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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF DOLUTEGRAVIR AND RILPIVIRINE IN PHARMACEUTICAL DOSAGE FORMS BY USING RP-HPLC METHOD

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ABSTRACT

A new, simple, precise, accurate and reproducible rp-hplc method for estimation of Dolutegravir and Rilpivirine in bulk and Pharmaceutical formulations. Separation of Dolutegravir and Rilpivirine was successfully achieved by using column like Inertsil ODS, column (200 x 4.6mm, 5µm) or equivalent in an isocratic mode utilizing mobile phase was optimized to 0.1% OPA : Acetonitrile in the proportion of 80: 20 v/v at a flow rate of 1.5ml/min and eluate was monitored at 230nm with a retention time of 3.417min and 4.392 for Dolutegravir and Rilpivirine. The method was validated and their response was found to be linear in the drug concentration range of 50µg/ml to150µg/ml for Dolutegravir and Rilpivirine. The values of the correlation coefficient were found to 0.999 for Dolutegravir and Rilpivirine. The LOD and LOQ for Dolutegravir was found to be 3.08 and 10.10 and LOD and LOQ for Rilpivirine was found to be 3 and 10.02 . This method was found to be good percentage recovery which indicates that the proposed method is highly accurate. This method was extensively validated according to ICH guidelines for accuracy, precision, linearity, robustness and system suitability

INTRODUCTION

Dolutegravir is an antiretroviral medication used, together with other medication, to treat HIV/AIDS. It may also be used as part of post exposure prophylaxis, to prevent HIV infection following potential exposure. Rilpivirine is chemically known as 4-{[4-({4-[(1E)-2-cyanoeth-1-en-1-yl]-2, 6-dimethylphenyl} amino) pyrimidin-2-yl] amino} benzonitrile . 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 diaryl 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.[(1)] Spectrophotometer, HPLC and HPTLC are the reported analytical methods for Compounds either individually or in combination with other dosage forms. Hence, it was felt that, there is a need of new analytical method development for the simultaneous estimation of Dolutegravir and Rilpivirine in pharmaceutical dosage forms. [(2-12)]

METHOD DEVELOPMENT:

Preparation of phosphate buffer: Take 1ml of Ortho phosphoric acid in 1000ml volumetric flask and make up with HPLC water and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Preparation of mobile phase: Accurately measured 800 ml (80%) of above Buffer and 200 ml (20%) of Acetonitrile were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation: The Mobile phase was used as the diluent.

Preparation of the dolutegravir & rilpivirine standard & sample solution:

Standard Solution Preparation: Accurately weigh and transfer 50 mg of Dolutegravir and 25 mg of Rilpivirine working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 3.0 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation: Accurately weigh and transfer equivalent to 50 mg of Dolutegravir and 25 mg of Rilpivirine sample into a 100 ml clean dry volumetric flask add about 7 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure: Inject 20 µL of the standard, sample into the chromatographic system and measure the areas for Dolutegravir and Rilpivirine peaks and calculate the % assay by using the formulae.

METHOD VALIDATION

ACCURACY

Preparation of Standard stock solution: Accurately weigh and transfer 50 mg of Dolutegravir and 25 mg of Rilpivirine working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 3.0 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation Sample solutions

For preparation of 50% solution (With respect to target Assay concentration): Accurately weigh and transfer 25 mg of Dolutegravir and 12.5 mg of Rilpivirine working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 3.0 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

For preparation of 100% solution (With respect to target Assay concentration) Accurately weigh and transfer 50 mg of Dolutegravir and 25 mg of Rilpivirine working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent(Stock solution).Further pipette 3.0 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

For preparation of 150% solution (With respect to target Assay concentration): Accurately weigh and transfer 75 mg of Dolutegravir and 37.5 mg of Rilpivirine working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).Further pipette 3.0 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure: Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions. Calculate the Amount found and Amount added for Dolutegravir & Rilpivirine and calculate the individual recovery and mean recovery values.

Acceptance Criteria: The % Recovery for each level should be between 98.0 to 102.0%

RESULT

Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated.

Acceptance Criteria: The percentage recovery was found to be within the limit (97-103%). The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

PRECISION

Preparation of stock solution: Accurately weigh and transfer 50 mg of Dolutegravir and 25 mg of Rilpivirine working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette 3.0 ml of the above stock solutions into

a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure: The standard solution was injected for six times and measured the area for all six. Injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Acceptance Criteria: The % RSD for the area of six standard injections results should not be more than 2%**.**

Acceptance criteria: %RSD for sample should be NMT 2. The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Intermediate Precision/Ruggedness: To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day.

Preparation of stock solution: Accurately weigh and transfer 50 mg of Dolutegravir and 25 mg of Rilpivirine working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette 3.0 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure: The standard solutions prepared in the precision was injected on the other day, for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Acceptance Criteria: The % RSD for the area of six standard injections results should not be more than 2%.

Acceptance criteria

%RSD of five different sample solutions should not more than 2

The %RSD obtained is within the limit, hence the method is rugged.

LINEARITY: Preparation of stock solution : Accurately weigh and transfer 50 mg of Dolutegravir and 25 mg of Rilpivirine working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I: 1ml of above stock solutions has taken in 10ml of volumetric flask, dilute up to the mark with diluent.

Preparation of Level – II : 2ml of above stock solutions has taken in 10ml of volumetric flask, dilute up to the mark with diluent.

Preparation of Level – III: 3ml of above stock solutions has taken in 10ml of volumetric flask, dilute up to the mark with diluent.

Preparation of Level – IV: 4ml of above stock solutions has taken in 10ml of volumetric flask, dilute up to the mark with diluent **.**

Preparation of Level – V : 5ml of above stock solutions has taken in 10ml of volumetric flask, dilute up to the mark with diluent **.**

Procedure: Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

Acceptance Criteria: Correlation coefficient should be not less than 0.999.

RESULT: The linearity range was found to lie from 50µg/ml to 250µg/ml of Dolutegravir, 25µg/ml to 125µg/ml of Rilpivirin and chromatograms are shown below.

Acceptance criteria: Correlation coefficient (R^2) should not be less than 0.999

The correlation coefficient obtained was 0.999 which is in the acceptance limit.

LIMIT OF DETECTION: (for Dolutegravir)

Preparation of 0.15 µg/ml solution: Accurately weigh and transfer 50 mg of Dolutegravir working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).Further pipette 3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Further pipette 0.1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Calculation of S/N Ratio: Average Baseline Noise obtained from Blank

Signal Obtained from LOD solution

S/N = 160/52 = 3.08

Acceptance Criteria: S/N Ratio value shall be 3 for LOD solution.

RESULT: The lowest concentration of the sample was prepared with respect to the base line noise and measured the signal to noise ratio. Signal to noise ratio shall be 3 for LOD solution

The result obtained is within the limit.

Limit of Quantification

Preparation of 0.50 µg/ml solution : Accurately weigh and transfer 50 mg of Dolutegravir working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette 3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. Further pipette 1 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Further pipette 0.33 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Calculation of S/N Ratio: Average Baseline Noise obtained from Blank

Signal Obtained from LOQ solution

S/N = 525/52 = 10.10

Acceptance Criteria

S/N Ratio value shall be 10 for LOQ solution.

LIMIT OF DETECTION: (for Rilpivirine)

Preparation of 0.11 µg/ml solution: Accurately weigh and transfer 25 mg of Rilpivirine working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. Further pipette 1 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents. Further pipette 0.15 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Calculation of S/N Ratio: Average Baseline Noise obtained from Blank

Signal Obtained from LOD solution

S/N = 156/52 = 3.00

Acceptance Criteria: S/N Ratio value shall be 3 for LOD solution.

Limit Of Quantification

Preparation of 0.36 µg/ml solution: Accurately weigh and transfer 25 mg of Rilpivirine working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. Further pipette 1 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Further pipette 0.48 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Calculation of S/N Ratio: Average Baseline Noise obtained from Blank

Signal Obtained from LOQ solution

S/N = 521/52 = 10.02

Acceptance Criteria: S/N Ratio value shall be 10 for LOQ solution.

Procedure for LOD and LOQ: The LOD and LOQ solutions was prepared injected, for three times and measured the area for all three injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Limit Of Quantification For Dolutegravir And Rilpivirine: The lowest concentration of the sample was prepared with respect to the base line noise and measured the signal to noise ratio.

Signal to noise ratio shall be 10 for LOQ solution

The result obtained is within the limit.

ROBUSTNESS: As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

A. The flow rate was varied at 1.35 ml/min to 1.65ml/min. Standard solution 150 ppm of Dolutegravir & 75 ppm of Rilpivirine was prepared and analysed using the varied flow rates along with method flow rate. On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.

Table 1(a): Accuracy (recovery) data for Dolutegravir

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	**Mean Recovery**
50%	809552.3	25	25.21	100.82	100.39
100%	1611682	50	50.18	99.36	
150%	2408440.7	75	74.99	99.98	

*Average of three determinations

Table 1(b): Accuracy (recovery) data for Rilpivirine

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	617877.7	12.5	12.59	100.75	100.04
100%	1224225.3	25	24.95	99.81	
150%	1831657.7	37.5	37.33	99.55	

*Average of three determinations

Table 2: Results of Precision for Dolutegravir and Rilpivirine

Injection	**Area for Dolutegravir**	**Area for Rilpivirine**
Injection-1	1610934	1228406
Injection-2	1609985	1223300
Injection-3	1619309	1213803
Injection-4	1608645	1201667
Injection-5	1610885	1228897
Injection-6	1618951	1220372
Average	1613118.2	1219407.5
Standard Deviation	4731.4	10327.1
%RSD	0.3	0.8

Table 3: Results of Intermediate precision for Dolutegravir and Rilpivirine

Injection	**Area for Dolutegravir**	**Area for Rilpivirine**
Injection-1	1604507	1214125
Injection-2	1594158	1210517
Injection-3	1591505	1212127
Injection-4	1601953	1211539
Injection-5	1598025	1219177
Injection-6	1604821	1203992
Average	1599161.5	1211912.8
Standard Deviation	5538.0	4950.5
%RSD	0.3	0.4

Table 4(a): Area of different concentration of Dolutegravir and Rilpivirine

S. No.	**Dolutegravir**		**Rilpivirine**	
	Concentration (µg/ml)	**Area**	**Concentration (µg/ml)**	**Area**
1	50	524876	25	380761
2	100	1059982	50	782401
3	150	1574201	75	1164038
4	200	2068062	100	1549472
5	250	2604868	125	1965315

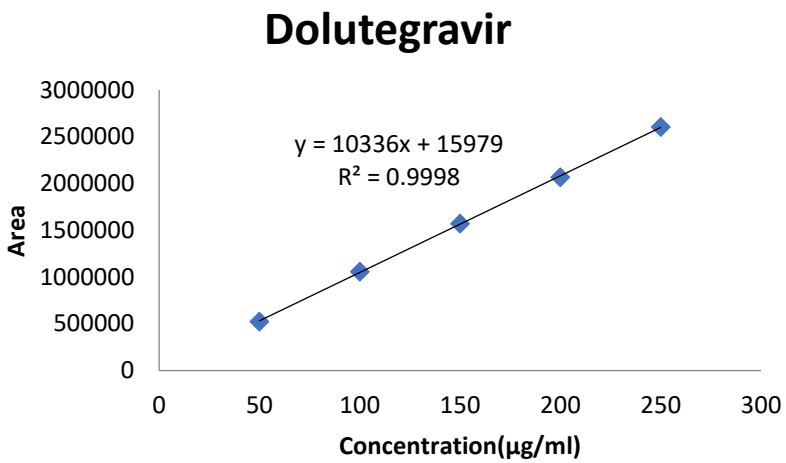

Fig.1 (a) : Linearity plot of Dolutegravir

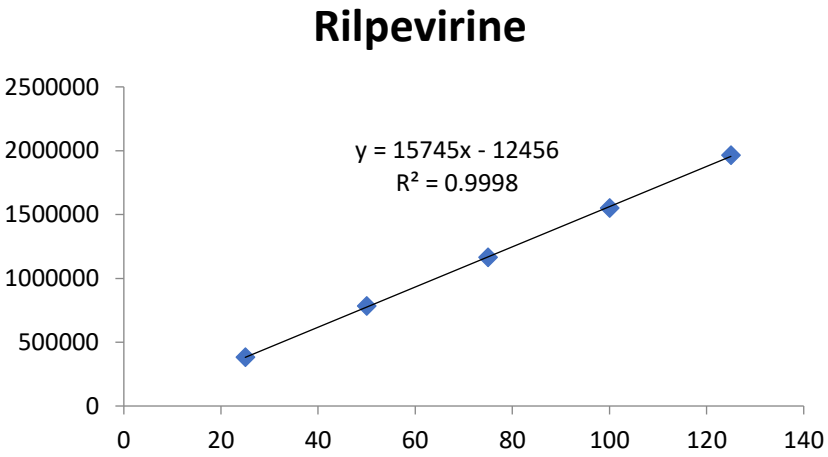

Fig.1(b) : Linearity plot of Rilpivirine

Table 4(b): Analytical performance parameters of Dolutegravir and Rilpivirine

Parameters	Dolutegravir	Rilpivirine
Slope (m)	10336	15745
Intercept (c)	15979	12456
Correlation coefficient (R^2)	0.999	0.999

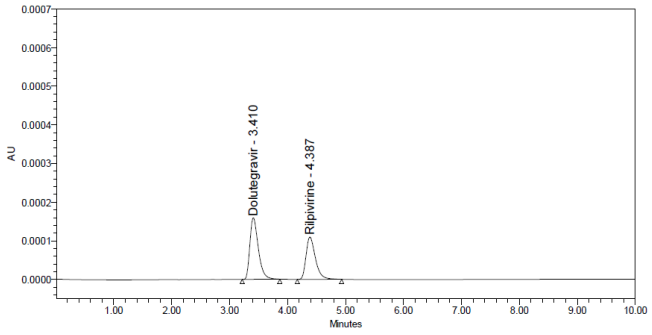

Fig.2 (a): Chromatogram of Dolutegravir, Rilpivirine showing LOD

Table 5(a): LOD values

Drug name	Baseline noise (µV)	Signal obtained (µV)	S/N ratio
Dolutegravir	52	160	3.08
Rilpivirine	52	156	3.00

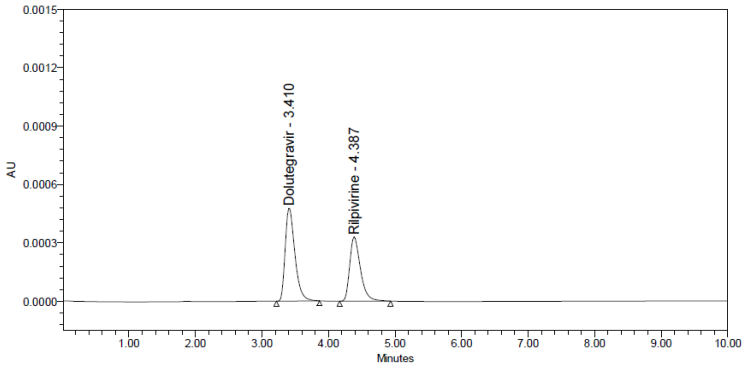

Fig.2(b): Chromatogram of Dolutegravir, Rilpivirine showing LOQ

Table 5(b): LOQ values

Drug name	Baseline noise (µV)	Signal obtained (µV)	S/N ratio
Dolutegravir	52	525	10.10
Rilpivirine	51	521	10.02

Table 6(a): Results for variation in flow for Dolutegravir

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	1.35	2630.90	1.45
2	1.5	2657.20	1.42
3	1.65	2611.73	1.35

Table 6(b): Results for variation in flow for Rilpivirine

S. No	Flow Rate (ml/min)	System Suitability Results		
		USP Resolution	USP Plate Count	USP Tailing
1	1.35	3.49	3726.83	1.45
2	1.5	3.52	3669.74	1.40
3	1.65	3.44	3385.64	1.30

***** Results for actual flow (1.5ml/min) have been considered from Assay standard

Table 6(c): Results for variation in mobile phase composition for Dolutegravir

S. No.	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	2569.17	1.39
2	*Actual	2657.20	1.42
3	10% more	2526.40	1.38

Table 6(d): Results for variation in mobile phase composition for Rilpivirine

S. No.	Change in Organic Composition in the Mobile Phase	System Suitability Results		
		USP Resolution	USP Plate Count	USP Tailing
1	10% less	3.48	3485.60	1.30
2	*Actual	3.52	3669.74	1.40
3	10% more	3.44	3416.12	1.34

*****Results for actual Mobile phase composition have been considered from Accuracy standard.

Table 6(e): Results for Stability of Dolutegravir and Rilpivirine

Sample Name	Dolutegravir		Rilpivirine	
	Area	% Degraded	Area	% Degraded
Standard	1602702		1224118	
Acid	1583722	1.18	1207822	1.33
Base	1528333	4.64	1173832	4.11
Peroxide	1558673	2.75	1146223	6.36
Thermal	1492533	6.87	1196732	2.24
Photo	1509356	5.82	1127897	7.86

B. The Organic composition in the Mobile phase was varied from ±10%.

Standard solution 150 ppm of Dolutegravir & 75 ppm of Rilpivirine was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method. On evaluation of the above results, it can be concluded that the variation in 10%.Organic composition in the mobile phase affected the method significantly. Hence it indicates that the method is robust even by change in the Mobile phase ±10

DEGRADATION STUDIES:

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the Dolutegravir and Rilpivirine using the proposed method.

Preparation of stock: Accurately weigh and transfer 50 mg of Dolutegravir and 25 mg of Rilpivirine working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Hydrolytic degradation under acidic condition: Pipette 3 ml of above solution into a 10ml volumetric flask and 3 ml of 0.1N HCl was added. Then, the volumetric flask was kept at 60ºC for 24 hours and then neutralized with 0.1 N NaOH and make up to 10ml with diluent. Filter the solution with 0.44 microns syringe filters and place in vials.

Hydrolytic degradation under alkaline condition: Pipette 3 ml of above solution into a 10ml volumetric and add 3ml of 0.1N NaOH was added in 10ml of volumetric flask. Then, the volumetric flask was kept at 60ºC for 24 hours and then neutralized with 0.1N HCl and make up to 10ml with diluent. Filter the solution with 0.44 microns syringe filters and place in vials.

Thermal induced degradation: Dolutegravir and Rilpivirine sample was taken in petridish and kept in hot air oven at 110^0 C fo 3 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analysed.

Oxidative degradation: Pipette 3 ml above stock solution into a 10ml volumetric flask and 1ml of 30% w/v of hydrogen peroxide added in 10 ml of volumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min. Filter the solution with 0.45 microns syringe filters and place in vials.

Photo degradation: Pipette 3 ml above stock solution into a 10ml volumetric flask and expose to sunlight for 24hrs and the volume was made up to the mark with diluent. Filter the solution with 0.45 microns syringe filters and place in vials.

Acceptance criteria: The Retention time, USP plate count, USP tailing factor obtained for change of flow rate, variation in mobile phase was found to be within the acceptance criteria. Hence the method is robust.
DEGRADATION STUDIES

SUMMARY AND CONCLUSION

The estimation of Dolutegravir and Rilpivirine was done by rp-hplc. The assay of Dolutegravir and Rilpivirine was performed with tablets and the % assay was found to be 100.08 and 99.94 which shows that the method is useful for routine analysis. The acceptance criteria of intermediate precision should be not more than 2.0% and the method show precision of 0.3 and 0.4 for Dolutegravir and Rilpivirine which shows that the method is repeatable when performed in different days also. The accuracy limit is the percentage recovery should be in the range of 97.0% - 103.0%. The total recovery was found to be 100.39% and 100.04% for Dolutegravir and Rilpivirine. The validation of developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility. The acceptance criterion for LOD and LOQ is 3 and 10. The LOD and LOQ for Dolutegravir was found to be 3.08 and 10.10 and LOD and LOQ for Rilpivirine was found to be 3 and 10.02 . The robustness limit for mobile phase variation and flow rate variation are well within the limit, the percentage degradation results are in limits which shows that the method is having good system suitability and precision under given set of conditions.

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