China Petroleum Processing and Petrochemical Technology

Catalyst Research Volume 23, Issue 1, January 2023 Pp. 523-533

DEVELOPMENT OF A RP-HPLC METHOD FOR ESTIMATION OF DOLUTEGRAVIR SODIUM IN RAT PLASMA

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Abstract

A simple, sensitive, and specific reversed phase liquid chromatographic method was described for the determination of dolutegravir sodium (DGS). The separation was achieved on a Phenomenex ODS C18 (250 mm x 4.6 mm, 5 µ) column at ambient temperature using isocratic water alliance 2695 HPLC system equipped with empower version 2.0 software and with UV-visible detector. The mobile phase consisted of potassium dihydrogen orthophosphate buffer (pH 3 ± 0.05) and acetonitrile in a ratio of 25:75v/v. The detection was carried out at wavelength of 258 nm. The method was validated for system suitability, linearity, accuracy, precision, robustness and stability tests. The results indicated that the reported method is highly specific and reproducible.

Keywords: — Dolutegravir sodium, HPLC, UV-visible detector, accuracy, precision and stability tests.

I. Introduction

Dolutegravir sodium (DGS), is chemically (3S, 7R)-N-[(2, 4-diflurophenyl) methyl]-11-hydroxy-7-methyl-9, 12-dioxo-4-oxa-1, 8-diazatricyclo, tetradeca-10, 13-diene;13-carboxide[1]. DGS is a newly developed human immune deficiency virus (HIV-1) integrase inhibitor; it binds to the active site, 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 [2].

Literature review revealed various analytical methods for the determination of DGS earlier are either in single or combination with other drugs analyzed in bulk and pharmaceutical dosage forms. These analytical methods were briefly reported; **Bala saheb *et al*** [3] reported a UV-spectrophotometric method for estimation of dolutegravir sodium in tablet dosage form. **Masthanamma *et al*** [4] reported a novel UV-Spectrophotometric method for the development and validation of dolutegravir in bulk and its laboratory synthetic mixture. **Naresh and Nagaraju** [5] reported UPLC method for simultaneous estimation of abacavir, lamivudine and dolutegravir from its tablet dosage form. **Joseph *et al*** [6] reported a RP-HPLC method for the estimation of dolutegravir and rilpivirine in both bulk and pharmaceutical dosage form. **Talari Kalpana *et al*** [7] reported a RP-HPLC method for determination of dolutegravir sodium, lamivudine and tenofovir disoproxilfumarate. **Rajkumar *et al*** [8] reported a RP-HPLC method for the determination of lamivudine, abacavir and dolutegravir in pharmaceutical dosage forms. **Devanna *et al*** [9] reported a method for the simultaneous estimation of dolutegravir and lamivudine in drug product by RP-HPLC.

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II. Experiment

A. Instrumentation

Liquid chromatography method was developed for determination of dolutegravir by using isocratic water alliance 2695 HPLC system equipped with empower version 2.0 software and with UV-visible detector. The separation was achieved on a Phenomenex ODS C18 (250 mm x 4.6 mm, 5 µ) column at ambient temperature.

B. Preparation of standard solution

Accurately weighed 20.0 mg of dolutegravir was transferred in 10.0mLvolumetric flask and added 2.0mL of methanol to dissolve and diluted up to the mark with methanol. The resultant solution was sonicated to dissolve the drug and filtered through 0.22 µm filter membrane. From the filtered solution 5 mL was pipetted out and diluted to 10 mL with methanol.

(1) Calibration curve dilutions: Different solutions of DGS were prepared from the stock solution (**Table I**) to get a concentration range of 1.11 - 101.65 µg/mL using the diluents (1:1 mixture of potassium dihydrogen phosphate buffer and acetonitrile. These solutions were further used for spiking the screened blank plasma.

Young, healthy male Wistar rats were used for the studies and all experiments were conducted in accordance with the guidelines laid by local ethical committee of the institute for animal experiments.

TABLE I. CALIBRATION CURVE DISSOLUTION DATA FOR DGS IN SPIKING SOLUTION

Stock/ SSID	**Stock/SS Concentrations (µg/mL)**	**Stock/SS Volume (mL)**	**Diluent Volume (mL)**	**Total Volume (mL)**	**Final Conc (µg/mL)**	**Spiking solution ID**
Stock solution	999.00	0.37	9.63	10	369.63	SS-7
SS-6	369.63	1.26	3.740	5	93.147	SS-6
SS-5	93.147	0.900	4.100	5	66.533	SS-5
SS-4	66.533	0.600	4.400	5	44.356	SS-4
SS-3	44.356	0.400	4.600	5	29.570	SS-3
SS-2	29.570	0.200	4.800	5	14.785	SS-2
SS-1	14.785	0.030	4.970	5	2.218	SS-1

(2) Spiked calibration curve plasma standards: The above calibration curve dilutions were used to spike the screened blank rat plasma matrix to prepare the plasma calibration curve standards in the range 110.889 – 4657.338 ng/mL as given in the **Table II**. Aliquots containing 0.50 mL of the above plasma calibration curve standards were taken in polypropylene vials, labeled properly, tightly closed and stored in a freezer at -70^0C for further use.

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TABLE II. CALIBRATION CURVE DISSOLUTION DATA FOR DGS IN SPIKING PLASMA STANDARDS

Stock/SSID	Stock/ SS Concentrations (µg/mL)	Stock/ SS volume (mL)	Plasma volume(mL)	Total Volume (mL)	Final Conc (µg/mL)	Spiking solution ID
SS-6	93.147	0.50	9.50	10	4.657	SS-6
SS-5	66.533	0.50	9.50	10	3.326	SS-5
SS-4	44.356	0.50	9.50	10	2.217	SS-4
SS-3	29.570	0.50	9.50	10	1.478	SS-3
SS-2	14.785	0.50	9.50	10	0.739	SS-2
SS-1	2.218	0.50	9.50	10	0.110	SS-1

C. Preparation of solutions

(1) Preparation of 0.01M potassium dihydrogen phosphate buffer: Accurately weighed 1.36 gm of potaasiumdihydrogen orthophosphate was transferred into a 1000 mL volumetric flask. To this 900 mL of milli Q water was added. The solution was sonicated for 2 minutes and the final volume was made up to 1000mLwith water. One mL of triethylamine was added and the pH was adjusted to 3.0 by using dilute orthophosphoric acid solution. The solution was stored at room temperature and was used up to three days after preparation.

(2) Preparation of mobile phase: The mobile phase was prepared by mixing 25 parts of buffer solution and 75 parts of acetonitrile in a reagent bottle. It was sonicated for 5 minutes and was filtered through a 0.45µ nylon filter. The mobile phase was stored at room temperature and was used up to three days after preparation.

(3) Preparation of diluents: A volume of 500 mL of acetonitrile was transferred into 1000 mL reagent bottle and 500 mL of buffer was added to it, mixed and sonicated for 5 minutes. The solution was stored at room temperature and used within seven days from the date of preparation.

(4) Rinsing solution: A volume of 500 mL of acetonitrile was transferred into a 1000 mL reagent bottle; 500 mL of buffer solution was added, it was mixed and sonicated for 5 minutes. The solution was stored at room temperature and was used within seven days from the date of preparation. This solution was used for rinsing the injection needle of the HPLC instrument.

D. Method development and optimization of the chromatography conditions

- For the development of RP-HPLC method for the assay of dolutegravir, different parameters were studied by altering one parameter at a time, keeping all the remaining parameter constant. A non polar Phenomenex ODS C18 (250 mm x 4.6 mm, 5 µ) column was chosen as the stationary phase for this study.

- The UV absorption spectrum was taken and the λ_{max} was found to be at 258 nm. Hence,

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further analysis and detection of the drug was carried out at 258 nm.

- **The mobile phase and the flow rate:** In order to get a sharp peak and baseline separation of the components, various trials have been taken by using different mobile phases (single solvent or combination of solvents like acetonitrile, water, methanol with or without buffer) on C18 column as a stationary phase. A binary mixture of potassium dihydrogen orthophosphate buffer (pH 3 ± 0.05) and acetonitrile in a ratio of 25:75v/v was found to be suitable mobile phase with well defined and well resolved peaks without tailing. A mobile flow rate 1.0 mL/min was found to be suitable when tried in the range of 0.5 to 1.5 mL/min.

- **Retention time of DGS:** A model chromatogram, showing the separation of DGS under the above optimized conditions at a retention time of 4.78 min was obtained as shown in **Fig2**.

E. Data acquisition and processing

The chromatograms were obtained and the data was processed by the peak area ratio method using the Empower software. The concentration of the unknown samples was calculated from the following equation of the regression analysis of the spiked plasma calibration graph using $1/X^2$ as the weighting factor.

$$\mathbf{Y= mX+ C}$$

Where,

X= Analyte concentration
Y= Analyte area ratio
m= Slope of the calibration curve
C= Intercept value

F. Extraction process of plasma samples and their dying

A volume of 400 µL of spiked plasma calibration curve standards was transferred to a set of prelabelled polypropylene tubes. To this 25 µL of DGS (approximately 500 µg/mL) was added and vortexed for 10 seconds. To this 1.2 mL of HPLC grade methanol is added to precipitate the plasma proteins. The samples are then centrifuged for 15 minutes at 4000 rpm in a refrigerated centrifuge. The supernatant is transferred to another set of pre labelled polypropylene tubes and evaporated to dryness under nitrogen at 40ºC. The dried sample is reconstituted with 300 µL of mobile phase, vortex thoroughly and transferred to auto sampler vials for analysis. 20 µL was taken as an injection volume during final analysis.

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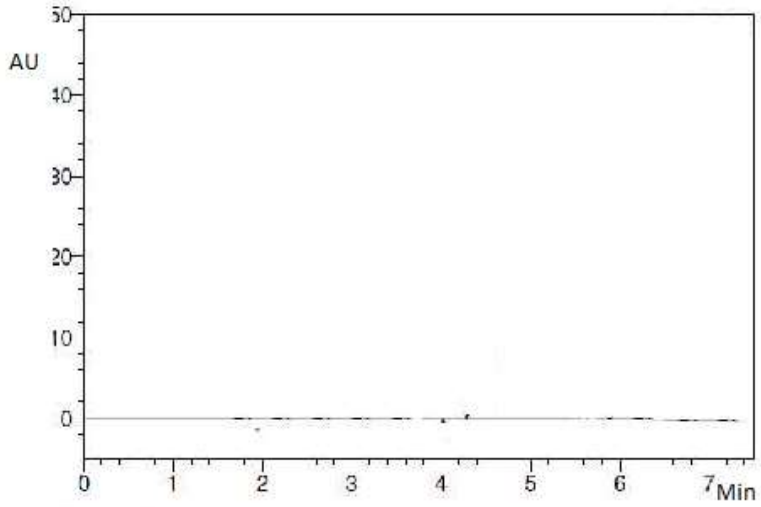

Fig 1. Chromatogram of extracted blank plasma sample

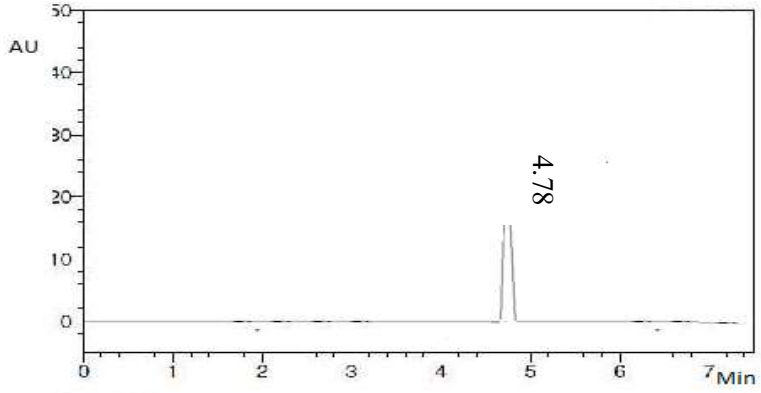

Fig 2. Chromatogram of dolutegravir with blank plasma

G. Validation of HPLC method

(1) **Specificity:** Specificity of the developed method was validated to check the interference of any compound from formulation matrix. To determine specificity the standard, sample and placebo were injected and the recorded chromatograms are depicted in **Fig 3 to 4**. No peaks were observed at the retention times of DGS under optimized conditions which confirm that the selected drug is evidently allotted and hence the proposed HPLC method is selected.

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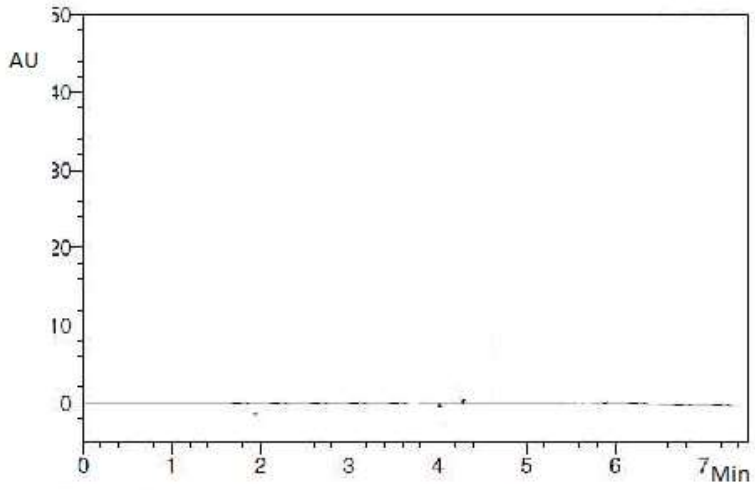

Fig 3. Chromatogram of placebo

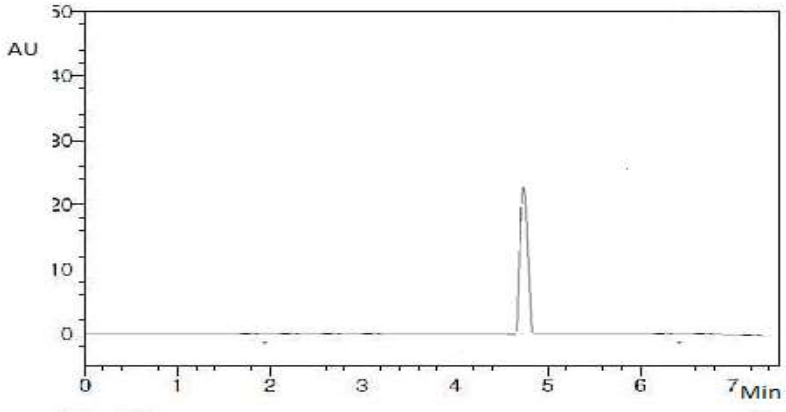

Fig 4. Chromatogram of standard DGS solution

(2) **Linearity range:** The standard concentrations were injected in given range and analyzed according to the method. Acceptance of linearity data is judged by examining the calibration curve equation and correlation coefficient (r) and shown in **Table III and Fig 5**. The standard solution containing 1000 µg/mL of dolutegravir was prepared and diluted to appropriate concentration from 10-50 µg/mL as a working concentration for each standard 20µL of each solution was injected and analyzed using developed chromatographic method. The chromatograms were recorded and the peak areas of the drug were calculated. The data obtained was subjected to least square regression analysis within microsoft excel to calculate calibration curve equation and correlation coefficient (r). Limit of detection (LOD) and Limit of quantitation (LOQ) were determined from the slope of calibration curve using following formula,

$LOD = 3.3\sigma / S$

$LOQ = 10\ \sigma / S$

Where,

σ is the standard deviation of the response

S is the slope of the calibration curve.

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TABLE III. LINEARITY STUDY OF DOLUTEGRAVIR SODIUM

Conc(µg/mL)	**Peak area (µ Volts × min)**
10	219519
20	368131
30	597321
40	793218
50	987497
Calibration curve equation	Y=19679X + 2297
Correlation coefficient (r)	0.998
LOD (µg /mL)	0.638
LOQ (µg /mL)	1.933

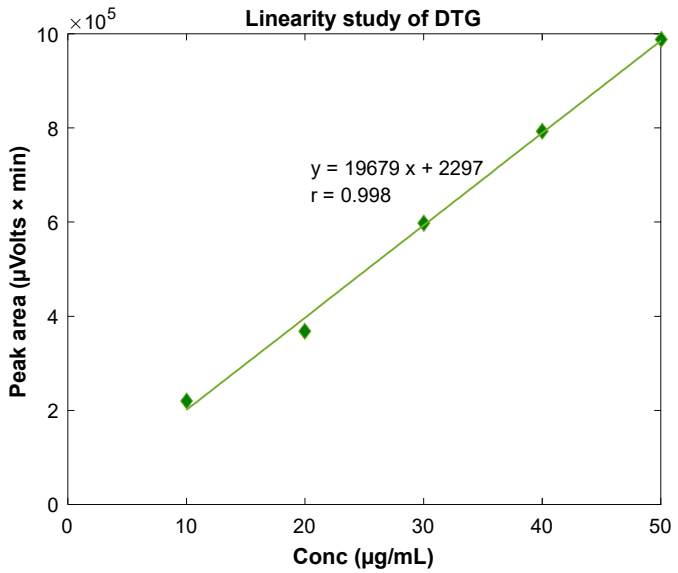

Fig 5. Linearity study of dolutegravir sodium

(5) Accuracy: The accuracy of the method was tested by calculating recoveries of dolutegravir by standard addition method. Correct amount of standard solution each 50%, 100% and 150% were spiked to pre-quantified solution, and the amount of compound recovered and estimated. The results are tabulated in **Table IV.**

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TABLE IV. ACCURACY STUDY OF DOLUTEGRAVIR SODIUM

S.No	%Concentration (at specification level)	% Recovery	Mean Recovery%
1	50	94.93	95.15
2	50	95.58	
3	50	94.93	
4	100	101.19	100.09
5	100	99.26	
6	100	99.83	
7	150	96.50	96.47
8	150	96.63	
9	150	96.28	

(6) Precision: Precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

(7) Repeatability: Six replicates of standard mixture were injected and analyzed using optimized method. The average peak area along with the % RSD is shown in the **Table V**. The % RSD for DGS was found to be 0.98.

TABLE V. REPEATABILITY OF DOLUTEGRAVIR SODIUM

Parameter	Dolutegravir*
Average Peak area	1916046 ± 18811
% RSD	0.98

* mean± S.D (n=3)

Intermediate precision: Intermediate precision was carried out by intraday and inter day assay method and results were tabulated in **Table VI**. The results showed no significant variation in % RSD of peak area of dolutegravir sodium.

TABLE VI. INTERMEDIATE ASSAY PRECISION OF DOLUTEGRAVIR SODIUM

Compound	*Intra day (%RSD)	*Inter day (% RSD)
Dolutegravir sodium	1.25	0.96

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(8) Robustness and ruggedness: The robustness of the method was unaffected when small, deliberate changes like, flow change, mobile phase composition, column temperature were performed at 100% test concentration. The ruggedness of the method was studied under different columns, analyst and instrument, laboratories analysis of the same sample and the method was found to be robust at different conditions and shown in **Table VII**.

TABLE VII: ROBUSTNESS STUDY OF DOLUTEGAVIR SODIUM

Change in parameters	**% RSD peak area**
Flow rate (0.9 mL/min)	1.4
Flow rate (1.1 mL/min)	1.2
Wave length 255 nm	1.5
Wave length 261nm	1.2

H. Stability Study

Forced degradation stability indicating studies like acidic, alkali, oxidative etc. were performed and from the degradation studies it was observed that dolutegravir is most sensitive for alkali stress than remaining stress studies as shown in **Table VIII.**

TABLE VII: DEGRADATION STUDIES OF DOLUTEGRAVIR SODIUM

S.No	**Type of Degradation**	**% Degradation**	**% Recovery**
1	Acid	17.37	82.63
2	Alkali	18.88	81.12
3	Hydrolysis	18.00	82.00
4	Peroxide	18.69	81.31
5	Photo	18.11	81.89
6	Reduction	18.34	81.66
7	Thermal	17.46	82.54

III. Results and Discussion

The RP-HPLC method developed was statistically validated in terms of selectivity, accuracy, linearity, precision, and robustness, stability of solution and mobile phase stability. The chromatograms were recorded for extracted blank sample, DGS with blank sample, placebo and standard DGS solution the peaks were well separated from each other.

The LOD and LOQ were found to be 0.638µg/mL and 1.933µg/mL,

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respectively. The linearity results in the specified concentration range are found satisfactory calibration curve was plotted with correlation coefficient (r) 0.998.

Accuracy studies were shown as % recovery for DGS at 50, 100 and 150%. The limit of % recovered shown is not less than 95% and the results obtained were found to be within the limits.

Hence the method was found to be accurate. For precision studies six replicate injections were performed. % RSD was determined from peak areas and the results were found to be within the acceptance limits. Intermediate assay precision of DGS results showed no significant variation in % RSD of peak area of dolutegravir sodium.

The result of the robustness study shows the method was robust at different conditions. Forced degradation studies were performed and were observed that dolutegravir is most sensitive for alkali stress than remaining stress studies. Hence, the chromatographic method developed for DGS is simple, rapid, sensitive, precise and accurate.

IV. Conclusion

A RP-HPLC method reported in literature was adopted and was modified for the purpose of the present work and was validated as per ICH guidelines. A simple, specific and reliable method reported in the literature was adopted studied by validation for estimation of the DGS. The total run time was 7 minutes where dolutegravir got separated at 4.78 minutes. There was no interference of any other peak with dolutegravir peak. When the same sample containing dolutegravir was injected 6 times, it did not affect the retention time of the drug. The developed method was validated for intraday and inter day variations. The results indicated that the reported method is highly specific and reproducible. Hence, it was concluded that the reported method may be used in the formulation development of the selected drug candidate, namely dolutegravir sodium.

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