International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 148-154 www.ijprajournal.com ISSN: 2456-4494

Development and Validation of Dolutegravir Sodium In Human Spiked Plasma Using HPLC

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Date of Submission: 05-07-2025 Date of Acceptance: 15-07-2025

ABSTRACT:
A rapid and simple high performance liquid chromatography (HPLC) method was developed and validated for the measurement of dolutegravir sodium from tampered human plasma using a UV detector. A mobile phase comprising Acetonitrile: Phosphate Buffer pH3 (50:50%, v/v) at a flow rate of 1 ml/min was used to develop and optimise chromatographic conditions on the RESTEK C18 Column (250 × 4.6 mm, 5 um). Internal standard pioglitazone and dolutegravir sodium were extracted from human plasma by protein precipitation using the organic solvent acetonitrile (ACN). Dolutegravir sodium was successfully extracted from plasma components and internal standards. The detection was performed at 258 nm. For the calibration curve, which was linear in the 0.2–8 µg/mL range, heteroscedasticity was minimised using weighted least square regression with a weighing factor (1/x). The November 2022 ICH M10 guidelines for bioanalytical method validation's standard curve requirements were met by this process. At all three tested levels (LQC, MQC, and HQC), the percentage RSD and %RE were less than 15 and between ± 15, respectively, indicating the statistical accuracy and precision of the approach. The results of recovery studies show that extraction is effective.The results of the stability evaluation indicate that the dolutegravir sodium concentrations in the stability samples varied between 85 and 115% of the nominal concentration with percentage RSD. below fifteen. The method is in compliance with ICH M10 guidelines and can be applied to dolutegravir sodium bioavailability and bioequivalence tests.

1. INTRODUCTION:

The separation method known as chromatography is mostly employed in chemical analysis. HPLC, or high-performance liquid chromatography, is a very flexible method. Whereby analytes are separated by passing through a column filled with particles as small as micrometres. In HPLC, reversed-phase chromatography is a frequently employed separation method.

DOLUTEGRAVIR

IUPAC name of Dolutegravir Sodium (4R,12aS)-N-(2,4-Difluorobenzyl)-7-hydroxy-4- methyl-6,8-dioxo-3, 4, 6, 8, 12,12ahexahydro- 2H-pyrido (1:,2':4,5) pyrazino (2,1-b) (1,3) oxazine-9-carboxamide. The molecular formula of C20H19F2N3O5 and molecular weight is 419.4gm/mol. Generally soluble in methanol, dimethyl formamide (DMF), Dimethyl sulphoxide (DMSO).

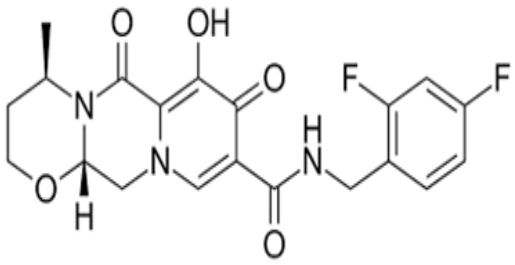

Fig. Structure of Dolutegravir

Dolutegravir (DTG), also known as Tivicay, is an antiretroviral drug [1] that is used in combination with other drugs to treat HIV-acquired immune insufficiency syndrome [2]. Additionally, it can be used to prevent HIV infection after possible exposure [4] as part of post-exposure prophylaxis [3]. It is consumed orally. HIV integrase strand transfer inhibitor DTG [5] prevents HIC integrates, which are essential for viral replication, from working.

Pioglitazone hydrochloride (HCL) is a thiazolidinedione anti-diabetic drug used to treat type 2 diabetes. By increasing the body's sensitivity to insulin, a crucial hormone involved in controlling glucose, it lowers blood sugar levels. Typically used as a pill, pioglitazone can be purchased in conjunction with other diabetes drugs, such as metformin.

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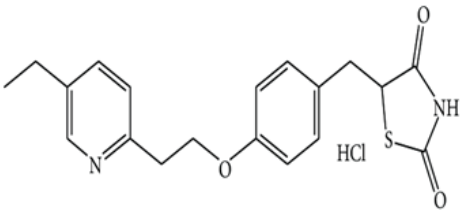

Fig. Structure of Pioglitazone

2. Material and method

Dolutegravir sodium and Pioglitazone HCl were received from Macleod's Pharmaceuticals Ltd. HPLC grade acetonitrile, Methanol and DMSO procured from Merck Pvt. Ltd, Mumbai. The HPLC JASCO instrument was used solvent delivery system using a RESTEK C18 (250×4.6mm) 5 µm and UV Visible detector(UV 2075). The software used was to Borwin . The mobile phase was sonicated using the Ultra Sonic Sonicator. The wavelength was fixed utilizing UV1800, Shimadzu.

Preparation of Mobile phase:

The isocratic mobile phase was Acetonitrile: 10MM Phosphate Buffer pH 3.The mobile phase was filtered through a 0.45 µm Millipore filter and degassed by sonication for 15 min.

Buffer Prepration :

10 milimolar of Phosphate Buffer pH 3 was prepared by dissolving 1.36 gm of Potassium dihydrogen phosphate in 1000 ml water and adjust PH by using orthophosphoric acid (OPA).
Upto 3.

Preparation of standard stock solution of Dolutegravir:

Quantityequivalentto10mgof was weighed and dissolved in methanol to make 10 mL. This gave 10 mg standard stock solution for Dolutegravir Sodium.Quantity equivalentto10 mg of the Pioglitazone was weighed and dissolved in methanol to make 10 mL. This 1000 µg/mL standard stock solution for internal standard.

Sample Preparation:

Before sample preparation, plasma was centrifuged in a polypropylene centrifuge tube at 7000 rpm for 10 minutes. It was defrosted. Plasma sample was divided throughout the validation process into 1.5 mL eppendorf tubes and kept in freezer at -20 °C until analysis in order to prevent repeated freezing and thawing cycles. Using a glass pipette, an aliquot of 975 µL of plasma samples was pipetted on the day of analysis into centrifuge tube with 25µL of DTG working Std solution and 25 µL of PGT working standard solution. The proteins were precipitated by adding 500 µL of Precipitating Solvent after 2 min of vortexing the tubes. Different precipitating solvents tried were Methanol, Acetonitrile, Acetone, Ethanol The tubes were vortexed for an additional 2 minutes. Before being extracted at 7000 rpm for 10 minutes at -5°C with centrifuge.1 mL supernatant liquid was filtered through 0.45 micron syringe filter and injected into HPLC system.

Optimization of Chromatographic condition:

Different mobile phases were tried, to select the ideal mobile phase. Among that Acetonitrile: Phosphate Buffer pH3 (50:50 v/v) was found to be ideal since it gave good resolution and peak shapes with perfect symmetry. The retention time of Pioglitazone and Dolutegravir was found to be 3.5 min and 5.7 min respectively. Stationary phase used was the Restek C18 (250X4.6)5µm column. The flow rate was found to be optimized at 1.0ml/min. detection was carried out at 258 nm by UV detection.

Analytical column	Restek C18 (250X4.6 mm)5µm
Mobile Phase	Acetonitrile: Phosphate Buffer pH 3 (50:50 v/v%)
Detection wavelength	258 nm
Flow rate	1.0 ml /min
Injection volume	20 µl
Retention time	5.7 min

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Quality control samples and calibration standards preparation

By adding each Dolutegravir sodium working solution (i.e., 0.2, 0.6, 1.6, 3.2, 4, 6.5, and 8 µg/ml) to the blank plasma, dolutegravir sodium was detected at seven distinct concentrations ranging from 0.2 to 8 µg/ml. The QC working solutions' low, medium, and high concentrations (0.6, 3.2, and 6.5 µg/ml of Dolutegravir) were appropriately introduced to human plasma.

3. HPLC Method Validation:

The suggested approach was validated using International Conference on Harmonisation (ICH M10) standards after the chromatographic method was optimised based on Dolutegravir sodium separation, an acceptable peak shape, and good resolution. Selectivity, sensitivity, linearity range, accuracy, and precision were all evaluated as validation parameters for the suggested approach The purpose of the technique validation was to make sure that the established procedure could yield reliable and repeatable findings when examined in different labs.

Selectivity:

This parameter was evaluated at the Lower Limit of Quantification (LLOQ) i.e. 10 ng per mL of plasma. The LLOQ sample analyzed and the drug peak area was noted. In the similar manner blank plasma sample was analyzed and the detector response for the Blank was noted at the retention time of the drug. The blank response was compared with the Peak area of the LLOQ sample. This experiment was performed six times using blank plasma. Samples from six different sources

Linearity

In order to evaluate linearity, plasma with a known concentration of Dolutegravir sodium solution was spiked over the 0.2–8 µg/ml concentration range. To create the regression equation, the peak area ratio of dolutegravir sodium /IS vs dolutegravir sodium concentrations was displayed. The precision and correctness of the regression equation might be ascertained by deducting the Dolutegravir sodium concentrations.

Accuracy and precision:

By spiking Dolutegravir sodium in Human plasma, the new method's accuracy and precision were evaluated both within and between days. To evaluate the intra-day precision and accuracy, the samples were spiked at concentrations of 0.2, 3.2, and 6.5 µg/ml. For three consecutive days, the inter-day precision and accuracy were measured using the same concentrations. Precision (CV) was determined using the coefficient of variation, whereas accuracy was measured using the percentage relative error.

Recovery:

The recovery of Dolutegravir sodium from rat plasma was assessed using QC (LQC, MQC, and HQC) standards. The peak areas of each extracted QC standard were compared to the peak areas of unextracted standard solutions that contained the proper amounts of Dolutegravir sodium in the mobile phase in order to determine the recovery of Dolutegravir sodium. Three separate recoveries were made.

Stability Studies :

It is important to assess the stability both Doluteravir sodium during sample collection and handling, as well as following both short-term and long-term storage. The QC samples (LQC, MQC, and HQC) were tested in triplicate during three freeze-thaw cycles in order to determine freeze-thaw stability. Three aliquots of each concentration were separated, frozen for 24 hours at -40 °C, and then allowed to defrost naturally at room temperature. After the samples had completely thawed, they were frozen for an additional twenty-four hours in the same way. The cycle of freeze-thaw was then done twice more, and the stability of the medication in plasma was examined.

In order to evaluate the long-term stability, four aliquots of the low, medium, and high concentrations in the plasma were maintained at 40 °C for 21 days, and the analyte concentration in the sample was measured. Three aliquots of each QC concentration were stored under these conditions, and four aliquots of each concentration were stored for one and eight hours at room temperature.

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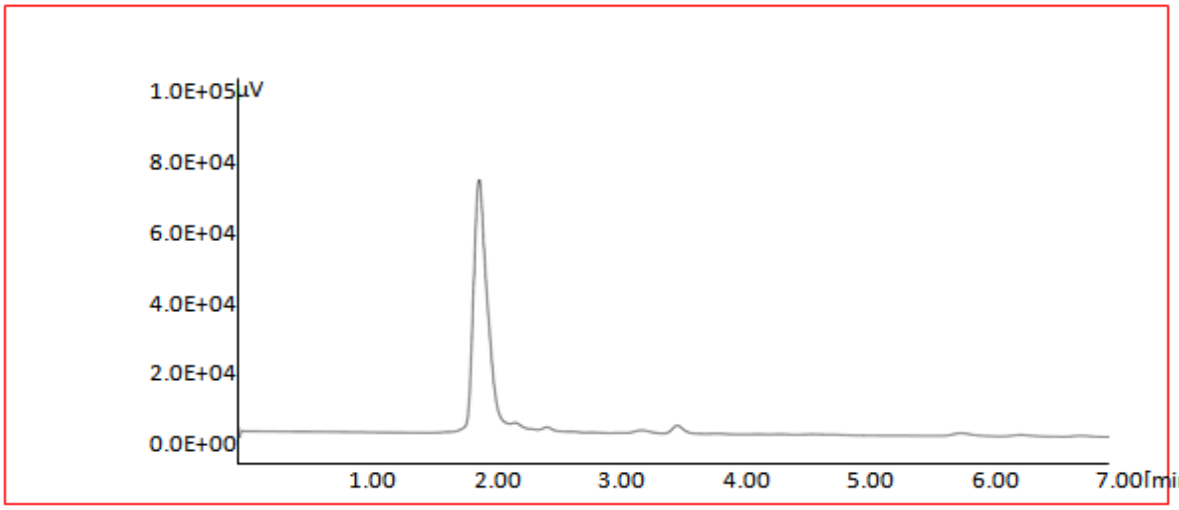
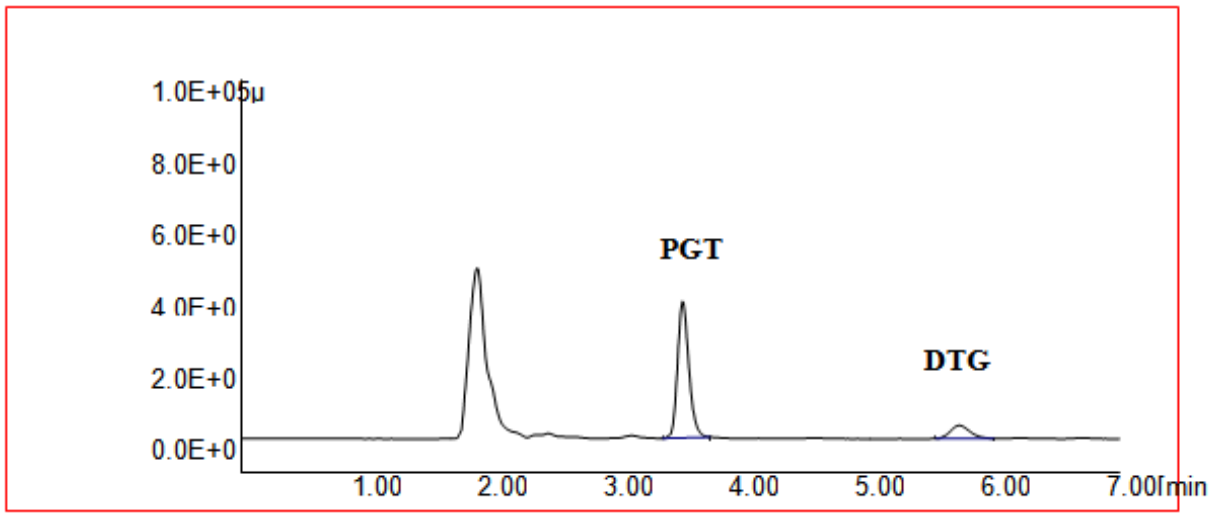
4. RESULTS:

Selectivity:

The method's selectivity was evaluated by looking at the plasma .Figure 1 shows the results of a single blank plasma sample, and the lack of interference is similar to that of other samples that were also looked at. At the3.5 minute Dolutegravir sodium retention period, no influence from endogenous components of blank plasma was detected.



Chromatogram of Blank Plasma



Chromatogram of LLOQ

Linearity:

A linearity equation of y = 0.0002x -0.0192 and a correlation coefficient of 0.9947 (Fig. 3) showed that the Dolutegravir calibration curve was linear and varied between 0.2 and 8 µg/ml. Dolutegravir sodium lower limit of quantification (LLOQ) was determined to be 0.2 µg/ml. The developed approach demonstrated good linearity in light of this. It was discovered that a standard curve was validated if the coefficient of correlation was close to one.

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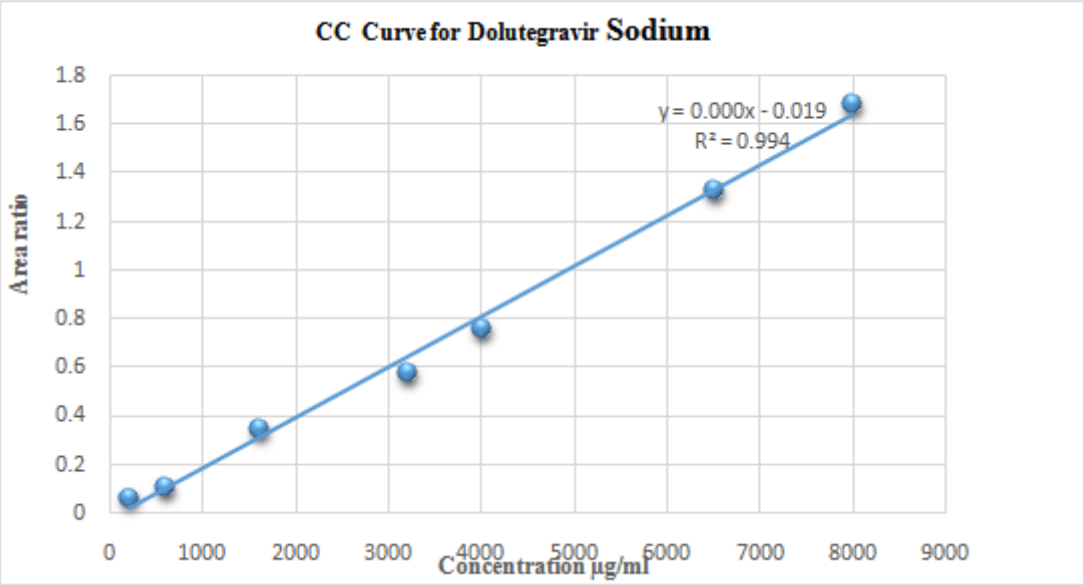

Accuracy and precision :

The within run accuracy and precision were expressed in terms in terms of % Accuracy and % R.S.D. respectively. At the LQC, MQC and HQC levels the maximum within run % Accuracy values were 102%, 100.49% and 100.69% respectively, while the maximum within run % R.S.D. values at the LQC, MQC and HQC levels were 5.479, 1.9160 and 4.658 respectively. All other within batch % Accuracy and % R.S.D. values were smaller than these.

The overall % RSD which was a measure of between run precision had values 3.274900, 1.4162 and 1.1979 respectively at the LQC, MQC and HQC levels. The overall % Accuracy which was a measure f between run accuracy had values 98.07%, 100.78% and 100.27% at the LQC, MQC and HQC levels respectively.

The % Accuracy at the three QC levels was thus between ± 15% while the % R.S.D. was less than 15 %. **Table 2**are representative chromatograms of LQC, MQC and HQC samples of Dolutegravir Sodium. ICH M10 guidance states that the % Accuracy at the three QC levels should be between ± 15% while the % R.S.D. should be less than 15 %. From **TABLE 2**, it can be concluded that the method meets this acceptance criteria.

QC level	Calculated conc. (µg/ml)	Accuracy	SD	% CV
0.6	0.59	99.81	3.9325	3.992
3.2	3.202	100.07	1.89884	1.8589
6.5	6.502	100.03	1.81172	1.81114

TABLE 2:Intraday accuracy and precision

QC level	Calculated conc (µg/ml)	Accuracy	SD	% CV
6	6.19	103.23	4.7736	4.6243
3.2	3.20	100.12	1.7894	1.7451
6.5	6.49	99.91	0.7730	0.7378

TABLE 3: Interday Accuracy and precision

Recovery

It was observed that the recovery of Dolutegravir Sodium was consistent over the three levels and that of Pioglitazone was comparable to that of Dolutegravir Sodium.**TABLE 4** gives % recovery for Dolutegravir Sodium at three QC levels as well as for Pioglitazone.

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TABLE 4:The recovery of Dolutegravir Sodium from human plasma

Samples	Unextracted	Extracted	% Recovery
LQC	31245	23564	75.41
MQC	169085	124567	73.67
HQC	359329	267894	75.57
IS	357894	264578	73.92

Stability Studies

The results of stability studies are given in **TABLE 5**. The ICH M10 requires that the mean % nominal between 85 – 115 and % R.S.D. be less than 15. From the results obtained, it was concluded that there was no systematic decrease in the concentration with time and the variability of the results was consistent with the acceptance criteria for accuracy. Thus, the results did not suggest instability of DTG, if the plasma under the conditions tested.

TABLE 5: Results of stability studies for Dolutegravir Sodium

Benchtop_8Hr Stability at room temperature						
QC level	**Unextracted**	**Extracted**	**Benchtop_8h Stability at room temperature**			
			% Nominal	**Average**	**SD**	**%RSD**
LQC	28431	22890	80.51	80.47	0.669	0.831
	28214	22508	79.78			
	28541	23150	81.11			
HQC	362483	298541	82.36	81.47	1.0103	1.2400
	364352.7	297642	81.69			
	372547	299432	80.37			

Result of Freeze Thaw stability

Freeze thaw stability_20° C _3 Cycles							
QC level	FTS	Unextracted	Extracted	Freeze thaw stability_20° C _3 Cycles			
				% Nominal	Average	SD	%RSD
LQC	FTS1	27321	22056	80.73	80.83	0.840	1.040
	FTS2	28656	23415	81.71			
	FTS3	27543	22045	80.04			
HQC	FTS1	354678.8	290451	81.89	82.39	0.4896	0.5942
	FTS2	365472.5	302865	82.87			
	FTS3	376542.1	310345	82.42			

Result of Stock Solution Stability

Stock solution stability (MQC)		
	Fresh Sample	**Stability sample (8h)**
Mean area	139375.7	132012.7
% RSD	1.917633	1.431495

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