dolutegravir Impurity, based on six measurements of same solution of Rilpivirine and Dolutegravir Impurity are depicted in table 14 and 15. The % RSD for Rilpivirine Impurity and Dolutegravir Impurity was found to be 1.735 and 1.545 respectively.

Table 14 Repeatability Data for Rilpivirine Impurity

Rilpivirine Impurity				
Sr. No.	**Conc (µg/ml)**	**Area**	**Mean ± S.D (n=6)**	**% R.S.D**
1	10	270.356	278.315±4.829	1.735
		281.254		
		274.854		
		279.475		
		283.479		
		280.475		

Table 15 Repeatability data for Dolutegravir Impurity

Dolutegravir Impurity				
Sr No.	**Conc (µg/ml)**	**Area**	**Mean ± S.D (n=6)**	**% R.S.D**
1	15	295.124	299.109±4.622	1.545
		303.247		
		299.784		
		294.586		
		305.758		
		296.158		

Intraday precision

The data for intraday precision for Rilpivirine and Dolutegravir Impurity is shown in table 16. The % R.S.D. for Intraday precision was found to be 1.572-0.765 for Rilpivirine Impurity and 2.065-0.158 for Dolutegravir Impurity.

Table 16 Intraday precision data for Estimation of Rilpivirine and Dolutegravir Impurity

Sr. No.	**Rilpivirine Impurity**			**Dolutegravir Impurity**		
	Conc. (µg/ml)	Area Mean ± S.D. (n=3)	% R.S.D	Conc. (µg/ml)	Area Mean ± S.D. (n=3)	% R.S.D
1	LOQ	8.714 ± 1.137	1.572	LOQ	7.648± 0.158	2.065
2	5	271.245± 5.135	1.893	10	297.648±3.463	1.163
3	7.5	408.348± 3.124	0.765	15	444.754±4.785	1.075

Interday precision

The data for intraday precision for Rilpivirine and Dolutegravir Impurity is shown in table17. The % R.S.D. for interday precision was found to be 1.526-0.676 for Rilpivirine Impurity and 2.572-1.065 for Dolutegravir Impurity.

Table 17 Interday Precision data for Estimation of Rilpivirine and Dolutegravir Impurity

Sr. No.	**Rilpivirine Impurity**			**Dolutegravir Impurity**		
	Conc. (µg/ml)	**Area Mean ± S.D. (n=3)**	**% R.S.D**	**Conc. (µg/ml)**	**Area Mean ± S.D. (n=3)**	**% R.S.D**
1	LOQ	8.124 ± 0.124	1.526	LOQ	6.142 ± 158	2.572
2	5	274.145± 1.854	0.676	10	297.245± 5.160	1.736
3	10	417.752± 5.144	1.231	15	448.368± 4.775	1.065

3.1.5. Accuracy

Accuracy of the method was confirmed by recovery study from marketed formulation at three level of standard addition. The results are shown in table 18 and 19. Percentage recovery for Rilpivirine Impurity was 98.33-100.75%, while for Dolutegravir Impurity, it was found to be in range of 98.43-100.67 %.

Table 18 Recovery Data for Rilpivirine Impurity

Sr. No.	Conc. Level (%)	Amount Added (µg/ml)	Amount recovered (µg/ml)	% Recovery	% R.S.D
1	LOQ	0.3	0.301	100.33	1.026
2		0.3	0.297	99.00	
3		0.3	0.295	98.33	
4	80%	4	4.03	100.75	0.978
5		4	3.955	98.88	
6		4	3.974	99.35	
7	100%	5	5.035	100.70	1.019
8		5	5.022	100.44	
9		5	4.941	98.82	
10	120%	6	6.045	100.75	1.134
11		6	5.953	99.22	
12		6	5.913	98.55	

Table 19 Recovery Data for Dolutegravir Impurity

Sr. No.	Conc. Level (%)	Amount Added (µg/ml)	Amount recovered (µg/ml)	% Recovery	% R.S.D
1	LOQ	0.15	0.148	98.67	1.021
2		0.15	0.15	100.00	
3		0.15	0.151	100.67	
4	80%	8	7.985	99.81	0.919
5		8	8.012	100.15	
6		8	7.874	98.43	
7	100%	10	10.054	100.54	0.793
8		10	9.923	99.23	
9		10	9.912	99.12	
10	120%	12	12.012	100.10	0.351
11		12	11.946	99.55	
12		12	11.934	99.45	

3.1.6. LOD and LOQ

Calibration curve was repeated for five times and the standard deviation (SD) of the intercepts was calculated. Results are shown in table 20, 21 Then LOD and LOQ were calculated as follows:

LOD = 3.3 * SD/slope of calibration curve

LOQ = 10 * SD/slope of calibration curve

3.1.7. Limit of Detection

Table 20 Limit of Detection Data for Rilpivirine Impurity and Dolutegravir Impurity

Rilpivirine Impurity	Dolutegravir Impurity.
LOD = 3.3 x (SD / Slope) = 3.3 x (0.754/31.245) = 0.079 µg/ml	LOD = 3.3 x (SD / Slope) = 3.3 x (0.871/19.754) = 0.145 µg/ml

3.1.8. Limit of Quantitation

Table 21 Limit of Quantitation Data for Rilpivirine Impurity and Dolutegravir Impurity

Rilpivirine Impurity	Dolutegravir Impurity
LOQ = 10 x (SD / Slope) = 10 x (0.754/31.245) = 0.241 µg/ml	LOQ = 10 x (SD / Slope) = 10 x (0.871/19.754) = 0.441 µg/ml

3.1.9. Robustness

The effect of changes was found to be within the acceptance criteria as shown in below**.** The % RSD should be less than 5%. Results are shown in table 22, 23.

Table 22 Robustness Rilpivirine Impurity

Sr No.	Area at Flow rate (+ 0.2 ml/min)	Area at Flow rate (- 0.2 ml/min)	Area at pH (-0.2)	Area at pH (+0.2)	Area at Mobile phase(-2)	Area at Mobile phase(+2)
1	248.345	304.78	278.45	273.34	247.67	279.64
2	244.654	298.48	271.65	278.12	238.41	281.67
3	251.345	301.57	268.45	281.35	241.34	285.64
% R.S.D	1.351	1.044	1.872	1.452	1.952	1.081

Table 23 Robustness data for Dolutegravir Impurity

Sr No.	Area at Flow rate (+ 0.2 ml/min)	Area at Flow rate (- 0.2 ml/min)	Area at pH (-0.2)	Area at pH (+0.2)	Area at Mobile phase(-2)	Area at Mobile phase(+2)
1	245.32	296.45	275.68	278.57	248.67	284.76
2	248.35	304.85	274.36	276.43	235.47	279.41
3	251.36	301.24	270.65	273.48	243.71	281.46
% R.S.D	1.216	1.401	0.953	0.925	2.748	0.958

3.1.10. Calculation of Known Impurities of Rilpivirine and Dolutegravir

Applicability of the proposed method was tested by analyzing the commercially available Tablet formulation Ricovir-EM. The results are shown in below table 24.

Table 23 Calculation of Known Impurities of Rilpivirine and Dolutegravir

Impurity	Conc (µg/ml)	Area	% Impurity	% R.S.D
Rilpivirine		150.021	0.051	
	10	151.234	0.052	1.92
		153.75	0.053	
		98.568	0.036	
Dolutegravir	15	99.514	0.037	1.57
		99.148	0.037	

The results indicate that the developed method is accurate, precise, simple and rapid. It can be used in the routine quality control of dosage form in industries.

3.2. Method Validation Summary

Table 24 Summary of Validation Parameters for Rilpivirine and Dolutegravir Related Impurities

Sr. No.	Parameter		Rilpivirine	Dolutegravir
1	Specificity		Specific	
2	Linearity & Range		5.0-15.0	7.5-22.5
3	Regression equation		y = 29.763x – 1.68	y = 18.62x – 2.25
4	Correlation co-efficient (r^2)		0.9974	0.9994
5	Precision	Repeatability	1.735	1.545
	(% RSD)	Interday	1.572-0.765	2.065-0.158
		Intraday	1.526-0.676	2.572-1.065
6	Accuracy (% recovery)		98.33-100.75	98.43-100.67
7	Limit of Detection(LOD)		0.079 µg/ml	0.145 µg/ml
8	Limit of Quantification(LOQ)		0.241 µg/ml	0.441 µg/ml
9	Robustness (% RSD)		The system suitability parameters were found well within the acceptance criteria as per system suitability	

4. Discussion

A new Related Impurities RP-HPLC method has been developed for estimation of Rilpivirine and Dolutegravir Impurity in tablet dosage form was rapid, accurate, precise, economic and easy to perform. The linearity was investigated in the range of 5.0-15.0 µg/mL (r^2 = 0.9974) for Rilpivirine Impurity and 7.5-22.5 µg/ml (r^2 = 0.9994) for Dolutegravir Impurity. The LOD were 0.079µg/ml and 0.145µg/ml for Rilpivirine and Dolutegravir Related Impurities, respectively. The LOQ were 0.241µg/mL and 0.441µg/mL for Rilpivirine and Dolutegravir Related Impurities, respectively. This method was found to be simple, accurate, robust and reproducible.

5. Conclusion

- There is no analytical work has been available regarding Related Impurities RP-HPLC method for Rilpivirine and Dolutegravir in a literature. Data regarding behavior of drug and its related impurities in chromatographic conditions and other relevant analytical properties are not available.
- A novel attempt in a field of research has been made to develop and validate Related Impurities method via RP-HPLC.
- Rilpivirine is an antiretroviral drug of the Non-nucleoside reverse transcriptase inhibitors (NNRTIs) class used in combination with Dolutegravir which is used to treat infection of human immunodeficiency virus (HIV).
- RP-HPLC method was developed for simultaneous estimation Rilpivirine and Dolutegravir. In RP-HPLC method, good resolution and separation of two drugs and its related impurities was achieved Water: Methanol (70:30 v/v) was used as mobile phase.
- Retention time of Rilpivirine and Dolutegravir were found to be 3.82 and 7.51 min respectively with a flow rate of 1 ml/min. The proposed method was accurate and precise. Therefore proposed method can be used for routine analysis of Rilpivirine and Dolutegravir in tablets.
- The suitability, performance and applicability of developed method has been validated as per ICH guideline by applying various validation parameters like specificity, linearity and range, accuracy precision and robustness.
- The RP-HPLC method developed for the determination of related impurities of Rilpivirine and Dolutegravir is found to be specific, linear, sensitive, precise, accurate and robust in nature.
- The method was successfully validated in terms of specificity, precision, linearity, accuracy and robustness as per ICH guidelines.
- It can be concluded that the proposed method can be used for routine analysis for estimation of related impurities of Rilpivirine and Dolutegravir in combined dosage form by RP-HPLC.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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