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Stability Indicating HPLC Method for the Quantification of (4*S*,12*aR*)-Enantiomer and (4*R*,12*aR*) Diastereomer in Dolutegravir Sodium

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ABSTRACT

A chiral HPLC method was developed for the quantification of dolutegravir enantiomer and dolutegravir diastereomer in Dolutegravir sodium drug substance. Both of these isomers are resolved on Lux cellulose-4, 250 mm x 4.6 mm, 5µ column using a mobile phase consisting of the mixture of acetonitrile, water, and orthophosphoric acid in the ratio of 980:40:2 v/v/v. The mobile phase was pumped through the column at the flow rate of 1.5 mL min^{-1}. The resolution between Dolutegravir enantiomer and Dolutegravir was found to be more than 3.0. The developed method was validated and proved to be specific, accurate, and precise according to ICH. The experimentally established limit of detection and quantification for dolutegravir enantiomer is 0.006 and 0.018% w/w respectively and for dolutegravir diastereomer are 0.007 and 0.021%w/w. The average percentage recoveries of enantiomer were ranged between 102.8% and 103.2 % and diastereomers was ranged between 97.5% and 96.2%. The linearity curve was found to be linear and correlation coefficient obtained was 0.9997 for enantiomer and 0.9993 for diastereomers.

1.0 INTRODUCTION

Dolutegravir Sodium [DGS] chemically known as (4*R*,12*aS*)-*N*-[(2,4-Difluorophenyl)methyl]-7-hydroxy-4-methyl-6,8-dioxo-3,4,6,8,12,12a-hexahydro-2*H*-pyrido[1',2':4,5]pyrazino[2,1-b] [1,3]oxazine-9-carboxamide sodium salt , its molecular formula is $C_{20}H_{18}F_2N_3O_5Na$.and molecular weight is 441.36. DGS is a new molecular entity in the integrase strand transfer inhibitor class, indicated for the treatment of HIV-1. [1-3]. Two other drugs in this same class, raltegravir, and elvitegravir (as a component of a fixed-dose combination product, Stribild® are approved and marketed in the U.S. The proposed dosing regimen of DGS, in combination with other antiretroviral therapy agents for the treatment of HIV 1 infection in adults and children ≥ 12 years of age (weighing at least 40 kg) is based on prior treatment experience [4]. DGS is a discovery of the second-generation integrase strand transfer inhibitor as a result of the collaborative efforts of scientists working for Shionogi (Japan) and GlaxoSmithKline (UK). It is formulated as 10, 25 and 50 mg tablets under *Tivicay* brand name [5]. DGS is not official in any pharmacopeias. DG Enantiomer is chemically known as (4*S*,12*aR*)-*N*-[(2,4-difluorophenyl)methyl]-7-hydroxy-4-methyl-6,8-dioxo-3,4,6,8,12,12a-hexahydro-2*H*-pyrido[1',2':4,5]pyrazino[2,1-b][1,3]oxazine-9-carboxamide and DG diastereomer is chemically known as (4*R*,12*aR*)-*N*-[(2,4-difluorophenyl)methyl]-7-hydroxy-4-methyl-6,8-dioxo-3,4,6,8,12,12a-hexahydro-2*H*-pyrido[1',2':4,5]pyrazino[2,1-b][1,3]oxazine-9-carboxamide. DGS, its enantiomer [DG Enantiomer] and diastereomers [DG diastereomer] are shown Figure. 1.

Fig 1a: DGS

Fig 1b : DG (4*S*, 12*aR*)-Enantiomer

Fig 1c : DG (4*R*, 12*aR*) Diastereomer

Fig 1: Chemical structures of DGS, DG enantiomer, and DG diastereomer

In literature, various methods are available for determination of DGS in human plasma by HPLC [6], for combined formulated dosage form quantification HPLC methods [7-8], for pure dosage form by HPTLC and HPLC [9], Bhavar *et al.* published in 2015, UV Spectroscopic method for tablets forms [10]. However, no chiral method is available for determination of enantiomer and diastereomers in DGS drug substance by HPLC or other analytical techniques. In this research work, an HPLC method was developed for resolving both enantiomer and diastereomer of DGS by considering 0.15%w/w as specification limit and validated the method accordance with ICH guidelines [11].

2.0 MATERIALS AND METHODS

2.1 Chemicals, reagents, and samples

DGS drug substance sample, DG Enantiomer, DG diastereomer and other known related substances (RS-1, RS-2, RS-3, RS-4) were procured in APL Research Centre-II (A division of Aurobindo Pharma Ltd., Hyderabad.). HPLC grade acetonitrile and Orthophosphoric acid (~ 88%w/w) procured from Merck; India and pure milli-Q water were used with the help of millipore purification system (Millipore®, Milford, MA, USA).

2.2 Instrumentation and Chromatographic conditions

Chromatographic separations were performed on HPLC system with Waters 2695 separations module and 2996 Diode array detector with Empower Pro data handling system [Waters Corporation, MILFORD, MA 01757, USA]. The mobile phase was a degassed mixture of acetonitrile, water and orthophosphoric acid in the ratio of 980:40:2 v/v/v. The analysis was

carried out on Lux cellulose-4, 250 mm long 4.6 mm internal diameter , 5µ particle diameter column (Make: Phenomenex) [i.e internal diameter filled with packing material cellulose derivative chemically bonded to silica particles of 5 µm diameter] maintained at temperature 25°C. Mobile phase was flushed through the column at a flow rate of 1.5 ml/min and pump was in an isocratic mode. The run time was 50 min. The injection volume was 15 µl and the analyte was monitored at 258 nm. A mixture of water and acetonitrile in the ratio of 50:50 v/v was used as diluent. The retention time of DG peak is at about 22 min and the DG enantiomer elutes at the relative retention time of 0.75 and DG diastereomer elutes at 1.33 with respect to DG peak. The USP resolution between DG enantiomer and DG peak is not less than 3.0 set as system suitability purpose.

2.3 Preparation of solutions

2.3.1 System suitability solution

Dissolve about 2 mg of DG *[enriched with DG-enantiomer, DG diastereomer]* reference sample in 2 ml of diluent.

2.3.2 Standard solution

Accurately weigh and transfer about 30 mg of DGS standard into a 100 mL volumetric flask, add 70 mL of diluent and sonicated to dissolve, makeup to the volume with diluent. Dilute 5 mL of this solution to 100 mL with the diluent, and then dilute 5 mL of this solution to 50 mL with diluent.

2.3.3 Sample solution

Accurately weigh and transfer about 50 mg of sample into a 50 mL volumetric flask, add 30 mL of diluent and sonicate to dissolve and make up to the volume of diluent.

3.0 RESULTS AND DISCUSSION

3.1 Method validation

To develop a rugged and suitable HPLC method for the separation of these two isomers, different stationary phases and mobile phases were employed. Finally, optimized good separation method with chromatographic parameters as mentioned in above sections. The optimized method was established through the validation experiments per the ICH guidelines

[11], individually in terms of specificity, LOD, LOQ, linearity, accuracy, precision(system precision, method precision, and intermediate precision or ruggedness), the stability of standard and sample solutions and robustness.

3.1.1 Specificity

Specificity parameter is the capability of the method to establish the interest analyte in the presence of other related substances of the drug substance. The blank solution, all the related substances of DGS including DG enantiomer, DG diastereomer solutions were prepared individually and injected into HPLC to confirm the retention times. Solutions of DGS drug substance, DGS drug substance spiked with DG enantiomer and DG diastereomer [control sample], DGS drug substance spiked with all related substances of DGS including DG enantiomer and DG diastereomer [spiked sample] were prepared and injected into HPLC and confirm any co-elution with analyte peak from blank, any of related substances peaks and the peak homogeneity was verified for analyte using waters empower software and found to be pure (purity angle should be less than purity threshold). The typical HPLC chromatograms of DGS spiked with DG enantiomer and DG diastereomer, (Control sample) and DGS spiked with DG enantiomer, DG diastereomer along with other known related substances (Spiked sample) shown in Fig 2. The specificity experiments data is given in Table 1.

Table 1: Specificity experiments data

	Retention time (min)	Relative retentive time	Purity angle	Purity threshold
Control sample				
DG enantiomer	16.278	0.74	0.374	0.861
DG	22.141	1.00	0.026	0.250
DG diastereomer	29.286	1.32	0.503	1.010
Spiked sample				
DG enantiomer	16.316	0.74	0.208	0.662
DG	22.176	1.00	0.003	0.249
DG diastereomer	29.327	1.32	0.232	0.852

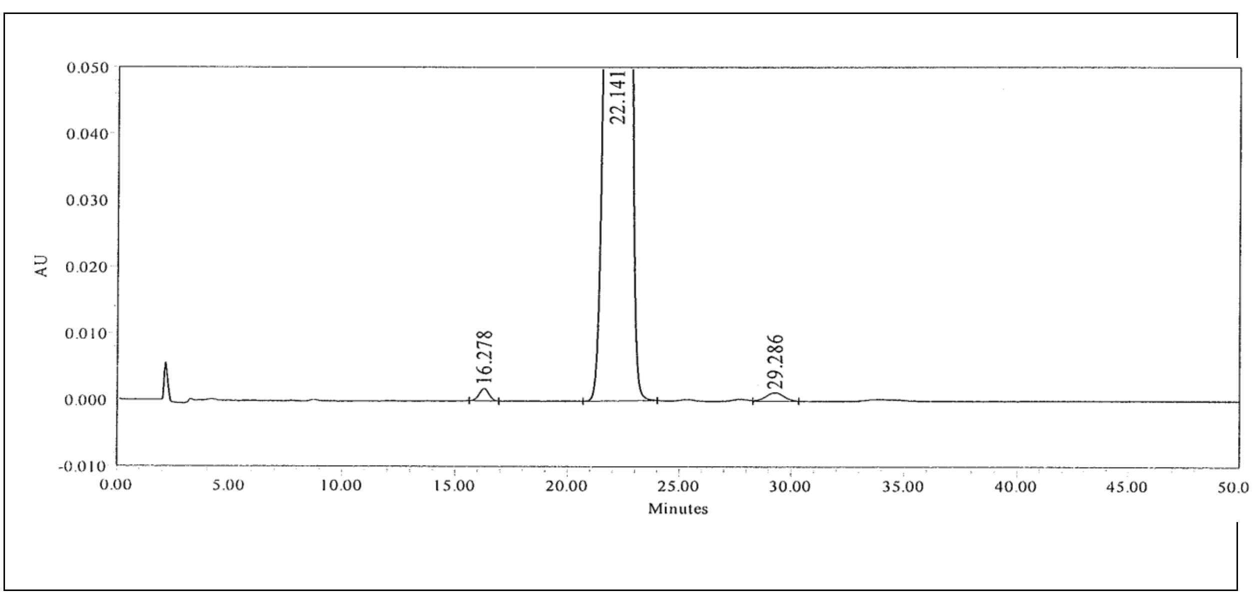

Fig. 2a A typical representative HPLC chromatogram of Control sample

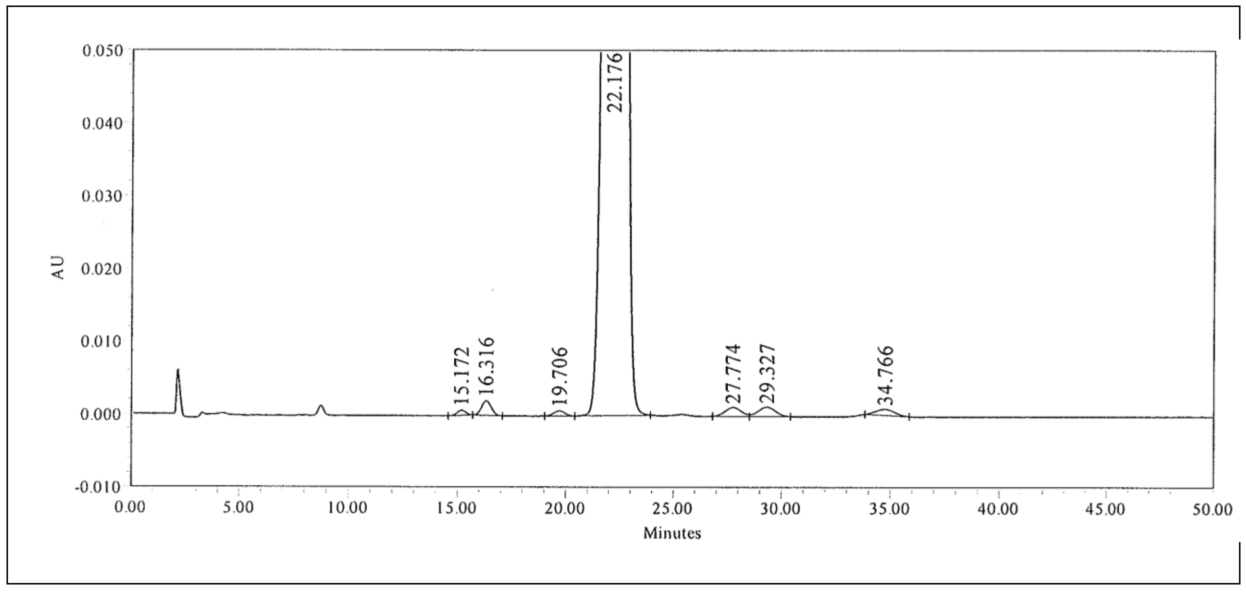

Fig. 2b: A typical representative HPLC chromatogram of Spiked sample

Moreover, the stability indicating nature of the method was verified through the forced degradation/stress studies of DGS drug substance. In this study, DGS drug substance was subjected to the following stress conditions. The undegraded sample and each stressed sample were prepared to the required concentration and injected into HPLC using the analytical conditions and the obtained data is given in Table 2.

Thermal stress	:	The drug substance was subjected to dry heat at 105°C for 120 hours.
Acid degradation	:	The sample was degraded under 5M Hydrochloric acid at 85°C temperature for 120 min.
Base degradation	:	The sample was degraded under 5M Sodium hydroxide at 85°C temperature for 120 min.
Photolytic degradation	:	The sample was exposed to photolytic degradation (white fluorescent light 1.2 million lux hours followed by UV light 200 watt-hours/m^2.
Humidity degradation	:	A sample was exposed to degrade under 90% RH at 25°C for 120 hours.
Peroxide degradation	:	0.01% Hydrogen peroxide at room temperature for 15 min.

Table 2: Forced degradation experiments data

Degradation	**Condition**	**Degradation (%w/w)**	**DG Peak**	
			Purity angle	**Purity threshold**
Undegraded	--		0.029	0.249
Acid	5M HCl/85°C/120 min	8.4	0.021	0.247
Base	5M NaOH/85°C/120min	1.6	0.007	0.248
Peroxide	0.01% H_2O_2 / room temperature/15 min	2.2	0.079	0.257
Thermal	105°C /120 hours	0.5	0.028	0.249
Photolytic	white fluorescent light 1.2 million lux hours followed by UV light 200 watt-hours/m^2	0.8	0.024	0.249
Humidity	90% RH/25°C/120hours	0.9	0.024	0.249

3.1.2 LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) values were determined using the values of slope, standard deviation, and responses that have been obtained from linearity study carried out from 10% to 150% of specification level (0.15%). The predicted concentrations of LOD and LOQ of DG enantiomer and DG diastereomer were verified for precision by analyzing the solutions containing DG enantiomer and DG diastereomers at about predicted concentrations and injected each solution six times into HPLC and the results are tabulated in Table 3.

3.1.3 Linearity

The linearity of the detector was determined by preparing a series of solutions using DGS, DGS enantiomer and DGS diastereomer at concentration levels from about LOQ level to 150% of specification level. The obtained data was subjected to statistical analysis by using a linear regression model. The statistical evaluations like slope, intercept, STEYX and correlation coefficient values of linearity data is given in Table 3.

Table 3: Linearity/LOD/LOQ experiments data

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Statistical parameters	DG Enantiomer	DG Diastereomer
No. of points covered	9	9
Concentration range (µg/mL)	0.167 – 2.276	0.195 – 2.180
Slope	36402	33765
Intercept	305	-1292
STEYX	659	988
Correlation coefficient	0.9997	0.9993
Limit of detection(%w/w)	0.006	0.007
Limit of quantification(%w/w)	0.018	0.021
Precision for Limit Of Detection (%R.S.D)	9.5	12.4
Precision for Limit Of Quantification (%R.S.D)	6.3	7.9

3.1.4 Accuracy

The accuracy of the method was performed by recovery experiments using standard addition technique. The recoveries were determined by spiking DG enantiomer and DG diastereomer at four different levels at LOQ level, 50, 100 and 150% level of the specification into DGS drug substance. These samples were prepared as per respective test procedure and analyzed in triplicate and the percentage recoveries were calculated. The recovery results are shown in Table 4.

Table 4: Accuracy experiments data

Level	DG enantiomer			DG diastereomer		
	Amount added (%w/w)	Amount found (%w/w)	% Recovery	Amount added (%w/w)	Amount found (%w/w)	% Recovery
LOQ-1	0.0181	0.0188	103.9	0.0213	0.0201	94.4
LOQ-2	0.0181	0.0185	102.2	0.0213	0.0209	98.1
LOQ-3	0.0180	0.0184	102.2	0.0212	0.0212	100.0
50%-1	0.074	0.076	102.7	0.076	0.075	98.7
50%-2	0.074	0.076	102.7	0.077	0.076	98.7
50%-3	0.074	0.077	104.1	0.077	0.077	100.0
100%-1	0.150	0.152	101.3	0.156	0.148	94.9
100%-2	0.149	0.153	102.7	0.155	0.145	93.5
100%-3	0.150	0.152	101.3	0.156	0.146	93.6
150%-1	0.222	0.231	104.1	0.230	0.219	95.2
150%-2	0.221	0.232	105.0	0.230	0.219	95.2
150%-3	0.222	0.232	104.5	0.230	0.220	95.7
	Overall % recovery		103.0	Overall % recovery		96.5

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3.1.5 Precision

System precision was demonstrated by preparing the standard solution of DGS as per methodology and analyzed by injecting six replicates. Method precision was demonstrated by preparing six sample solutions individually using a single batch of DGS drug substance spiked with DG enantiomer and DG diastereomer at specification level and determined the both of these contents. Ruggedness was demonstrated by following the same procedure as mentioned for method precision experiment by another analyst using another lot of column, different system on a different day. Achieved results like %RSD and 95% confidence interval for six determinations and cumulative of twelve determinations are summarized in Table 5.

Table 5: Precision experiments data

	System precision (n=6)			
%RSD for DG peak area	3.2			
95% Confidence interval(±)	1566			
	DG enantiomer content(%w/w)		***DG diastereomer content(%w/w)***	
	Method Precision	Intermediate Precision or Ruggedness	Method Precision	Intermediate Precision or Ruggedness
Sample-1	0.170	0.167	0.209	0.206
Sample-2	0.171	0.164	0.206	0.208
Sample-3	0.170	0.163	0.207	0.210
Sample-4	0.170	0.166	0.207	0.217
Sample-5	0.171	0.166	0.204	0.213
Sample-6	0.172	0.172	0.204	0.208
Mean	0.171	0.166	0.206	0.210
SD	0.001	0.003	0.002	0.004
%RSD	0.6	1.8	1.0	1.9
95% Confidence interval(±)	0.001	0.003	0.002	0.004
Overall mean(n=12)	0.169		0.208	
Overall SD	0.003		0.004	
Overall %RSD	1.8		1.9	
Overall 95% Confidence interval(±)	0.002		0.003	

3.1.6 Solution stability

For the determination of stability of the standard and sample solutions, standard solution and sample solution spiked with DG enantiomer and DG diastereomer at specification level were prepared and analyzed initially and at different time intervals up to 24hours by keeping the solutions at room temperature (≈25°C). The % difference in the peak areas of DG enantiomer and DG diastereomer obtained at initial and different time intervals was found to be less than 5.8. The results concluded that standard and sample solutions are stable for at least 24 hours at room temperature.

3.1.7 Robustness

For the determination of robustness of the method, system suitability solution and sample solution spiked with DG enantiomer and DG diastereomer at specification level were prepared as per methodology and injected into HPLC at different deliberately varied conditions to evaluate the method's ability like flow rate (±10%), detection wavelength (±3nm), composition of mobile phase (±2% absolute water variation) and column oven temperature (±5°C) from methodology values. The results indicated that there was no variation in relative retention time (RRT) of DG enantiomer and obtained at each of the varied conditions with respect to methodology conditions and system suitability requirement is also complied as per methodology. The robustness results are given in Table 6.

Table 6: Robustness experiments data

Condition	Variation	RRT		USP Resolution
		DG enantiomer	DG diastereomer	
As per Method	-	0.74	1.32	6.1
Flow	-10%	0.74	1.32	6.4
	+10%	0.74	1.32	6.0
Column Oven Temperature	-5°C	0.74	1.32	6.1
	+5°C	0.74	1.32	6.0
Composition of mobile phase (water variation)	-2% absolute	0.74	1.35	5.8
	+2% absolute	0.74	1.29	6.0
	+1% absolute	0.74	1.30	6.0
Wavelength	-3 nm	0.74	1.32	6.0
	+3 nm	0.74	1.32	6.0

4.0 CONCLUSION

The HPLC chromatography method was developed, optimized and validated for the determination of DG enantiomer and DG diastereomer contents in DGS drug substance and the results of various validation parameters demonstrated that the methods are specific, sensitive, linear, precise, accurate, rugged and robust and the method can be introduced into routine testing.

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