Development Of New Simple Validated Rp- HPLC Method for The Estimation of Dolutegravir in Bulk and Tablet Dosage Form

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Abstract - A rapid, selective, precise, simple and accurate Reverse Phase High Performance Liquid Chromatographic method has been developed and validated for the estimation of Dolutegravir in bulk and tablet formulation. The chromatographic separation was achieved by using Symmetry C_{18} (4.6 X 150 mm; 5µm Waters) with the mobile phase comprising of methanol: water in the ratio of 60: 40 v/v. The flow rate was 1ml/min and the separated Dolutegravir was detected by UV detections at 240 nm. The retention time of Dolutegravir was found to be 2.273 minutes. The column temperature was 30±0.8ºC with injection volume of 10µl. The linearity data showed good linear relationship within the concentration range of 10-50 µg/ml and the regression coefficient was found to be r2= 0.9998. The method obeyed ICH guidelines. The method was successfully validated in accordance to the ICH guidelines for accuracy, precision, specificity, linearity, system suitability, LOD & LOQ. The proposed method was found to be sensitive, accurate, precise, economic, reproducible and consistent.

Keywords: Dolutegravir, RP-HPLC, validation, method development.

I. INTRODUCTION: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography is a very sensitive analytical technique most widely used for quantitative and qualitative analysis of pharmaceuticals. The principal advantage of HPLC compared to classical column chromatography is improved resolution of the separated substance, faster separation times and the increased accuracy, precision and sensitivity[8].

Principle of Separation and its type

There are four types of chromatography in which the mobile phase is a liquid. The mobile phase is pumped through the packed column, under high pressure.

a. Partition chromatography
   i. Normal phase chromatography
   ii. Reverse phase chromatography
b. Adsorption or liquid solid chromatography
c. Ion exchange chromatography
d. Size exclusion or gel permeation chromatography

Normal Phase Chromatography

In normal phase mode, the stationary phase (e.g. silica gel) is polar in nature and the mobile phase is non-polar in this technique, non-polar compounds travel faster and are eluted first. This is because less affinity between solute and stationary phase. Polar compounds are retained for longer time in the column because more affinity towards stationary phase and takes more time to be eluted from the column. This is not advantageous in pharmaceutical applications since most of the drug molecules polar in nature and takes

longer time to be eluted and detected. Hence this technique is not widely used in pharmacy.

Solute Polarity
Adsorption strengths increased with increasing solute polarity and this is favorable interaction between the polar solutes and the polar stationary phase increases the elution time (note:-the interaction strength not only depends on the functional groups in the enlight molecule, but also stearic factors).

Reverse phase chromatography
In RP-HPLC the stationary phase is non-polar often a hydrocarbon and the mobile phase is relatively polar such as water, methanol or Acetonitrile. In RPC the solutes are eluted in the order of their decreasing polarities. These are prepared by treating the surface of silanol group with an organochlorosilane reagent.

Reverse phase mode
Non-polar stationary phase and polar mobile phase is used here.

Mechanism
Retention by interaction of the stationary phase non-polar hydrocarbon chain with non-polar parts and sample molecules.

Adsorption or liquid solid chromatography
The principle of separation is adsorption; separation of components takes place because of difference in affinity of compounds towards stationary phase. This principle is seen in normal phase as well as reverse phase mode, where adsorption will take place.

Dolutegravir is chemically designated as Isopropyl (4R,12aS)-N-(2,4-difluorobenzyl)-7-hydroxy-4-methyl6,8-dioxo-3,4,6,8,12,12a-hexahydro-2H-pyrido [1',2':4,5] pyrazino[2,1-b][1,3] oxazine-9-carboxamide. Its molecular formula is C20H19F2N3O5, and its molecular weight is 441.37 g/mol.

Dolutegravir is an HIV-1 antiviral agent. It inhibits HIV integrase by binding to the active site and blocking the strand transfer step to retroviral DNA integration. This is an essential step of the HIV replication cycle and will result in an inhibition of viral activity. Dolutegravir has a mean EC50 value of 0.5 nM(0.21 ng/mL) to 2.1 nM (0.85 ng/mL) in peripheral blood mononuclear cells (PBMCs) and MT-4 cells.

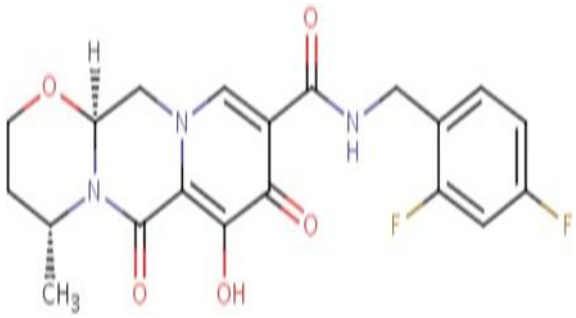

Fig: 1 Dolutegravir Structure

Based on new evidence assessing benefits and risks, the WHO recommends the use of the ANTI- HIV drug Dolutegravir (DTG) as the preferred first-line and second-line treatment for all populations, including pregnant women and those of childbearing potential.

II. MATERIALS AND METHODS

Chemicals and Reagents: Pharmaceutical grade (>99%) drug was obtained as gift sample from Aadhar Life Sciences Pvt. Ltd., (Solapur, Maharashtra State, India). Water and Methanol (HPLC grade) were obtained from Merck Specialties Pvt. Ltd., Mumbai, India. All other chemical of analytical grade were procured from local sources unless specified. Tablet Instgra 50 mg Emcure Pvt. Ltd. was purchased from local market.

Instruments Used:
Waters HPLC with Alliance with Auto sampler with Empower 2.0 software Column: Waters C_{18}, 150 X 4.6mm, 5µm, Labindia pH – Meter Vaccum Pump, Afcoset Digital Balance.

Selection of wavelength: (λ_{max})
From the UV Visible spectrophotometric results, the detection wavelength of 240 nm (for Dolutegravir) was selected because at this wavelength they show maximum absorbance. At 240 nm the chromatogram was observed in PDA detector. So the chromatographic condition was optimized at 240 nm.

UV Spectrum for Dolutegravir:

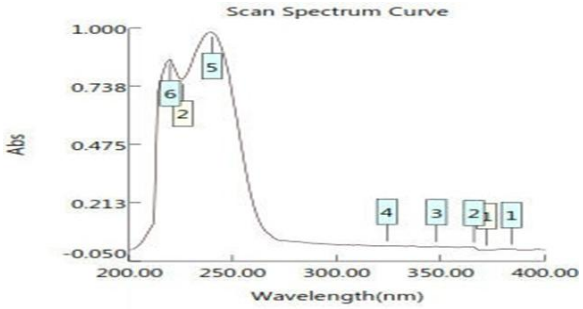

Fig : 2 UV Spectrum for Dolutegravir

Selection of initial chromatographic conditions:
Appropriate selection of chromatographic method depends upon the characteristic nature of the sample (ionic or ionisable or neutral), its molecular weight and solubility. The nature of Dolutegravir is polar. Hence reverse phase chromatography is used. The reverse phase HPLC was selected for initial chromatographic condition because of its simplicity and suitability[1].

Optimized Chromatographic conditions:
Column : Symmetry C_{18} (4.6 X 150 mm; 5µm Waters).
Column temperature : 250C
Flow rate : 1 ml/min.
Injection volume : 20 µl.
Wavelength : 240nm.
Run time : 10 min.
Diluent : mobile phase.
Mobile phase composition : methanol: water (60:40%v/v). Injector : Rheodyne.
Stationary phase : C_{18} (4.6 X 150 mm; 5µm Waters)
Operating temperature : Room temperature.

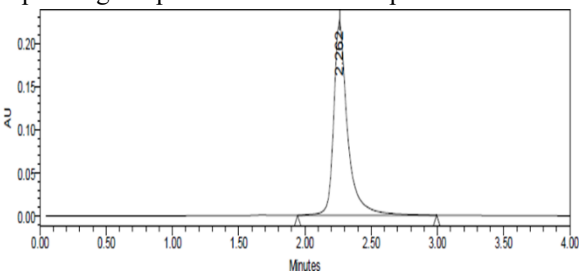

Fig:3 Optimized Chromatogram

III.ASSAY OF PROPOSED METHOD

Preparation Mobile phase:
Mix a mixture of above methanol (60%), 400 mL of HPLC water (40%) and degas in ultrasonic water bath for 5 minutes. Filter through 0.45 µ filter under vacuum filtration[2, 3].

Standard Solution Preparation
Accurately weigh and transfer 10 mg of Dolutegravir working standard into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation:
Accurately weigh and transfer equivalent to 368.0 mg of Dolutegravir sample into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)[4].

Further pipette 0.3ml of Dolutegravir of the above stock solution 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 peaks and calculate the %Assay by using the formulae.

System Suitability:
Tailing factor for the peaks due to Dolutegravir in Standard solution Should not be more than 2.0 Theoretical plates for the Dolutegravir peaks in Standard solution Should not be less than 2000

VALIDATION PARAMETERS

S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	2396.0	1.3
2	*Actual	2804.8	1.5
3	10% more	2218.0	1.4

Table 1: SYSTEM SUITABILITY PARAMETERS

Results for actual Mobile phase composition (60:40 methanol: water) have been considered from Accuracy standard.

ACCURACY

Sample preparation:

The accuracy shell be carried out using samples prepared for assay accuracy studies was conducted using triplicate determination as per the test method[6,7]

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	823686.2	5.0	5.0	100.1%	99.5%
100%	1634793	10	9.93	99.3%	
150%	2451939	15.0	14.9	99.3%	

Table 2: Accuracy for Dolutegravir

Linearity and Range: Standard preparation:

Dolutegravir working standard solutions were prepared across the range of the analytical method with a minimum of 5 concentrations that are within the specified range (10-50 µg/ml) low level (10 µg/ml)and higher level (50µg/ml) for 5 replicating injections were taken and calculated the %RSD

The degree of linearity was estimated by calculating the correlation coefficient, Y-intercept, slope of the regression line and residue some of squers a plot of data for analyte response Vs its concentration was established.

S.No	Linearity Level	Concentration	Area
1	I	10ppm	682741
2	II	20ppm	1201305
3	III	30ppm	1627183
4	IV	40ppm	2180552
5	V	50ppm	2716958
Correlation Coefficient			0.999

Table 3: Linearity Data for Dolutegravir

Parameters	Dolutegravir
Linearity Range	10-50 µg/ml
Correlation Coefficient	0.999
Slope (m)	

Table 4: Linearity Data for Dolutegravir

Linearity curve for Dolutegravir:

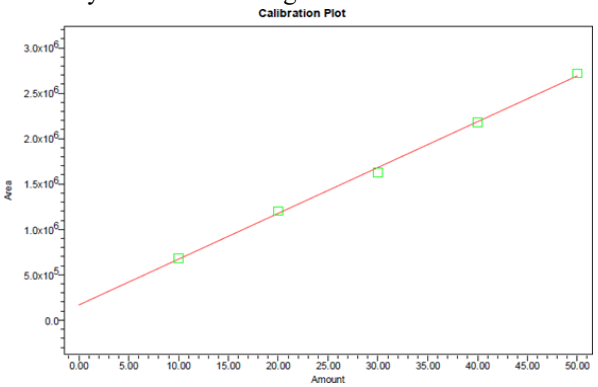

Fig 4 Linearity curve

PRECISION

Precision:

The system precision of the test method was performed by injecting 5 replicate determination of standard preparation injections were injected and the % RSD was calculated.

For Dolutegravir:

Injection	Area
Injection-1	1631295
Injection-2	1630511
Injection-3	1636464
Injection-4	1628557
Injection-5	1635684
Average	1632502.2
Standard Deviation	3420.4
%RSD	0.2

Table 5 Precision readings

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

Intermediate Precision:
The system precision of the test method was performed by injecting 5 replicate determination of standard preparation injections were injected and the % RSD was calculated

Injection	Area
Injection-1	1639701
Injection-2	1645897
Injection-3	1640705
Injection-4	1637036
Injection-5	1638609
Average	1640389.4
Standard Deviation	3365.9
%RSD	0.2

Table 6 Intermediate Precision readings

ROBUSTNESS
Effect of flow rate
Robustness of assay method was carried out with variation of flow rate. Standard preparation was prepared and performed analysis as per test method and evaluated the system suitability parameters.

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.8	3353.0	1.5
2	1	2804.8	1.5
3	1.2	2384.0	1.4

Table 7 Effect of flow rate readings

Effect of Organic Solvent:
Robustness of assay method was carried out with variation of Organic Solvent. Standard preparation was prepared and performed analysis as per test method and evaluated the system suitability parameters [11].

S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	2396.0	1.3
2	*Actual	2804.8	1.5
3	10% more	2218.0	1.4

Table 8 effect of organic solvent

LIMIT OF DETECTION (LOD):
The lowest amount of analyte in sample that can be detected, but not necessary quantified was determined by comparison of measured signal with 0.02 µg/ml of Dolutegravir standard solutions with those of blank (mobile phase).

LIMIT OF QUANTITATION (LOQ):
The lowest amount of analyte in the sample that can be determined with acceptable precision and accuracy was determined by the comparison of measured signal with 0.05 µg/ml of Dolutegravir.

IV.RESULT AND DISCUSSION

A simple, precision and accuracy HPLC method was developed the estimation of Dolutegravir analysis of uncoated formulation, consisting of an Methanol: water (60: 40 % v/v). The chromatographic condition was set at a Flow rate of 1 ml/min with the UV detector at 240 nm. The above method was optimized with a view to develop an assay method for Dolutegravir.
Several mobile phase compositions were tried to resolve the peaks of Dolutegravir. The optimum mobile phase containing methanol: water (60: 40 % v/v) was selected because it was found ideal to resolve the analyte peaks of the drug. Quantification was achieved with UV detections at 240 nm based on peak

area and absorbance. As per USP requirements system suitability studies were carried out and freshly prepared standard solutions of Dolutegravir. Various parameters obtained with 20 µl of injection volume are summarized in the table given below.

Validation and system suitability parameters

S.NO	PARAMETERS	LIMIT	OBSERVATION
1	System suitability (%RSD of tailing factor)	suitable	1.0
2	Precision: A) Precision B)Intermediate Precision	RSD NMT 2.0%	0.2 0.2
3	Linearity	Correlation coefficient NLT 0.999	0.998
4	Accuracy	%Recovery range98-102 %	99.5%
5	Robustness	RSD NMT 2%	Robustted
6	LOD	S:N Ratio should be more than 3:1	2.92
7	LOQ	S:N ratio should be more than 10:1	9.95

Table: 9 Validation and system suitability parameters

The system is suitable for tailing factor, theoretical plate, and resolution. The method was specific for the drug.

The data obtained from the precision experiments. The R.S.D. value for precision was indication that the method was efficiently precise.

It is evident that the response for Dolutegravir was strictly linear in the studied concentration range, which is evident from the R.S.D values, slope, intercept and correlation. The method worked well in the range from 10µg/ml to 50µg/ml which suggests full capacity for the quantification of Dolutegravir. The regression coefficient was found to be 0.998.

Percentage recovery was calculated from 50% to 150% by injecting to HPLC. The excellent recovery was made at each added concentration.

There is allowable variation in flow rate, wave length which indicates that method is robust enough.

The LOD for Dolutegravir was found to be 0.02µg/ml.

The LOQ for Dolutegravir was found to be 0.05 µg/ml.

The chromatogram of sample showed a single peak at the retention time (2.273) of Dolutegravir indicating that there is no interference of the changing the persons for injecting the sample to the instrument.

V.SUMMARY AND CONCLUSION

- The reliability and suitability of the method could be seen from recovery studies. Further there is no interference due to excipients.
- System suitability parameters were calculated which includes efficiency, resolution and tailing factor.
- Precision of the methods were studied by making repeated injections of the samples and system precision values were determined.
- The method was validated for linearity, accuracy, precision, robustness.
- The method is simple, specific & easy to perform and requires short to analyse the samples.
- Low limit of Quantification and limit of detection makes this method suitable for Quality control.
- The method was found to be accurate, precise and robusted.
- Hence it was concluded that the RP-HPLC method developed was very much suit for routine analysis. Dolutegravir in tablet formulations and future planings use this method for estimation Dolutegravir in clinical trials.

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