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Short Communication

Development of Validation and Stability Indicating Method of Anti-HIV Dolutegravir Drug and its Related Impurities by Using RP-HPLC

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ABSTRACT

A simple, rapid and robust reverse phase HPLC method was developed and validated for the determination of impurities in Dolutegravir drug substance. The main aim of this study is to reduce the run time of the HPLC method by developing and validating a new, less expensive HPLC method. The chromatographic separation of Dolutegravir and its related impurities is carried out by using C8 column (150 × 4.6 mm), 5 µm with 0.1% trifluoroacetic acid in water as mobile phase A, methanol as mobile phase B. The flow rate is 1.0 mL/min with gradient elution mode. The wave length for detection is 240 nm (UV detector). The developed method was validated and proved that the method was specific, accurate and precise as per ICH. The system suitability criteria found to be within the limits. The limit of detection and limit of quantification demonstrate that the method is sensitive. The linearity curve was found to be linear and the correlation coefficient obtained is not less than 0.998. The average percentage recoveries of impurities were in the range of 97 to 101%. The proposed method was found to be suitable and accurate for quantitative determination of impurities in Dolutegravir drug substance.

Keywords: Dolutegravir; Impurities; Method development; Method validation; RP-HPLC; ICH

INTRODUCTION

Dolutegravir belongs to a group of HIV drugs called '*integrase*' inhibitors. Integrase inhibitors block HIV enzyme called *integrase*. By blocking *integrase*, *integrase* inhibitors prevent HIV from multiplying and can reduce the amount of HIV in the body. Dolutegravir is a prescription medicine approved by the U.S. Food and Drug Administration (FDA) for the treatment of HIV infection in combination with Rilpivirine (brand name: Edurant). Dolutegravir is chemically known as (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, its molecular formula is $C_{20}H_{19}F_2N_3O_5$ and molecular weight is 419.38 g/mol. Dolutegravir (DTG), sold under the brand name Tivicay, 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 [1-3]. The structures of Dolutegravir and its impurities are shown in Figure 1.

Impurity profiling of drug substances and products is a very critical task in the pharmaceutical industry under regulatory conditions [4-7]. The presence of unknown impurities, unwanted solvents, even at very low levels, may change the effect of drug efficiency and cause side effects. Therefore, impurity profile of drug substance and product should be carried out by using stability indicative analytical method.

High Performance Liquid chromatography is one of the effective separation analytical tools to determine and quantitate the impurities. By using HPLC, we can separate a mixture of compounds to identify and quantify into individual components. In literature survey, various quantification methods are available for the determination of Dolutegravir in combined dosage form [8-11].

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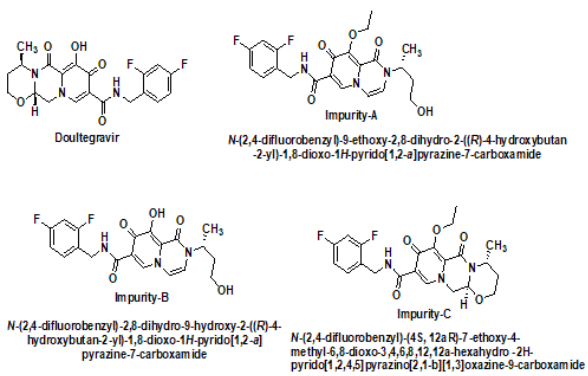

Figure 1: Structures of Dolutegravir and its impurities.

Chandra Sekhar et al. separated Dolutegravir enantiomer and Diastereomer by using chiral RPLC [11]. Srinivas et al. reported the separation of Dolutegravir optical isomers by using chiral HPLC [12].

There is no literature available for the determination of impurities in Dolutegravir drug substance. In this present research work, a stability indicative HPLC method was developed for separation of impurities and degradation products by considering 0.1% as specification limit along with short run time and validated the method according to ICH guidelines [13-15].

OPTIMIZATION EXPERIMENTS

In the process of developing impurities separation by HPLC method, different parameters were studied which influence the separation such as using different columns, different mobile phases and column temperatures. HPLC columns used for development of method were C_{18}, C_8 with different combination of mobile phases. In this development separation between impurities and Dolutegravir was not achieved. Hence we tried with Kromasil C_8 column, trifluoroacetic acid buffer in different combination of mobile phase with organic solvents acetonitrile and methanol. Finally specific method was optimized in this column. Method was finalized with satisfactory resolution among all impurities with Mobile phase A is 0.1% of trifluoroacetic acid in water and mobile phase B is methanol with gradient elution.

MATERIALS AND REAGENTS

Dolutegravir drug substance and related impurities were procured from Clearsynth laboratory. The HPLC grade methanol and acetonitrile procured from Rankem India Pvt. limited. Trifluoro acetic acid procured from Thermo scientific, India and Pure milli-Q water is used with the help of Millipore purification system (Millipore®, Milford, USA).

INSTRUMENTATION AND CHROMATOGRAPHIC CONDITIONS

WATERS HPLC, Model: 2695 equipped with 2996 photo diode array detector was used for development and method validation, with an automated sample injector. Kromasil C_8, 150 × 4.6 mm, 5 µm column was used for the separation. 0.1% of trifluoroacetic acid in water is used as mobile phase A and methanol is used as mobile phase B. Analysis was carried out in Gradient mode with flow rate of 1.0 mL/min and injection volume was 10 µL. The column temperature was 35°C, the run time was 20 min and the gradient programme is shown in Table 1. The data was acquired at 240 nm. The output signal was monitored and integrated using Empower 2 software.

Table 1: Gradient Programme for separation of impurities from Dolutegravir.

Time	Mobile phase A	Mobile phase B
0	45	55
10	40	60
12	25	75
14	25	75
15	45	55
20	45	55

PREPARATION OF SOLUTIONS

Diluent

Mixed water and acetonitrile in the ratio of 50:50 v/v.

Sample solution preparation

A 25 mg of Dolutegravir was weighed, transferred to 50 mL volumetric flask, dissolved in 20 mL of diluent by sonication, made up to the mark with diluent and the resulting concentration of the solution is 0.5 mg/mL.

Impurities solution preparation (0.1%)

Stock solution-1: Each 10 mg of impurity-A, impurity-B and impurity-C were weighed and transferred to 100 mL volumetric flask, dissolved and made up to mark with diluent to give stock solution-1 (0.1 mg/mL).

Stock solution-2: From the above solution 1 mL is transferred into a 10 mL volumetric flask, volume was made up to mark with diluent to give a solution containing 0.01 mg/mL of Impurities. It is further diluted to 2.5 mL in 50 mL of diluent to give a solution containing 0.0005 mg/mL.

System suitability solution preparation

25 mg of Dolutegravir sample is weighed and transferred into 50 mL volumetric flask, dissolved in 20 mL of diluent by sonication, 2.5 mL of impurity stock solution-2 is added and is made up to the volume with diluent solution containing concentration of 0.5 mg/mL of Dolutegravir and 0.0005 mg/mL of each impurity.

RESULTS AND DISCUSSION

Method validation

The validation of HPLC method was carried out for the determination of Impurity-A, Impurity-B and Impurity-C in Dolutegravir drug substance as per the ICH guidelines to demonstrate that this method is stability indicative for intended use.

System suitability: The system suitability was performed for every validation parameters by injecting of system suitability solution containing 0.5 mg/mL of Dolutegravir and 0.0005 mg/mL of each impurity.

Specificity (Selectivity): The blank, system suitability solution and spiked sample solutions were prepared as per methodology. Individual impurities were prepared at 0.1 mg/mL and spike sample solutions were prepared as per specification limit and injected into HPLC system. The retention time of all peaks obtained in the resulting chromatograms were recorded. Based on the results, all impurities are eluted at different retention times and adequately resolved from each other and also from main peak. Representative chromatogram is shown in Figure 2 and experimental data is given in Table 2.

Table 2: Specificity experimental data.

Sample name	Retention time (min)	Relative retention time
Impurity B	5.947	0.8
Dolutegravir	7.406	1
Impurity A	8.642	1.17
Impurity C	9.489	1.28
Blank	-	-

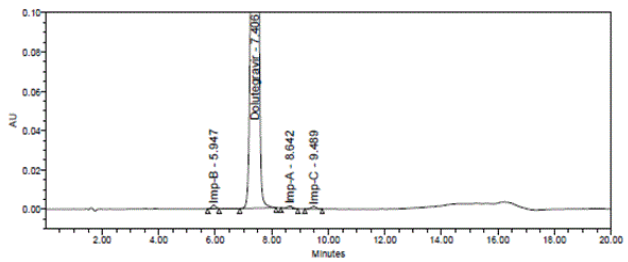

Figure 2: A typical representative HPLC chromatogram of spiked sample.

From the above chromatogram, there was no interference observed due to blank at the retention times of Dolutegravir and its related impurities. All impurities are separated with good resolution.

In order to assess the stability indicating nature of the HPLC method, Dolutegravir samples were stressed by acid, base, oxidation, heat, light (Fluorescent overall illumination with UV) and humidity. The degraded samples were analyzed by using a photodiode-array detector. The peak purity of Dolutegravir and its related impurities were passed. The forced degradation conditions are mentioned in Table 3 and the results are mentioned in Table 4.

Table 3: Forced degradation conditions of Dolutegravir.

Stress condition	Solvent	Temp (°C)	Exposed time in hours
Acid	1N HCL	60	6
Base	1N NaOH	60	6
Oxidation	5% H_2O_2	-	24
Hydrolytic	Water	60	6
Thermal	Diluent*	105	24
Photolytic	Diluent*	-	-

*Water: Acetonitrile (50:50, v/v)

From the results, no degradation was observed when Dolutegravir sample was exposed to acid, base, hydrolysis, light, humidity and heat. Slight degradation was observed in peroxide conditions. According to the stress study, none of the degradants co-eluted with the Dolutegravir peak and its related impurities formed.

Limit of detection (LOD) and Limit of quantitation (LOQ): The detection limit is considered as very low level of concentration of an analyte in a sample that can be detected, but not necessarily quantitated. The detection limit was determined as the lowest concentration for which the response is approximately three times greater than the baseline noise. The limit of quantitation is considered as 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 method. The LOD values obtained for Dolutegravir and its impurities are listed in Table 5 and corresponding representative chromatogram is shown in Figure 3.

Table 4: Degradation profile results.

Degradation condition	% Area normalization				Peak purity for Dolutegravir	
	Dolutegravir	Impurity B	Impurity A	Impurity C	Purity angle	Purity threshold

Control Sample	99.96	0.04	ND	ND	0.045	0.356
Acid sample (1.0N HCl) 60°C, 6 Hours	99.95	0.04	ND	ND	0.048	0.366
Base sample (1.0N NaOH) 60°C, 6 Hours	99.95	0.03	ND	ND	0.085	0.489
5% H_2O_2 at RT 24 Hours	94.71	0.12	ND	ND	0.059	0.726
Thermal/105°C 24 Hours	99.96	0.04	ND	ND	0.043	0.351
Photolytic	99.94	0.06	ND	ND	0.04	0.366
Humidity at 90 ± 5% RH	99.95	0.04	ND	ND	0.084	0.453

Table 5: Summary of limit of detection.

Component	Concentration (mg/mL)	LOD w.r.t Test Concentration (%w/w)	S/N ratio
Impurity B	0.000033	0.0066	3.2
Dolutegravir	0.000025	0.005	3.6
Impurity A	0.000033	0.0066	3
Impurity C	0.000033	0.0066	3.1

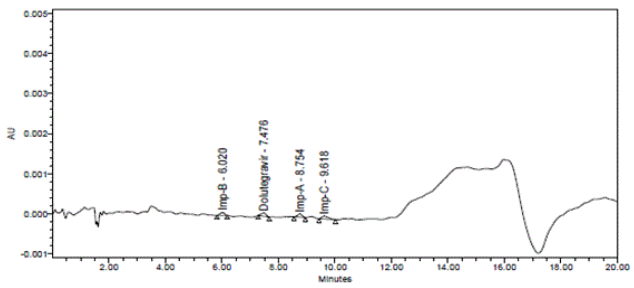

Figure 3: A typical representative HPLC chromatogram of LOD solution.

Based on above results for LOD, S/N ratio of each component was within the limit.

The LOQ values obtained for Dolutegravir and its impurities are listed in Table 6 and corresponding representative chromatogram is shown in Figure 4.

Table 6: Summary of limit of Quantitation.

Component	Concentration (mg/mL)	LOQ w.r.t Test Concentration (%w/w)	S/N ratio
Impurity B	0.000099	0.02	10.2
Dolutegravir	0.000076	0.015	11.5
Impurity A	0.000099	0.02	10.5
Impurity C	0.000099	0.02	10.1

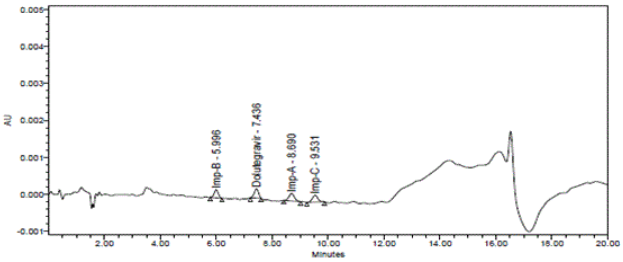

Figure 4: A typical representative HPLC chromatogram of LOQ solution.

Based on above results for LOQ, S/N ratio of each component was within the limit.

Precision at LOQ: The precision at LOQ was performed by analyzing six replicate injections of Dolutegravir and each impurity at LOQ level. Results of peak area of Dolutegravir and each impurity were summarized in Table 7.

Table 7: Summary of LOQ precision.

Injection	Imp B	DTG	Imp A	Imp C
1	2413	3382	2577	2225
2	2339	3258	2522	2111
3	2282	3349	2372	2273
4	2463	3313	2514	2215
5	2261	3411	2403	2147
6	2402	3310	2502	2340
Mean	2349	3328	2462	2217

SD	83.9	56.5	69.8	92.7
%RSD	3.57	1.7	2.83	4.18
DTG-Dolutegravir				

Based on the above results, it was observed that the % RSD for the peak areas of Dolutegravir and its impurities obtained from QL level was within the acceptable limit.

Linearity: The linearity of the method was demonstrated for Dolutegravir and its related impurities by analyzing the solutions ranging from LOQ to 150% of the specification limit (Table 8).

Table 8: Linearity data.

Level	Impurity B		Dolutegravir		Impurity A		Impurity C	
	Conc (mg/mL)	Peak area	Conc (mg/mL)	Peak area	Conc (mg/mL)	Peak area	Conc (mg/mL)	Peak area
LOQ	0.000099	2245	0.000076	2678	0.000099	2759	0.000099	2529
50	0.0005	7139	0.0005	11354	0.0005	7815	0.0005	7543
75	0.00075	11083	0.00075	16401	0.00075	11284	0.00075	12451
100	0.001	15227	0.001	22138	0.001	15887	0.001	17246
125	0.00125	19800	0.00125	28504	0.00125	19967	0.00125	21505
150	0.0015	23298	0.0015	33428	0.0015	24476	0.0015	26526
Correlation coefficient	0.9996		0.9995		0.9994		0.9997	

The correlation coefficient for impurity B was 0.9996, Dolutegravir was 0.9995, Impurity A was 0.9994 and Impurity C was found to be 0.9997 which indicates good linearity (Figures 5-8).

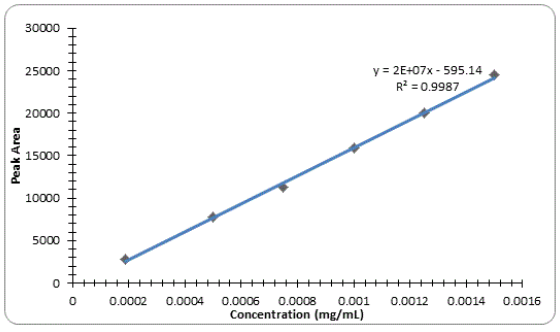

Figure 5: Linearity plot of Impurity B.

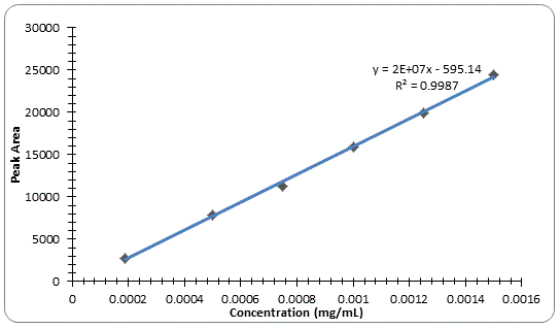

Figure 6: Linearity plot for Dolutegravir.

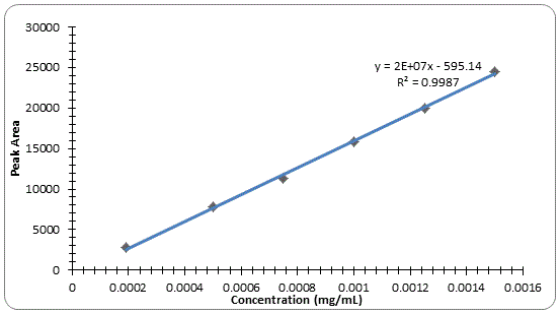

Figure 7: Linearity plot for Impurity A.

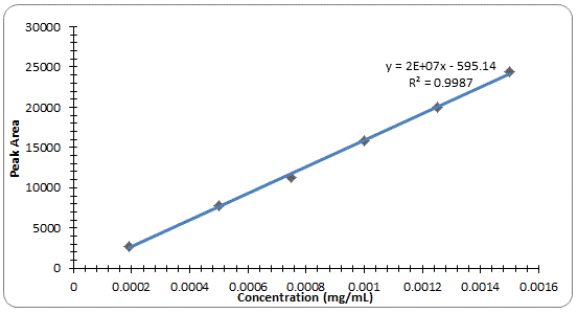

Figure 8: Linearity plot for Impurity C.

Accuracy: The accuracy of the method was determined by using solutions containing Dolutegravir sample spiked with each impurity at LOQ, 50%, 100% and 150% of the working strength of Dolutegravir. All the solutions were prepared in triplicate and analyzed. The percentage recovery results obtained for each impurity was listed in Table 9.

Table 9: Summary of percentage recoveries for each impurity.

Level	Percentage Recovery (%)		
	Impurity B	Impurity A	Impurity C
LOQ	103.2	107.6	101.3
	108.8	106.7	97.5
	99.7	100	99.1
50%	101.9	100.5	99.9
	104	99.6	101
	105.8	100.6	99.8
100%	98.8	101.7	100.4
	98.7	100.6	100.5
	98.9	101.2	100.6
150%	101.4	100.8	100.4
	104	101.7	99.6
	101.6	100.8	100.7

The percentage recovery values obtained for each impurity at LOQ level, 100% and 150% were within the acceptable limit.

System Precision: The system precision was performed by analyzing six replicate injections of standard solution at 100% of the specified limit with respect to the working strength of Dolutegravir. Results of peak area of Dolutegravir and its impurities are summarized in Table 10.

Table 10: Summary of system precision.

Injection	Imp B	DTG	Imp A	Imp C
1	14836	20928	15056	16682
2	14849	21291	15031	16677
3	14930	20961	15262	16569
4	15091	21060	15264	16382
5	15126	20875	15048	16564
6	14781	21245	14992	16666
Mean	14955	21086	15119	16571
%RSD	1	0.85	0.88	0.71

DTG-Dolutegravir

%RSD-Percentage Relative Standard Deviation

The % RSD for the peak areas of Dolutegravir and its impurities obtained from six replicate injections of standard solution was within the limit.

Method Precision: The precision of the method was determined by analyzing a sample of Dolutegravir spiked with each impurity at 100% of the specification limit (Six individual sample preparations). Data obtained is summarized in Table 11.

Table 11: Summary of method precision results.

Sample No.	% w/w		
	Impurity B	Impurity A	Impurity C
1	0.1	0.102	0.1
2	0.099	0.101	0.1
3	0.1	0.101	0.101
4	0.1	0.102	0.1
5	0.098	0.101	0.1
6	0.098	0.101	0.101
Mean	0.099	0.101	0.1
%RSD	1.71	1.71	1.71

From the above results, the % RSD for the impurities from method precision study was within the limit.

Robustness: The chromatographic conditions were deliberately changed to evaluate the robustness of the existing method. To determine the robustness of method, system suitability solution is prepared as per methodology and injected into HPLC at different altered conditions to check the method's ability like flow rate (± 10%), column oven temperature (± 5°C) and

wavelength (± 3 nm) from actual method conditions. No significant change is observed by changing flow, temperature, wavelength and system suitability also complied as per methodology. The robustness results are summarized in Table 12.

Table 12: Summary of robustness results.

Condition		Variation	USP resolution between Imp-A and Dolutegravir
Actual		~	3.92
Flow		-10%	3.56
		10%	4.16
Column temperature	Oven	-5°C	4.05
		+5°C	3.95
Wavelength		-3 nm	3.93
		+3 nm	3.92

From the robustness study, system suitability criteria comply with the results.

CONCLUSION

From the above experimental results it was concluded that, the newly developed method for the simultaneous estimation of related substances (i.e. Impurity A, Impurity B and Impurity C) in Dolutegravir was found to be simple, precise and accurate with high resolution and shorter retention time. The present proposed methodology makes is cost effective which can be implemented for routine analyses in pharmaceutical industry.

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