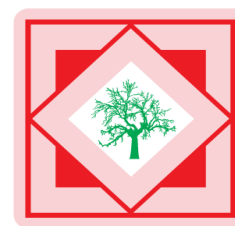




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## **Development and validation of high performance liquid chromatographic method for the determination of Dolutegravir in human plasma**

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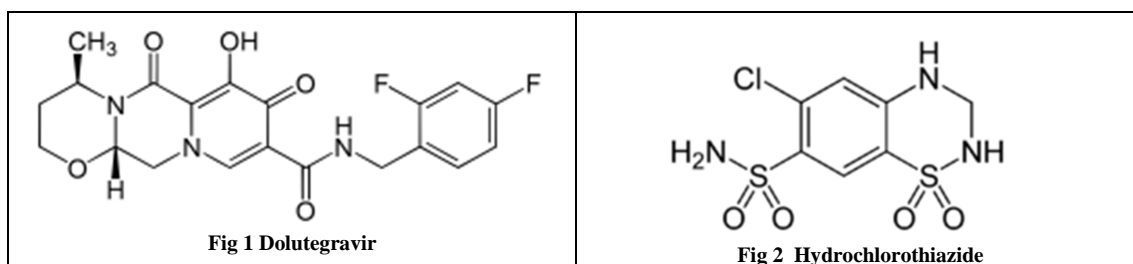
### **ABSTRACT**

*A rapid, specific and accurate high performance liquid chromatographic method for the determination of dolutegravir in human plasma using hydrochlorothiazide as internal standard was developed and validated by UV detection. The extraction process involved a liquid-liquid extraction using methyl-t-butyl ether. Both dolutegravir and the internal standard were eluted under isocratic mode using a 150 X 4.6 mm i.d, 5 µm Phenomenex ODS 2 C18 column. The mobile phase composed a mixture of 30:70 % v/v 20 mM Sodium acetate buffer (pH 4.0) and Methanol at a flow rate of 1.0 mL/minute. The wavelength of detection is 254 nm. The injection volume is 20 µL. The runtime of the method is 6 minutes with retention times 2.08 minutes and 4.16 for Hydrochlorothiazide and Dolutegravir respectively. The method showed good linearity in the range of 101.90 to 7004.49 ng/mL. The recovery of Dolutegravir is 59.21 % with a co-efficient of variation 3.72 % and recovery of internal standard was 60.61 % with a co-efficient of variation 3.33 %.*

**Keywords:** Dolutegravir, HIV Treatment, Antiretroviral drug, integrase inhibitor, HPLC-Method.

### **INTRODUCTION**

Dolutegravir(DOLU),(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. Doluteg -ravir is integrase inhibitor used in the treatment of HIV<sup>[1]</sup> and was approved by FDA<sup>[2]</sup>. Dolutegravir [Figure 1] works by blocking integrase, an HIV enzyme. This prevents HIV from replicating and lowers the amount of HIV in the blood. It can be used in the treatment of HIV in both adults and children of 12 years age and older and weighing atleast 40 kilograms. A thorough review of literature revealed that only a very few methods were reported for the determination of Dolutegravir in human plasma<sup>[3]</sup> and a number of methods were reported for the determination of hydrochlorothiazide<sup>[4-18]</sup>. Hence it is decided to develop a new RP-HPLC method for the determination of Dolutegravir in human plasma. To access the reproducibility and wide applicability of the developed method, it was validated as per FDA guidelines<sup>[2]</sup>.



## MATERIALS AND METHODS

### *Solvents and Chemicals*

Dolutegravir (purity 99.90 % w/w) was used as received from M/S Laurus Pharma Ltd. Hydrochlorothiazide (used as Internal Standard, Purity 99.0 % w/w) is gifted by M/S Roorkee Drugs Pvt Ltd. HPLC grade Methanol, Methyl-*t*-butyl chloride was purchased from Merck Ltd (Mumbai, India). Deionized water was processed through a Milli-Q water purification system (Millipore, USA). All other chemicals and reagents were of analytical grade.

### *Chromatographic System*

The Chromatographic system consisted of a Shimadzu Class VP Binary pump LC-10ATvp, SIL-10ADvp Auto sampler, CTO-10Avp Column Temperature Oven, SPD-10Avp UV-Visible Detector. All the components of the system are controlled using SCL-10Avp System Controller. Data acquisition was done using LC Solutions ver. 1.23 SP 1 software. The detector is set at a wavelength of 254 nm. Chromatographic separations were accomplished using a Phenomenex ODS-2, C<sub>18</sub>, 5 μm, 150 mm×4.6 mm column. The mobile phase consists of a mixture of 70 parts of Methanol and 30 parts of 20 mM Sodium acetate buffer (pH 4.0±0.05). The pH of the buffer adjusted to 4.0 using glacial acetic acid. The mixture was filtered through 0.45 μm membrane (Millipore, Bedford, MA, USA) under vacuum, and then degassed by flushing with nitrogen for 5 min. The mobile phase was pumped isocratically at a flow rate of 1.0 ml/min during analysis, at ambient temperature. The rinsing solution consists of a mixture of 50: 50 % v/v of acetonitrile: HPLC Grade Water.

### *Preparation of Standard Solutions*

A stock solution of Dolutegravir was prepared in methanol such that the final concentration is approximately 2.5 mg/mL. Stock solution of Hydrochlorothiazide (approx 10.0 mg/mL) is prepared in HPLC Grade methanol. The solutions were stored at 10°C and they were stable for at least two weeks. Aqueous stock dilution of Dolutegravir is prepared in diluent solution (mixture of 50: 50 % v/v of methanol: HPLC Grade water).

### *Sample Preparation*

Aqueous stock dilutions were prepared initially. 0.5 ml of each aqueous stock dilution is transferred into a 10 mL volumetric flask. The final volume is made up with screened drug-free K<sub>2</sub>EDTA human plasma and mixed gently for 15 minutes to achieve the desired concentration of calibration curve standards. The final calibration standard concentrations are 0.0 (Blank; no Dolutegravir added), 101.90, 203.80, 1018.98, 2037.96, 3056.94, 4585.41, 6419.57 and 7005.49 ng/ml. Each of these standard solutions was distributed in disposable polypropylene micro centrifuge tubes (2.0 ml, eppendorf) in volume of 0.6 ml and stored at -70°C until analysis. Similarly quality control samples were prepared in plasma such that the final concentrations were 106.99, 331.17, 3617.38 and 5604.39 ng/ml respectively and labeled as Lower limit of quantification (LLOQ), Low quality control (LQC), median quality control (MQC) and high quality control (HQC) respectively.

The extraction of the plasma samples involved Liquid-Liquid Extraction process. For processing, the stored spiked samples were withdrawn from the freezer and allowed to thaw at room temperature. An aliquot of 400 μL of the spiked plasma calibration curve standard and the quality control samples were transferred to pre-labeled 2.0 mL polypropylene centrifuge tubes. 50 μL of internal standard dilution (100 μg/mL) is then added and mixed. 1.0 mL diethyl ether extraction solvent is then added to extract the drug and internal standard. The samples were then subjected to flash-freezing using a mixture of dry-ice and acetone. The supernatant was then transferred into another labeled polypropylene tube and evaporated to dryness under nitrogen at 40<sup>o</sup> C The dried residue was then dissolved in 0.3 mL of mobile phase, vortexed thoroughly and transferred into auto sampler vial for analysis. 20 μL of the samples was then injected into the system for analysis. The autosampler temperature is maintained at 4 °C throughout the analysis. The column temperature oven is maintained at ambient temperature.

**Validation of quantitative HPLC method**

The quantitative HPLC-UV method was validated to determine selectivity, calibration range, accuracy and precision, limit of detection (LOD), limit of quantitation, % recovery, freeze–thaw and auto sampler stability. The initial assay was fully validated for Dolutegravir analysis in human plasma according to FDA guidelines.

**Selectivity**

The selectivity of the method was evaluated by analyzing six independent drug-free K<sub>2</sub>EDTA human plasma samples with reference to potential interferences from endogenous and environmental constituents.

**Calibration curve**

Calibration curves were generated to confirm the relationship between the peak area ratios and the concentration of Dolutegravir in the standard samples. Fresh calibration standards were extracted and assayed as described above on three different days and in duplicate. Calibration curves for Dolutegravir were represented by the plots of the peak-area ratio (Dolutegravir/Hydrochlorothiazide) versus the nominal concentration of the Dolutegravir in calibration standards. The regression line was generated using  $1/\text{concentration}^2$  factor as the mathematical model of best fit. Dolutegravir concentrations in QC samples, recovery and stability samples were calculated from the resulting area ratio and the regression equation of the calibration curve.

**Accuracy and precision**

Intra-day accuracy and precision were evaluated by analysis of QCs at four levels (LLOQ, LQC, MQC and HQC; n = 6 at each level) on the same day. Inter-day precision and the accuracy were determined by analyzing four QC levels on 3 separate days (n = 6 at each level) along with three separate standard curves done in duplicates.

The accuracy of an analytical method describes how close the mean test results obtained by the method are to the nominal concentration of the analyte. Accuracy was calculated by the following equation, expressed as a percentage:

$$\text{Accuracy (\%)} = \text{mean observed concentration/nominal concentration} \times 100$$

The precision was expressed by co-efficient of variation (CV). The CV % indicates the variability around the mean in relation to the size of the mean, and is defined as:

$$\text{CV (\%)} = \text{standard deviation/mean observed concentration} \times 100$$

**Stability Studies**

Autosampler, and freeze–thaw stability of Dolutegravir was determined at low, medium and high QC concentrations. To determine the impact of freeze–thaw cycles on Dolutegravir concentration, samples were allowed to undergo 3 freeze (–70 °C) thaw (room temperature) cycles. Following sample treatment/storage conditions, the Dolutegravir concentrations were analyzed in triplicates and compared to the control sample that had been stored at –70 °C. Autosampler stability of extracted samples was determined by comparing Dolutegravir concentration in freshly prepared samples and samples kept in autosampler at 4 °C for 24 h.

**Recovery**

Recovery was determined by comparing the area under the curve (AUC) of extracted QC samples (LQC, MQC and HQC) with direct injection of extracted blank plasma spiked with the same nominal concentration of Dolutegravir as in the QC samples. This should highlight any loss in signal due to the extraction process. IS recovery was determined for a single concentration of 40 µg/mL.

**Data analysis**

HPLC data acquisition and processing was performed by Shimadzu LC Solutions Ver 1.23 SP 1 software. Standard curves for quantitation of Dolutegravir were constructed using a  $1/\text{concentration}^2$  weighted linear regression of the peak area ratio versus Dolutegravir concentration. Unknown and QC sample concentrations were back-calculated from the standard curves.

**RESULTS AND DISCUSSION****Method Development**

The HPLC procedure was optimized with a view to develop a sensitive and reproducible method for the determination of Dolutegravir in Human Plasma. Since both Dolutegravir and internal standard hydrochlorothiazide are highly non-polar<sup>[13, 14, 15]</sup> we employed the usage of liquid-liquid extraction process with diethyl ether. To get a better response the pH of the mobile phase is set to the acidic side. During our observation, a pH value around 7.4

resulted in better peak shape for the internal standard while that of the drug is not acceptable. Also, alkaline mobile phase characteristics causes deterioration of the bonded phase in the column due to alkaline hydrolysis of end-capped silica<sup>16, 17</sup>. Compared to acid catalyzed hydrolysis, the hydrolysis of end-capped silica in alkaline conditions is usually very rapid. Therefore experiments were performed using 20mM sodium acetate in a limited pH range of 4.0 to pH 5.5. The response was checked at the detector using a connector (without the column). A pH value of  $4.0 \pm 0.5$  gave maximum response for the analyte at 254 nm. A similar response was observed with the usage of 70 % methanol. Therefore the final mobile phase consisted of 30: 70 % v/v 20 mM sodium acetate (pH adjusted to  $4.0 \pm 0.5$  using glacial acetic acid) and methanol. The run time of analysis is higher when a longer reverse phase column (250 X 4.6 mm id) is used. The resolution between the peaks was decreased and peaks were not acceptable peak shape when the experiment is performed using a shorter column (50 X 4.6 mm id). However better resolution, less tailing and high theoretical plates are obtained with a Phenomenex column C18 150 X 4.6 cm 5  $\mu$ m column.

The flow rate of the method is 1.0 ml/min. The column temperature is maintained at ambient. At the reported flow rate, peak shape was acceptable, however increasing or decreasing the flow rate increased the tailing factor and resulting in poor peak shape and decreased resolution between the drug and internal standard. There was no interference in the drug and internal standard, from the extracted blank. The peak symmetry were found to be good when the mobile phase composition of 30:70 v/v of 20 mM sodium acetate and methanol leading to better resolution of the drug and internal standard. Increasing the organic portion of the mobile phase caused hydrochlorothiazide to elute early. A mobile phase containing aqueous portion greater than 30 % led to very late elution and very poor peak shape for Dolutegravir. The peaks were also broad with unacceptable asymmetry factor.

Extraction methods were initially attempted using Protein precipitation technique. The use of organic solvents as reagents for protein precipitation was well known. Precipitation technique was adopted using Acetonitrile and or Methanol. Initial experiments of protein precipitation were done using 1: 3 ratio of plasma : Organic solvents. The recovery of the Dolutegravir was poor while that of the internal standard is relatively unchanged as compared with liquid-liquid extraction.

Since the noise effects in solid phase extraction (SPE) method are similar to that of liquid-liquid extraction, we have done the final analysis using liquid-liquid extraction (LLE). SPE methods although render a neat sample for final analysis, polar interferences do enter into the final sample during reconstitution. SPE is further expensive as compared to LLE technique.

Various solvents such as Ethyl acetate, Diethyl Ether, 100 % t-butyl methyl ether and combinations of t-butyl methyl ether and Dichloromethane were used for extraction. The recovery of Dolutegravir and internal standard was poor when Ethyl acetate or t- butyl ether was used individually. The highest recovery from the plasma samples is obtained with diethyl ether.

#### **Detection and chromatography**

Fig. 4 shows the typical chromatograms of a blank human plasma sample (A), a zero blank sample with Hydrochlorothiazide (B), LLOQ sample extracted using hydrochlorothiazide as internal standard and (D) with a sample containing ULOQ sample extracted using hydrochlorothiazide as internal standard indicating the specificity of the method. The retention times for Dolutegravir and IS were 4.16 and 2.08 minutes, respectively.

#### **Method validation**

##### **Selectivity**

The method was found to have high selectivity for the analytes; since no interfering peaks from endogenous compounds were observed at the retention time for Dolutegravir in any of the six independent blank plasma extracts evaluated (Fig. 4A).

##### **Calibration curves**

A system suitability exercise is performed before the initiation of the validation. A system is assumed to be suitable for analysis if and only if the % CV for the retention times of Dolutegravir and internal standards is less than 2 %. The results are tabulated in Table 1. Calibration curves for Dolutegravir in human plasma were fitted by weighted  $1/\text{concentration}^2$  quadratic regression, with the  $r^2$  values of  $>0.99$  for all curves generated during the validation. The calibration curve accuracy for plasma is presented in Table 2 demonstrating that measured concentration is within  $\pm 15\%$  of the actual concentration point (20% for the lowest point on the standard curve, the LLOQ). Results were calculated using peak area ratios. A representative calibration curve showing the regression equation and  $r^2$  value is depicted in Figure – 3.

### Accuracy and precision

A detailed summary of the intra-day and inter-day precision and accuracy data generated for the assay validation is presented in Table 3. Inter-assay variability was expressed as the accuracy and precision of the mean QC concentrations (LLOQ, LQC, MQC, and HQC) of three separate assays. Intra-assay variability was determined as the accuracy and precision of the six individual QC concentrations within one assay. The inter- and intra-assay accuracy and precision was <5% for all QC concentrations, which was within the general assay acceptability criteria for QC samples according to FDA guidelines [2].

### Limit of detection and limit of quantification

LOD is defined as the lowest concentration that produced a peak distinguishable from background noise (minimum ratio of 3:1). The approximate LOD was 55 ng/mL. The LLOQ has been accepted as the lowest points on the standard curve with a relative standard deviation of less than 20% and signal to noise ratio of 5:1. Results at lowest concentration studies (100 ng/mL) met the criteria for the LLOQ (Table 3). The method was found to be sensitive for the determination of RIF in human plasma samples. The ULOQ has been accepted as the highest points on the standard curve with a relative standard deviation of less than 15%.

### Carryover test

A critical issue with the analysis of many drugs is their tendency to get absorbed by reversed phase octa-decyl-based chromatographic packing materials, resulting in the carryover effect. However in this analysis no quantifiable carryover effect was obtained when a series of blank (plasma) solutions were injected immediately following the highest calibration standard.

### Stability studies

The results of short-term, long term and freeze–thaw stability are presented in Table 4. Determination of Dolutegravir stability following three freeze–thaw cycles showed that for all QC samples there was a minor change in the Dolutegravir concentration.

### Recovery

Percentage recovery of Dolutegravir was measured by dividing the peak area values of extracted QC samples with direct injection of solution containing the same nominal concentration of compounds as the QC samples in extracted blank plasma. The mean recovery of Dolutegravir from plasma spiked samples of Dolutegravir at LQC, MQC and HQC levels was 57.61 %, 61.72 % and 58.29 % respectively. The overall recovery is 59.21 % with a % Coefficient of variation of 3.72 %, respectively. IS recovery at 40 µg/mL of Hydrochlorothiazide was 60.61 % with a % Coefficient of variation of 3.33%.

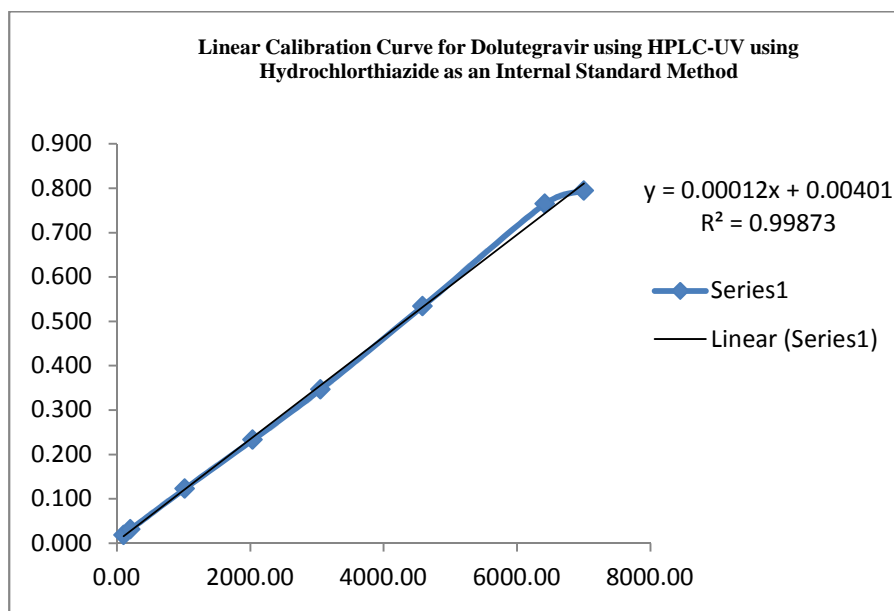


Figure 3: Calibration Curve of Dolutegravir (Curve – 1)

Figure 4: Chromatograms of (A) Extracted Blank Sample (B) Zero Blank Containing Hydrochlorothiazide as Internal standard (C) Dolutegravir containing hydrochlorothiazide as Internal Standard

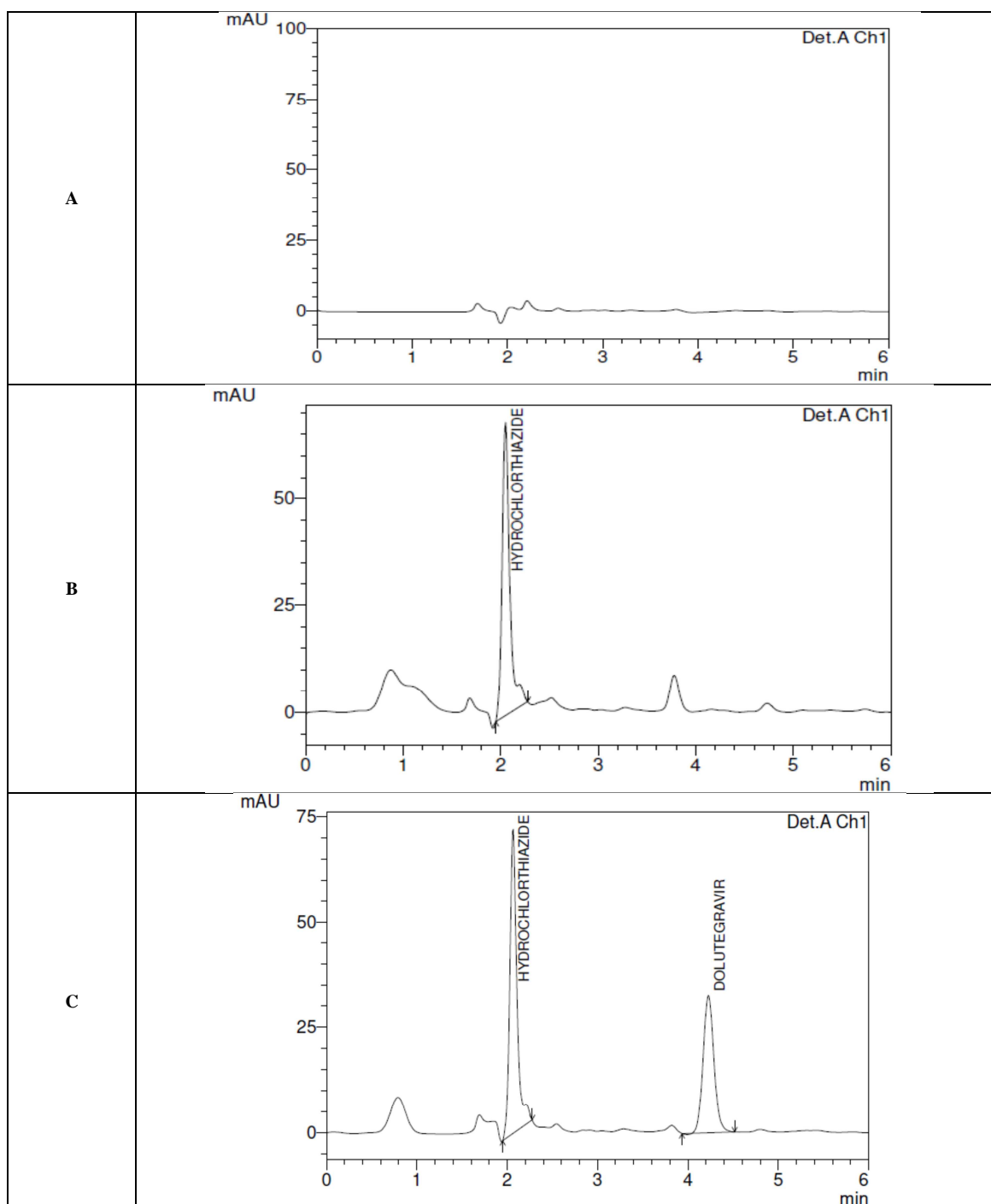


Table 1 System Suitability Study

	Hydrochlorothiazide Internal Standard (40 µg/mL)		Dolutegravir (2500.00 ng/mL)	
	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area
Mean (n = 6)	2.08	951862	4.16	580126
S. D.	0.02	12761.84	0.03	8185.00
% CV	0.44	4.66	0.36	5.22

Table 2 Results of regression analysis of the linearity data

Linearity parameters	Mean ± SD (n = 6)
Slope	0.00012
Intercept	0.00867
Correlation coefficient (r <sup>2</sup> )	0.99879

**Table 3** Intra and Inter day accuracy and precision of HPLC assay

	Nominal Concentration ( ng/mL)			
	106.99	331.17	3617.38	5604.39
<b>DAY 1</b>				
Mean	113.00	334.39	3906.91	5749.38
S.D.	11.863	19.335	326.487	274.401
% CV	10.34	5.78	8.36	4.77
<b>DAY 2</b>				
Mean	113.72	336.20	3923.07	5775.63
S.D.	13.438	22.114	274.065	195.721
% CV	11.82	6.58	6.99	3.39
<b>DAY 3</b>				
Mean	112.89	333.80	3895.60	5735.05
S.D.	13.116	21.945	282.542	220.075
% CV	11.62	6.57	7.25	3.84

Each mean value is the result of triplicate analysis

**Table 4** Short Term, long term and Freeze Thaw stability of Dolutegravir

	Nominal Concentration ( ng/mL)	
	331.17 (LQC)	5604.39 (HQC)
<b>Short-term stability (8 hours)</b>		
Mean Accuracy (%)	100.76	96.59
S.D.	11.50	61.24
% CV	3.50	1.06
<b>Long-term stability (11 Days)</b>		
Mean Accuracy (%)	99.46	94.88
S.D.	18.01	188.54
% CV	5.41	3.19
<b>Freeze – Thaw stability (3 Cycles)</b>		
Mean Accuracy (%)	101.46	97.44
S.D.	5.25	33.19
% CV	1.61	0.58

Each mean value is the result of triplicate analysis

## CONCLUSION

A HPLC method was developed and validated for the determination of Dolutegravir in human plasma. The extraction process was a single-step liquid–liquid extraction procedure employing the use of diethylether. LLE method is usually devoid of polar interferences thus rendering the sample clean for final analysis. The noise is usually absent or at minimum as compared to precipitation or SPE techniques. This assay requires only a small volume of plasma (500 µL). There is no carryover effect. Due to the LLE method of extraction, baseline noise is minimal. Matrix effects are not observed. In conclusion, method validation following FDA guideline indicated that the developed method had high sensitivity with an LLOQ of 106 ng/mL, acceptable recovery, reliability, specificity and excellent efficiency with a total running time of 6.0 min per sample, which is important for large batches of samples. Thus this method can be suitable for pharmacokinetic, bioavailability or bioequivalence studies of Dolutegravir in human subjects. This method has been successfully applied to analyze Dolutegravir concentrations in human plasma.

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