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Happening and confirmation of a RP-HPLC form for belief of Dolutegravir in API form and linked spoken stable portion of drug or other consumable form

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Abstract

An efficient and simple HPLC method has been developed and validated for the determination of Dolutegravir in bulk and was applied on marketed Dolutegravir products. The mobile phase used for the chromatographic runs consisted of HPLC Grade Methanol and Acetonitrile in the ratio of 70: 30% v/v. The separation was achieved on a Symmetry C18, 250 mm x 4.6 mm i.d.5µm particle size column using isocratic mode. Drug peak were well separated and were detected by a PDA detector at 245 nm. The method was linear at the concentration range 6–14µg/ml for Dolutegravir. The method has been validated according to ICH guidelines with respect to system suitability, specificity, precision, accuracy and robustness. Dolutegravir limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.507µg/ml and 1.539µg/ml respectively. Results of analysis were validated statistically and by recovery studies.

Keywords: Dolutegravir, RP-HPLC, Accuracy, Precision, ICH Guidelines

Introduction

Dolutegravir is a HIV-1 intergrase inhibitor that blocks the strand transfer step of the integration of the viral genome into the host cell (INSTI).1 The effect of this drug has no homology in human host cells which gives it an excellent tolerability and minimal toxicity. Dolutegravir [1] was developed by ViiV Healthcare and FDA approved on August 12, 2013.15 On November 21, 2017, Dolutegravir, in combination with Rilpivirine, was approved as part of the first complete treatment regimen with only two drugs for the treatment of adults with HIV-1 named Juluca. Dolutegravir [2] is indicated in combination with other antiretroviral agents for the treatment of patients with HIV-1 infection that comply with the characteristics of being adults or children aged 12 years and older and present at least a weight of 40 kg.7 The FDA combination therapy approval of Dolutegravir and Rilpivirine is indicated for adults with HIV-1 infections whose virus is currently suppressed (< 50 copies/ml) on a stable regimen for at least six months, without history of treatment failure and no known substitutions associated to resistance to any of the two components of the therapy. Dolutegravir[3] is an HIV-1 antiviral agent. It inhibits HIV integrase by binding to the active site and blocking the strand transfer step of retroviral DNA integration in the host cell. The strand transfer step is essential in the HIV replication cycle and results in the inhibition of viral activity. Dolutegravir has a mean EC50 value of 0.5nM (0.21ng/mL) to 2.1nM (0.85ng/mL) in peripheral blood mononuclear cells (PBMCs) and MT-4 cells. The IUPAC Name of Dolutegravir is (3S, 7R)-N-[(2, 4-difluorophenyl) methyl]-11-hydroxy-7-methyl-9, 12-dioxo-4-oxa-1, 8-diazatricyclo [8.4.0.03,8] tetradeca-10,13-diene-13-carboxamide. The Chemical Structure of Dolutegravir is following.

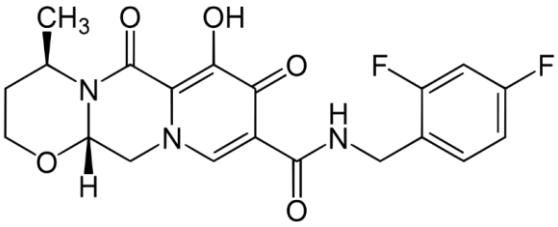

Fig 1: Chemical Structure of Dolutegravir

Many HPLC methods [33-36] have been developed for quantitative determination of Dolutegravir in bulk form and various pharmaceutical dosage forms. Spectrophotometric and HPTLC methods are reported for estimation of Dolutegravir in pure form and in marketed formulations. But, more accurate, simple, and widely used HPLC method has been not reported for the simultaneous estimation of Dolutegravir in bulk form and marketed formulations.

Materials and Methods

The HPLC system (WATERS with Empower2 Software with Isocratic with UV-Visible Detector.), consisted of 20AT pump, Symmetry C18, 250 mm x 4.6 mm i.d.5µm particle size column, UV visible absorbance detector, a automatic injector. Dolutegravir powder with 99.71% pure was used as standard. Tablet dosage form (Paracetamol 50 mg per tablet) of Tivicay Tablet (ViiV Healthcare) was used for the analysis. HPLC grade methanol and acetonitrile were purchased from Sigma-Aldrich (Germany). The water for HPLC was used and filtered through nylon 0.45 µm membrane filter.

HPLC Instrumentation & Conditions

The HPLC system[4] employed was HPLC WATERS with Empower 2 Software with Isocratic with UV-Visible Detector.

Standard preparation for UV-spectrophotometer analysis

The standard stock solutions-10 mg of Dolutegravir standard was transferred into 10 ml volumetric flask, dissolved & make up to volume with Methanol. Further dilutions were done by transferring 1 ml of the above solution into a 10ml volumetric flask and make up to volume with methanol to get 10ppm concentration.

It scanned in the UV spectrum [5] in the range of 200 to 400nm. This has been performed to know the maxima of Dolutegravir, so that the same wave number can be utilized in HPLC UV detector [6] for estimating the Dolutegravir.

Preparation of Mobile Phase

The mobile phase used in this analysis containing of a mixture of Methanol and Acetonitrile in the ratio of 70:30 v/v was prepared in the volume of 1000ml in which 700ml of Acetonitrile was mixed with 300ml of Methanol.

Preparation of Standard Solution

Accurately weigh and transfer 10 mg of Dolutegravir working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate [7] to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette out 0.1ml of Dolutegravir from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Method Validation

1. Accuracy

The accuracy of an analytical method is the closeness of the test results obtained by that method to the true value. This is sometimes termed trueness. It is recommended that accuracy[8] should be determined using a minimum of nine determinations over a minimum of the three concentration levels, covering the specified range (3 concentrations/3 replicates each of total analytical procedures).

It is measured as the percent of analyte recovered by assay [9]. The recovery can be determined by the equation:

$$\text{Recovery} = \frac{\text{Analytical Result}}{\text{True Value}} \times 100\%$$

The recovery [10] should be in the range of Control limit.

2. Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is repeated to multiple samplings of a homogeneous sample. The precision [11] of an analytical procedure is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements.

3. Specificity

Specificity [12] is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the sample matrix such as impurities, degradation products and matrix components. It must be demonstrated that the analytical method is unaffected by the presence of spiked materials (impurities and/or excipients).

4. Linearity

Linearity [13] is the ability of the method to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to analyte concentration within a given range [14]. It should be established initially by visual examination of a plot of signals as a function of analyte concentration of content. If there appears to be a linear relationship, test results should be established by appropriate statistical methods. Data from the regression line provide mathematical estimates of the degree of linearity. The correlation coefficient [15], y-intercept, and the slope of the regression line should be submitted.

5. Detection Limit and Quantitation Limit

The Detection Limit [16] is defined as the lowest concentration of an analyte in a sample that can be detected, not quantified. The Quantitation Limit [17] is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the analytical procedures.

6. Range

The range of an analytical procedure is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated to be determined with a suitable level of precision, accuracy, and linearity using the procedure as written. The range [18] is normally expressed in the same units as test results (e.g., percent) obtained by the analytical procedure.

7. Robustness

The robustness [19] of an analytical procedure is a measure of

Its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the procedure documentation and provides an indication of its suitability [20] during normal usage. Robustness may be determined during development of the analytical procedure.

System Suitability Testing

System suitability testing [21] is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system [22] that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated. They are especially important in the case of chromatographic procedures [23].

Results and Discussion

Method Development

Wavelength Selection

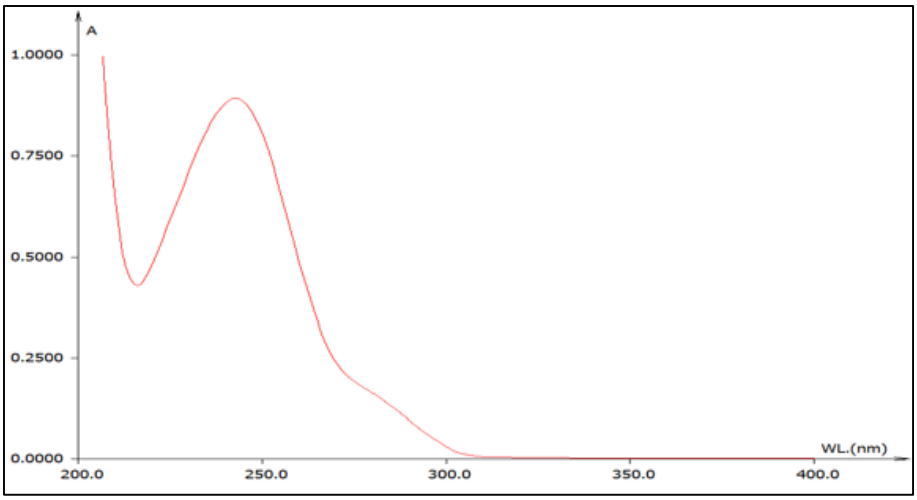

Fig 2: UV-Spectrum for Dolutegravir

Optimized Chromatographic Conditions

Column	:	Symmetry C18, 250 mm x 4.6 mm i.d.5µm particle size
Mobile Phase: Methanol	:	Acetonitrile (70: 30% v/v)
Flow Rate	:	1.0ml/minute
Wave length	:	245 nm
Injection volume	:	10 µl
Run time	:	7 minutes
Column temperature	:	Ambient

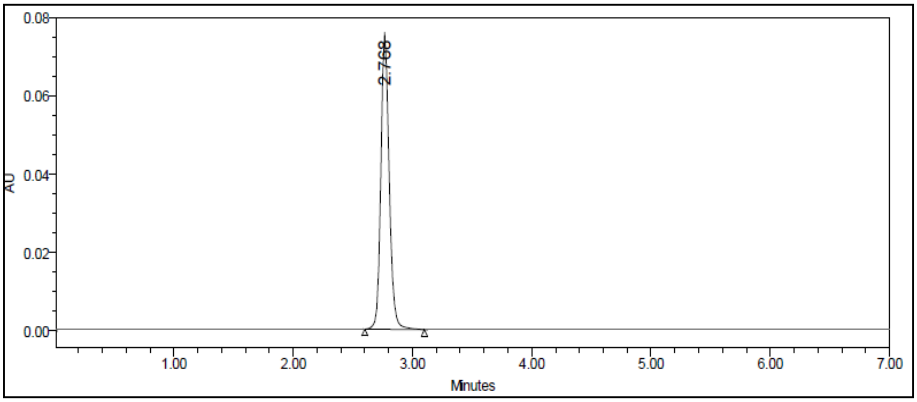

Fig 3: Optimized Chromatogram for Dolutegravir

Conclusion

The selected and optimized mobile phase [24] was Methanol: Acetonitrile (70: 30% v/v) and conditions optimized were flow rate (1.0 ml/minute), wavelength (245nm), Run time was 07 mins. Here the peak has shown better theoretical plate count and symmetry [25]. The proposed chromatographic conditions were found appropriate for the quantitative determination of the drug.

Validation of Method

System Suitability Test

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analysed constitute an integral system that can be evaluated as such. Following system suitability test [26] parameters were established. The data are shown in Table-1.

Table 1: Data of System Suitability Test

S. No.	Injection No.	RT	Area	Height	USP Plate Count	USP Tailing
1	Injection 1	2.786	715268	47844	5857	1.36
2	Injection 2	2.784	716584	46985	5986	1.38
3	Injection 3	2.768	715364	47258	5784	1.35
4	Injection 4	2.789	714895	47152	5896	1.34
5	Injection 5	2.784	716587	47258	5749	1.36
6	Injection 6	2.781	718549	47985	5657	1.39
Mean			**716207.8**		**5821.5**	**1.36**
S.D			**1347.976**			
%RSD			**0.18821**			

Table 2: Acceptance Criteria and Result

S. No.	Parameter	Limit	Result
1	Tailing factor	T ≤ 2	1.36
2	Theoretical plate	N > 2000	5821.5

Accuracy

Recovery Study: To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Dolutegravir were taken and 3 replications of each has been injected to HPLC system. From that percentage recovery values [27] were calculated from the linearity equation y = 74143x + 7294.9. The results were shown in table-3.

Table 3: Accuracy Readings

Sample ID	Concentration (µg/ml)		Peak Area	% Recovery of Pure drug	Mean % Recovery	
	Amount Injected	Amount Recovered				
S_1 : 80 %	8	8.013	601425	100.162	Mean = 100.195%	% Mean Recovery = 100.364%
S_2 : 80 %	8	8.012	601396	100.150		
S_3 : 80 %	8	8.022	602123	100.275		
S_4 : 100 %	10	10.038	751584	100.380	Mean = 100.356	
S_5 : 100 %	10	10.039	751642	100.390		
S_6 : 100 %	10	10.030	750969	100.300		
S_7 : 120 %	12	12.057	901253	100.475	Mean = 100.541	
S_8 : 120 %	12	12.073	902431	100.608		
S_9 : 120 %	12	12.065	901864	100.541		

Observation: From the Accuracy Method, we observed that the mean %Recovery of the drug is 99.686 which are within the range of 98-102%.

Precision

Repeatability: The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount [28] of drug Dolutegravir (API). The percent relative standard deviation was calculated for Dolutegravir.

Table 4: Results of Repeatability readings

HPLC Injection Replicates of Dolutegravir	Retention Time	Peak Area	Theoretical Plates	Tailing Factor
Replicate – 1	2.777	716984	5986	1.36
Replicate – 2	2.795	715698	5897	1.37
Replicate – 3	2.789	716859	5869	1.39
Replicate – 4	2.797	718548	5967	1.37
Replicate – 5	2.797	714895	5984	1.35
Replicate – 6	2.799	715986	5879	1.38
Average		**716495**	**5930.333**	**1.37**
Standard Deviation		**1268.126**		
% RSD		**0.17699**		

Observation: From the Precision method, we observed that the %RSD of the Peak Area is 0.176 which are within the acceptable range as per ICH guidelines.

Intermediate Precision

The Intermediate Precision [29] consists of two methods:-

Intra Day: In Intra Day process, the 80%, 100% and 120% concentration are injected at different intervals of time in same day.

Inter Day: In Inter Day process, the 80%, 100% and 120% concentration are injected at same intervals of time in different days.

Table 5: Peak results for Intra-Day Precision

S. No.	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Dolutegravir	2.784	716587	48685	1.38	5954	1
2	Dolutegravir	2.768	717845	48698	1.39	5935	2
3	Dolutegravir	2.786	716857	46989	1.36	5798	3
4	**Average**		**717096.3**	**48124**	**1.376**	**5895.66**	
5	**S.D**		**662.2698**				
6	**% RSD**		**0.092354**				

Table 6: Peak results for Inter-Day Precision

S. No.	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Dolutegravir	2.780	716987	49867	1.34	5968	1
2	Dolutegravir	2.794	718695	48574	1.33	5998	2
3	Dolutegravir	2.775	718542	48569	1.39	5859	3
4	**Average**		**718074.7**	**49003.33**	**1.353333**	**5941.667**	
5	**S.D**		**945.0483**				
6	**% RSD**		**0.131609**				

Observations: The intra & inter day variation of the method was carried out for standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Dolutegravir revealed that the proposed method is precise.

Linearity & Range

To evaluate the linearity, serial dilution of analyte were prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 6-14µg/ml. The prepared solutions were sonicated. From these solutions, 10µl injections of each concentration were injected into the HPLC system and chromatographed under the optimized conditions [30]. Calibration curve was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

Table 7: Linearity Concentrations of Dolutegravir

S. No.	Concentration (in ppm)	Peak Area
1	0	0
2	6	457896
3	8	607574
4	10	752268
5	12	896587
6	14	1036579

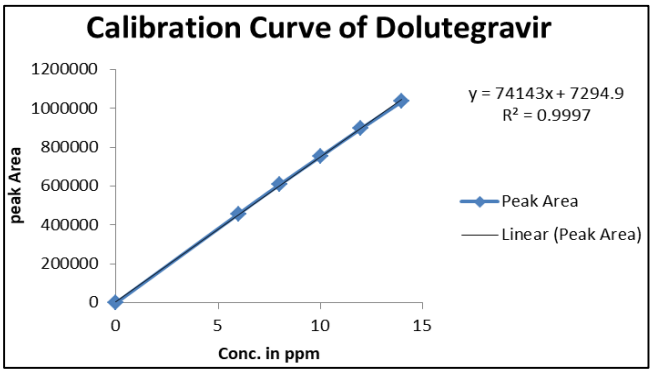

Fig 4: Calibration Curve of Dolutegravir

Observation: We observed that the calibration curve showed good linearity in the range of 6-14 µg/ml, for Dolutegravir with correlation coefficient (R^2) of 0.9997. A typical calibration curve has the regression equation of y = 74143x + 7294.9 for Dolutegravir.

Method Robustness: Influence of small changes in chromatographic conditions [31] such as change in flow rate 1ml (± 0.1ml/min), Wavelength of detection 245nm (±2nm) & organic phase content in mobile phase 60 (±5%) studied to determine the robustness of the method are also in favour of (Table-8, % RSD <2%) the developed RP-HPLC method for the analysis of Dolutegravir (API).

Table 8: Results of Method Robustness Test

Change in Parameter	Theoretical Plates	Tailing Factors
Flow (1.1 ml/min)	5954	1.35
Flow (0.8 ml/min)	6188	1.39
More Organic (70+5)	5748	1.41
Less Organic (70-5)	6185	1.48
Wavelength of Detection (250 nm)	6184	1.69
Wavelength of detection (240nm)	6247	1.47

LOD & LOQ: The detection limit (LOD) and quantization limit (LOQ) may be expressed as:

L.O.D. = 3.3(SD/S)

L.O.Q. = 10(SD/S)

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

The slope S may be estimated from the calibration curve[32] of the analyte.

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.507 & 1.539 µg/ml respectively.

Estimation of Dolutegravir in Pharmaceutical TABLET Dosage Form

Zetpril 50mg

Twenty tablets were taken and the I.P. method was followed to determine the average weight. Above weighed tablets were finally powdered and triturated well. A quantity of powder equivalent to 10 mg of drug were transferred to 10 ml volumetric flask, and 8 ml of mobile phase was added and solution was sonicated for 15 minutes, there after volume was made up to 10 ml with same solvent. Then 1ml of the above solution was diluted to 10 ml with HPLC grade methanol. The solution was filtered through a membrane filter (0.45 µm) and sonicated to degas. From this stock solution (1.0 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system.

The solution prepared was injected in five replicates into the HPLC system and the observations were recorded.
A duplicate injection of the standard solution was also injected into the HPLC system and the peak areas were recorded. The data are shown in Table-9.

ASSAY
% Assay=AT/AS×WS/DS×DT/WT×P/100×AW/LC×100
Where:
AT = Peak Area of Dolutegravir obtained with test preparation
AS = Peak Area of Dolutegravir obtained with standard preparation
WS = Weight of working standard taken in mg
WT = Weight of sample taken in mg
DS = Dilution of Standard solution
DT = Dilution of sample solution
P = Percentage purity of working standard
Results obtained are tabulated below:

Table-9: Assay of Dolutegravir Tablets

Brand name of Tablets	Labelled amount of Drug (mg)	Mean (±SD) amount (mg) found by the proposed method (n=5)	Assay + % RSD
Tivicay Tablets	50	49.875 (±0.234)	99.769% (±0.746)

Result & Discussion: The %Purity of Tivicay Tablets containing Dolutegravir was found to be 99.769% (±0.746).

Stability Studies
The results of the stress studies indicated the specificity of the method that has been developed. Dolutegravir was stable in Acidic, Photolytic & Oxidative conditions. The result of forced degradation studies are given in the following table-10.

Table 10: Results of forced degradation studies of Dolutegravir

Stress Condition	Time	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	87.635	12.365	100
Basic Hydrolysis (0.1N NaOH)	24Hrs.	94.154	5.846	100
Thermal Degradation (60^0C)	24Hrs.	90.311	9.689	100
UV (254nm)	24Hrs.	91.205	8.795	100
3% Hydrogen peroxide	24Hrs.	89.346	10.654	100

Summary and Conclusion
To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Dolutegravir, different chromatographic conditions were applied & the results observed are presented in previous chapters.
Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution.
In case of RP-HPLC various columns are available, but here Symmetry C18, 250 mm x 4.6 mm i.d.5µm particle size Column was preferred because using this column peak shape, resolution and absorbance were good.
Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Dolutegravir it is evident that most of the HPLC work can be accomplished in the wavelength range of 245 nm conveniently. Further, a flow rate of 1 ml/min & an injection volume of 10µl were found to be the best analysis.
The result shows the developed method is yet another suitable method for assay and stability related impurity studies which can help in the analysis of Dolutegravir in different formulations.

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