ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF DOLUTEGRAVIR AND RILPIVIRINE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN BULK AND TABLET DOSAGE FORMS

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ABSTRACT

A new simple, precise, robust, rapid and accurate reversed-phase high performance liquid chromatography method for the simultaneous determination of Dolutegravir and Rilpivirine was developed and validated as per ICH Guidelines. Chromatography was carried out by isocratic technique on a X-Bridge C_{18} column (150 x 4.6 mm, 3.5µm) Column with mobile phase mixture of Phosphate Buffer: Acetonitrile (65:35% v/v) was used as a mobile phase and the pH was adjusted into 4.7 by using with ortho-phosphoric acid, at a flow rate of 1.0 ml/min. The UV range was detected at 286 nm for Dolutegravir and Rilpivirine respectively. The different analytical performance parameters such as linearity, precision, accuracy, and specificity, limit of detection (LOD) and limit of quantification (LOQ) were determined according to International Conference on Harmonization ICH Q2B guidelines. The linearity of the calibration curves for each analyte in the desired concentration range is good (r2 >0.9). The recovery of the method was between 98% and 120% for Dolutegravir and Rilpivirine respectively. Hence the proposed method is highly sensitive, precise and accurate and it successfully applied for the reliable quantification of API content in the commercial formulations of Dolutegravir and Rilpivirine.

Keywords: Dolutegravir and Rilpivirine, RP-HPLC, ICH Guidelines, Accuracy, Precision.

INTRODUCTION

High performance liquid chromatography

High-performance liquid chromatography (HPLC) [5] is the fastest growing analytical technique for analysis of drugs. Its simplicity, high specificity and wide range of sensitivity make it ideal for the analysis of many drugs in both dosage forms and biological fluids.

The HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages.

- Speed (many analysis can be accomplished in 20 min or less)
- Greater sensitivity (various detectors can be employed)
- Improved resolution (wide variety of stationary phases)
- Reusable columns (expensive columns but can be used for many analysis)
- Ideal for the substances of low viscosity
- Easy sample recovery, handling and maintenance.
- Instrumentation leads itself to automation and quantification (less time and less labour)
- Precise and reproducible
- Integrator itself does calculations.

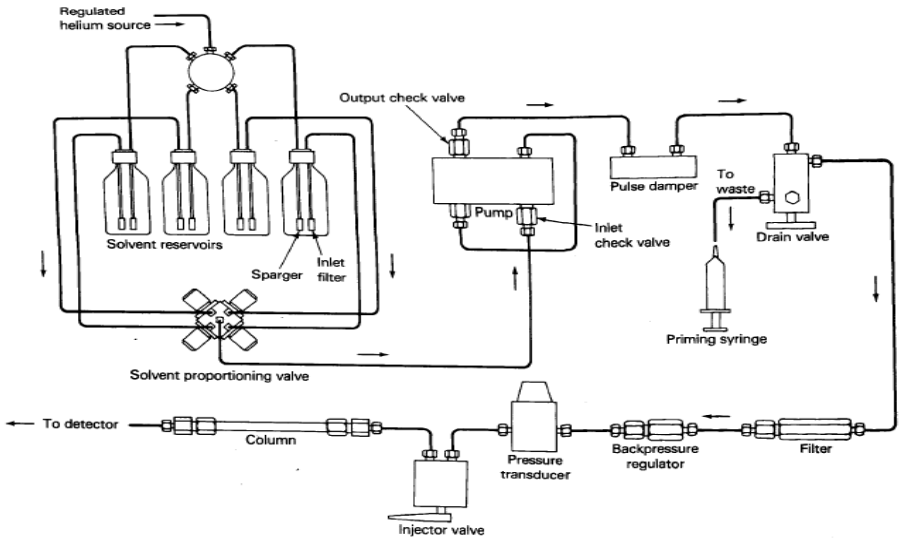

Fig-: Typical diagram of HPLC

HPLC Method Development:[5]

A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result. Finally method development should be as simple as possible, and it should allow the use of sophisticated tools such as computer modelling. During initial method development, a set of initial conditions (detector, column, mobile phase) is selected to obtain the first "scouting" chromatograms of the sample. In most cases, these are based on reversed-phase separations on a C_{18} column with UV detection. The important factors, which are to be taken into account to obtain reliable quantitative analysis, are

- Careful sampling and sample preparation.
- Appropriate choice of the column.
- Choice of the operating conditions to obtain the adequate resolution of the mixture.
- Reliable performance of the recording and data handling systems.
- Suitable integration/peak height measurement technique.
- The mode of calculation best suited for the purpose.
- Validation of the developed method.

Before beginning method development, it is need to review what is known about the sample in order to define the method goals.

Analytical method validation[4,5]

Method validation can be defined as per ICH,"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". For chromatographic methods used in analytical applications there is more consistency in validation practice. The Q (2) R1 of ICH guidelines for validation for analytical procedures includes various parameters.

MATERIALS

Dolutegravir and Rilpivirine (API), Potassium dihydrogen ortho phosphate, Ortho phosphoric Acid, HPLC Grade Methanol, HPLC Grade Acetonitrile, Double Distilled Water. Commercial formulations (tablets) containing Dolutegravir and Rilpivirine 25 and 50mg were procured from the local market.

METHODOLOGY :

METHOD DEVELOPMENT

Preparation of standard stock solution

Accurately weigh and transfer 25 mg of Dolutegravir and 50mg of Rilpivirineworking standard into a 100ml clean dry volumetric flask add about 70ml of Diluents (mobile phase) and sonicated to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 1ml of Dolutegravir & Rilpivirinethe above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of sample drug solution from pharmaceutical dosage form

Twenty tablets containing the drug were taken and powdered. The powder equivalent to 25mg of the tablet was accurately weighed and taken in a 100ml volumetric flask and mobile phase was add up to 70 ml and sonicated for 45 minutes to effect complete dissolution of drug and the solution was made up to volume with mobile phase and filtered through Whatman filter paper (0.45 µm) made up of cellulose nitrate. Aliquots solutions were prepared by taking 4ml and of the filtered solution into 10ml volumetric flasks, separately and made up to volume with mobile phase to yield concentrations of Dolutegravir and Rilpivirine in range of linearity previously described.

Preparation of mobile phase

Weigh 7.0 grams of Potassium dihydrogen ortho phosphate in to a 1000ml beaker, dissolve and diluted to 1000ml with HPLC water. Adjusted the pH to 7 with ortho - phosphoric acid. Mix a mixture of above buffer 650mL (65%) and 350mL of Acetonitrile HPLC (35%) and degas in ultrasonic water bath for 5 minutes. Filter through 0.45 µ filter under vacuum filtration.

Optimized Chromatographic conditions

Mobile phase **:** Phosphate buffer pH 7: Acetonitrile (65:35)

Flow rate **:** 1.0ml/min

Column **:** XBridgeC_{18} column (150 x 4.6 mm, 3.5µ)

Detector wave length **:** 286nm

Column temperature	**:**	Ambient
Injection volume	**:**	20µl
Run time	**:**	15min
Diluent	**:**	Mobile phase

METHOD VALIDATION

Evaluation of System Suitability:

The HPLC system was stabilized for thirty min by the above optimized Chromatographic conditions to get a stable base line. One blank followed by six replicates of a single standard solution was injected to check the system suitability. The column efficiency as determined from drug peak is not less than 3000 USP plate count and the tailing factor for drug peak is not more than 2.0.

The relative standard deviation for the peak areas of the six replicate injections is not more than 2.0 %.

A) For bulk samples:

Separately inject 20µl of the Blank, and Standard stock (five injections) into the liquid chromatography and record the chromatograms. The figure was shown in Fig.The retention time and average peak areas were recorded. Calibration graph was plotted by taking concentration of Dolutegravir and Rilpivirine on X-axis and peak areas on Y-axis.

B) For pharmaceutical formulations:

The content of twenty tablets was accurately weighed. From this powder an amount equivalent to 10 mg was taken and the drug was extracted in 10 ml of mobile phase as diluent by sonication for a period of 5min. This solution was filtered through 0.45 µm nylon disc filter. The filtered solution was suitably diluted for analysis and injected into the liquid chromatography and the chromatogram was recorded.

SPECIFICITY

The effect of wide range of excipients and other additives usually present in the formulations in the determinations under optimum conditions were investigated. The excipients present in Dolutegravir

and Rilpivirine have been added to the sample solution and injected and showed no peaks were observed at the retention time of both drugs and also over the range of 9.0min.

LINEARITY

The linearity of Dolutegravir and Rilpivirine responses was determined by preparing and injecting solutions at concentrations in the range of 12.50-37.50µg/mL and 25-75µg/mL respectively. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient. The data of the calibration curve was given in Table.

PRECISION

The precision of the method was ascertained from the peak areas of five replicate injections of a fixed standard concentration. Initially five sets of standard solution having a middle concentration were prepared by using standard stock solution. Inject 20 µL of the above solutions & record their respective peak areas.

ACCURACY

To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (50%, 100%, and 150%) of bulk samples of Dolutegravir and Rilpivirine to pre-analyzed amount of formulation. From this percentage recovery values were calculated.

ROBUSTNESS

Robustness of the method reflects the reliability of an analysis with respect to deliberate variations in the method parameters. Here, the flow rate and mobile phase composition were slightly changed to lower and higher sides of the actual values to find if the change in the peak area and retention time were within limits.

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ANALYSIS OF MARKETED FORMULATION

The assay of Dolutegravir and Rilpivirine were calculated by comparing the area of standard solution and tablet sample along with the consideration of average tablet weight and weight of powder bled taken and dilution factor if any. The assay was found to be within the limits and the present LC conditions can be used for the assay of Dolutegravir and Rilpivirine in different commercially available Marketed formulations.

RESULTS AND DISCUSSION

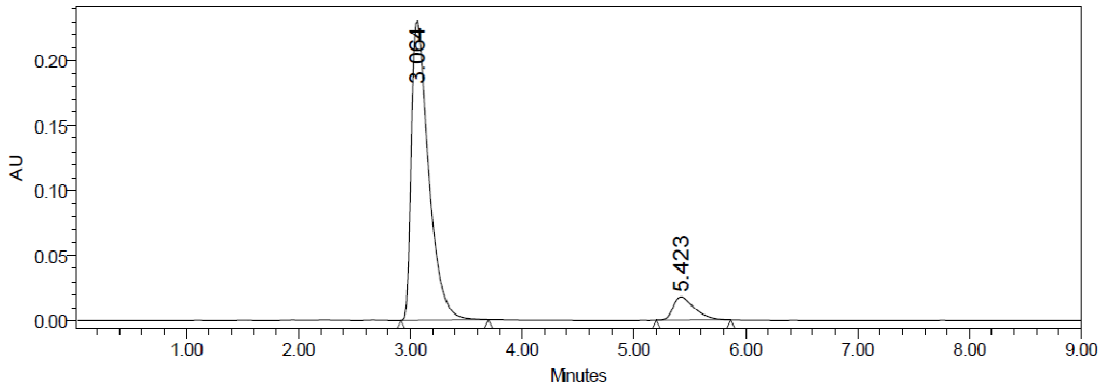

Fig-: Chromatogram for Optimized Chromatogram

Observation: The Resolution of peaks below 2.The retention time, good peak shapes and symmetry. Hence this method was finalized for simultaneous estimation of Dolutegravir and Rilpivirine.

METHOD VALIDATION

SPECIFICITY

The effect of wide range of excipients and other additives usually present in the formulations in the determinations under optimum conditions were investigated. The excipients present in Dolutegravir and Rilpivirine have been added to the sample solution and injected and showed no peaks were observed at the retention time of both drugs and also over the range of 9.0min.

Acceptance Criteria:

- Peak purity values should be greater than 0.999.

- Purity angle should be less than purity threshold without having any signs of purity flags.

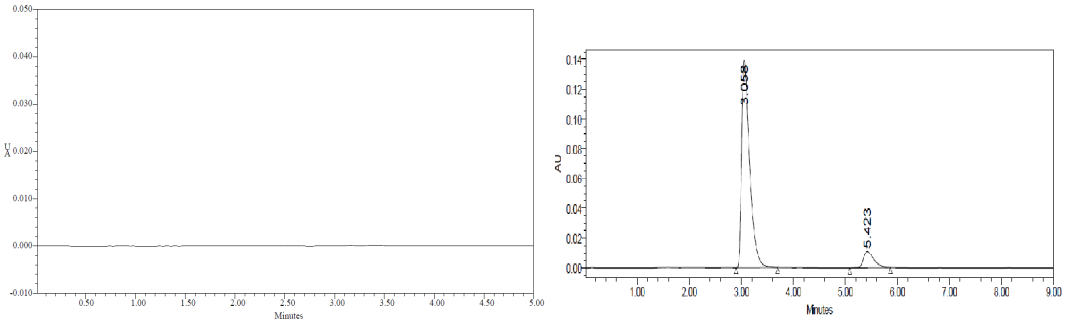

Fig: Typical Chromatogram of Blank SolutionFig:Standard Chromatogram of Dolutegravir and Rilpivirine

Observation:

- ✓ The placebo was not showed any drug peaks or interference peak during study and Peak purity values should be within the limits.

LINEARITY

Table : Linearity Range of Dolutegravir and Rilpivirine

S.No.	Linearity Level	Conc. (µg/ml)	Area of Dolutegravir	Conc.(µg/ml)	Area of Rilpivirine
1	I	12.50	1104857	25	114953
2	II	18.75	1629849	37.50	170406
3	III	25	2147234	50	223417
4	IV	31.25	2682376	62.50	277343
5	V	37.50	3207271	75	329787
Correlation Coefficient			**0.999**		**0.999**

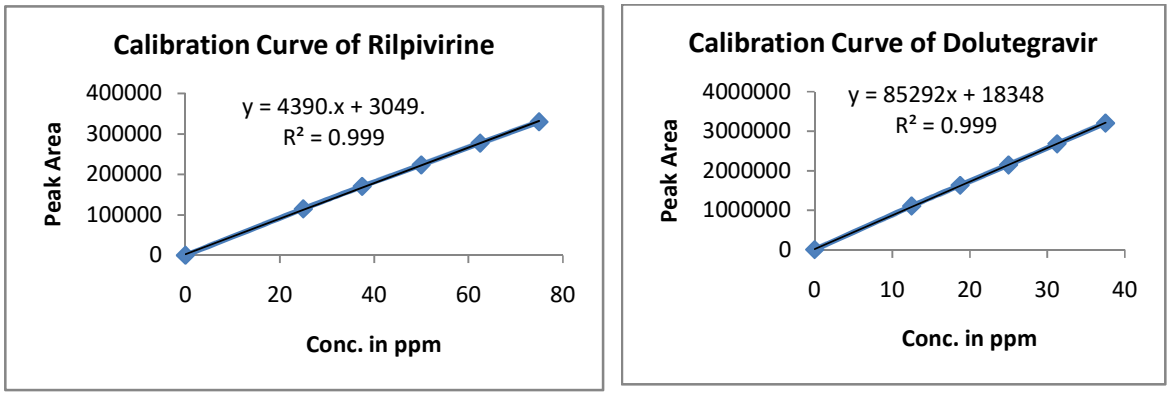

Fig: Linearity plot of RilpivirineFig: Linearity plot of Dolutegravir

Observations:

- ✓ The correlation coefficient and %RSD was found to be within the limits.

PRECISION

Repeatability:

Table. Day-1:System precision

Injection	**Area of Dolutegravir**	**Area of Rilpivirine**
Injection-1	2185685	205865
Injection-2	2187547	206587
Injection-3	2185659	205479
Injection-4	2187462	206857
Injection-5	2185974	207859
Average	2186465.4	206529.4
Standard Deviation	957.0592	925.1004
%RSD	0.043772	0.447927

Table Day-2: Method Precision

Injection	**Area of Dolutegravir**	**Area of Rilpivirine**
Injection-1	2098546	199858
Injection-2	2089878	198758
Injection-3	2098568	198659
Injection-4	2087547	199667
Injection-5	2078989	196858
Injection-6	2085478	197456
Average	2089834	198542.7
Standard Deviation	7668.152	1189.376

%RSD	0.366926	0.599053

ACCURACY

Table: Accuracy Table for Dolutegravir

% Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	1090448	12.5	12.569	100.552%	100.31%
100%	2155784	25	25.060	100.240%	
150%	3221344	37.50	37.553	100.141%	

Table: Accuracy Table for Rilpivirine

%Concentration(at specification Level)	Area	Amount Added(mg)	Amount Found(mg)	%Recovery	Mean Recovery
50%	112845	25	25.010	100.040	100.285%
100%	223584	50	50.235	100.470	
150%	333437	75	75.259	100.345	

ROBUSTNESS

Table-: Robustness Data of the RP-HPLC Method at Different flow rate for Dolutegravir

S. No.	Parameter	Condition	Theoretical Plates	Tailing Factor
1	Flow rate (ml/min)	0.8	3854	1.59
		1.0	**3675**	**1.48**
		1.1	4158	1.52
2	Mobile phase ratio	45:55	3746	1.54
		35:65	**3675**	**1.48**
		25:75	3896	1.46

Table-: Robustness Data of the RP-HPLC Method at Different Flow rate for Rilpivirine

S. No.	Parameter	Condition	Theoretical Plates	Tailing Factor
1	Flow rate (ml/min)	0.8	3727	1.56
		1.0	**3675**	**1.48**
		1.1	3715	1.47
2	Mobile phase ratio	45:55	3553	1.73
		35:65	**3675**	**1.48**
		25:75	3029	1.62

Observation:

- ✓ 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 not robust even by change in the flow rate ±10% and method is robust only in less flow condition.

SYSTEM SUITABILITY

System suitability parameters 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 has been completed. The USP (2000) defines parameters that can be used to determine system suitability prior to analysis. The system suitability parameters like Theoretical plates (N), Asymmetry (A), LOD (µg/ml) and LOQ (µg/ml) were calculated and compared with the standard values to ascertain whether the proposed RP-HPLC method for the estimation of ESO and NAP in pharmaceutical formulations was validated. The results were given in table 3.6.

Acceptance Criteria

- Theoretical plate should be N > 2000.
- Tailing factor should be T < 2.

Table: System Suitability Parameters

Parameters	Obtained Values	
	Dolutegravir	Rilpivirine
Theoretical plates (N)	3675	2896
Asymmetry (A_s)	1.48	1.79
LOD (µg/ml)	0.07	0.21
LOQ (µg/ml)	0.09	0.27

ANALYSIS OF FORMULATION

Table. Analysis of Commercial Formulation

Formulation	Labelled Amount (mg)		% Recovery by Proposed Method		%RSD	
	DOLU	RILP	DOL	RILP	DOLU	RILP
JULUCA	50	25	99.86 ± 0.08	100.05 ±0.07	0.09	0.06

CONCLUSION

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of Dolutegravir and Rilpivirine in bulk drug and pharmaceutical dosage forms. Dolutegravir is soluble in organic solvents such as DMSO and dimethyl formamide (DMF), which should be purged with an inert gas. Dolutegravir is also slightly soluble in ethanol. Rilpivirine is soluble in organic solvents such as DMSO and dimethyl formamide (DMF), which should be purged with an inert gas. Rilpivirine is sparingly soluble in aqueous buffers.

Phosphate buffer pH 7: Acetonitrile (65:35v/v) was chosen as the mobile phase. The solvent system used in this method was economical. The %RSD values were within 2 and the method was found to be precise.The results expressed inTablesfor RP-HPLC method was promising. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods.This method can be used for the routine determination of Dolutegravir and Rilpivirine in bulk drug and in Pharmaceutical dosage forms.

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