Research Article

IDENTIFICATION AND QUANTIFICATION OF POTENTIAL IMPURITIES USING LC-PDA COUPLED WITH NEW QDa MASS DETECTOR IN A NEW SINGLE TABLET REGIMEN CONTAINING DOLUTEGRAVIR, EMTRICITABINE AND TENOFOVIR DISOPROXIL FUMARATE TABLETS USED IN HIV-1 PREVENTION

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ABSTRACT

The objective of this current work was to develop and validate a rapid, sensitive, rugged, simple, single method for the determination and quantification of degradation products as well as process impurities by HPLC coupled with PDA, QDA mass detector and same method was also scalable to UPLC for a fixed dose combination containing dolutegravir, emtricitabine and tenofovir disoproxil fumarate. Chromatographic separation was achieved using a new Core-Shell Bi-phenyl column for both HPLC and UPLC and a gradient programme consisting of Mobile phase A: Ammonium acetate (pH 3.0 buffer) and Mobile phase B: Ammonium Acetate (pH 3.0 buffer) with Methanol and Acetonitrile. The run time was 120 minutes for HPLC and 45 minutes for UPLC within which dolutegravir, emtricitabine, tenofovir disoproxil fumarate and its fourteen related compounds were eluted. Drug was subjected to hydrolysis (acid and base), oxidative, photolytic and thermal stress conditions. The unknown degradation products were identified by PDA/QDA mass detector. Which revealed protonated molecular ion peaks [M=H at m/z DP1-418.21 and DP2-418.18 for sample preparation in acid and base hydrolysis, at m/z DP3-416.04, DP4-663.17, DP5-490.14, DP6-819.21 and DP7-606.14 for thermal degradation sample and at m/z DP8-438.18 and DP9-438.18 for Dolutegravir API in acid hydrolysis condition. A cogent mechanism for the formation of degradation and process impurities was proposed. The performance of the developed method was validated as per International Conference on Harmonization guidelines (ICH) with respect to specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision and robustness.

Key words: RP-HPLC/UPLC/QDA mass detector, Dolutegravir, Emtricitabine, Tenofovir disoproxil fumarate, Stress degradation, Method validation

INTRODUCTION

Combination antiretroviral therapy (cART) has evolved considerably over the past two decades leading to better control of human immunodeficiency virus (HIV), preservation of the immune system and decreased incidence of opportunistic infections, malignancies and deaths. But successful implementation of cART has been hampered by complicated regimens, high pill burden, drug-drug interactions and frequent short- and long-term adverse effects, leading to decreased adherence to prescribed regimens. Combination products consisted initially of partial regimens mostly combining two nucleoside reversed transcriptase inhibitors (NRTIs) such as zidovudine/lamivudine (3TC), abacavir (ABC)/3TC or tenofovir (TDF)/emtricitabine (FTC) or a boosted protease inhibitor (PI) lopinavir/ritonavir (RTV), but, in 2006, the first single-tablet regimen (STR), a combination of TDF/FTC/efavirenz (EFV) became available¹ and, since then, STRs have been regarded as relevant tools to manage chronic HIV infection. The most advanced regimens used nowadays involve a single pill administered daily. The US guidelines now recommend that providers, when choosing between regimens of similar efficacy and tolerability, use once-daily (OD) regimens for treatment-naïve patients beginning cART, switch treatment-experienced patients receiving complex or poorly tolerated regimens to OD regimens, and use fixed-dose combinations (FDCs) and STRs to decrease pill burden^{2,3}. In the present study a FDC containing a dolutegravir 60 mg (INSTI's), emtricitabine

200 mg (NRTIs) and tenofovir disoproxil fumarate 300mg (NRTIs) have been used for identification and quantification of degradation product as well process impurities.

Chemical Formula and molecular weight of dolutegravir sodium is C₂₀H₁₈F₂N₃NaO₅ (441.36 g/mol as DTG sodium, 419.38 as DTG free acid). Chemical name (IUPAC name) Sodium(4R,12aS)-9-[[{(2,4-difluorophenyl)methyl]carbamoyl}-4-methyl-6,8-dioxo-3,4,6,8,12,12a-hexahydro-2H-pyrido [1',2':4,5]pyrazino[2,1-b][1,3]oxazol-7-olate[1,2].

Dolutegravir sodium is a polycyclic, nitrogen containing heterocycle possessing amide functionality. It is a HIV integrase inhibitor^{4,5,6,7}. The chemical name of emtricitabine is 5-fluoro-1-(2R,5S)-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine. It has a molecular formula of C₈H₁₀FN₃O₃S and a molecular weight of 247.24. Emtricitabine, a synthetic nucleoside analog of cytidine, is phosphorylated by cellular enzymes to form emtricitabine 5'-triphosphate. Emtricitabine 5'-triphosphate inhibits the activity of the HIV-1 RT by competing with the natural substrate deoxycytidine 5'-triphosphate and by being incorporated into nascent viral DNA which results in chain termination. It is indicated for HIV infection in combination with other anti-retroviral drugs. It is also active against Hepatitis B virus^{8,9}.

Tenofovir DF is a fumaric acid salt of the bisisopropoxycarbonyloxymethyl ester derivative of tenofovir.

The chemical name of tenofovir disoproxil fumarate is 9-[(R)-2[[bis[[[(isopropoxycarbonyl)oxy]-methoxy]phosphinyl]methoxy]propyl]adenine fumarate (1:1). It has a molecular formula of $C_{19}H_{30}N_5O_{10}P \cdot C_4H_4O_4$ and a molecular weight of 635.52. Tenofovir Disoproxil Fumarate (TDF) is a fumaric acid salt of the bisisopropoxycarbonyloxymethyl ester derivative of tenofovir. Tenofovir is nucleotide analogue reverse transcriptase inhibitor (NtRTI) active against HIV¹⁰⁻¹⁴. It is used for the treatment of HIV infection and chronic hepatitis B infection.

A literature survey revealed that few methods available for the quantitative determination of Dolutegravir¹⁵⁻¹⁸, Emtricitabine¹⁹ and Tenofovir disoproxil fumarate²⁰ alone, a very few methods available for the ascertainment of emtricitabine and tenofovir disoproxil tablets^{21, 22, 23}, tenofovir disoproxil fumarate, lamivudine, and efavirenz in combined tablet dosage form²⁴, efavirenz, emtricitabine and tenofovir tablets²⁵. For the first time a method which used for the simultaneous quantitative determination of potential impurities in a fixed dose combination of dolutegravir, emtricitabine and tenofovir disoproxil fumarate by HPLC and UPLC. A RP-HPLC method has been reported for determination of Emtricitabine, Tenofovir and Efavirenz in Emtricitabine, Tenofovir and Efavirenz fixed dosage form. Degradation impurities and process impurities were considered developing stability-indicating method by HPLC and UPLC. Pharmacopoeial monographs available only for the estimation of organic impurities for tenofovir tablets but not for emtricitabine and dolutegravir. It is therefore, necessary to develop a single, simple, rugged and stability indicating method for the determination of process and degradation related compounds in dolutegravir, emtricitabine and tenofovir disoproxil fumarate tablets. It is also required to identify the degradation impurities for dolutegravir, emtricitabine and tenofovir disoproxil fumarate.

Hence the primary objective of this study was to develop a single HPLC/UPLC method that could separate dolutegravir, emtricitabine, tenofovir disoproxil fumarate (Figure 1) from its process and potential impurities namely DOL.RC01, DOL.RC02, DOL.RC03, DOL.RC04, ECT.RC01, ECT.RC02, ECT.RC03, ECT.RC04, TNF.RC01, TNF.RC02, TNF.RC03, TNF.RC04, TNF.RC05, TNF.RC06 (Figure 2) and unknown degradation impurities formed during forced degradation^{26, 27, 28} studies and stability studies²⁹. This method is a mass compatible, same method can also be used for the determination of mass of known and unknown degradation impurities formed during acid, base, peroxide, thermal and photolytic³⁰ stress conditions using a LC connected with a PDA detector followed by QDA mass detector^{31, 32, 33}. This method was successfully validated according to the ICH guidelines³⁴. Simultaneously a scalable UPLC^{35, 36, 37} method was also developed using same stationary phase, buffer and mobile phase, which can extensively reduce the analysis time three folds less than HPLC³⁸. As the product is a triple combination, a single method by HPLC/UPLC would allow for a much more cost effective and less time consuming analysis for dolutegravir, emtricitabine, tenofovir disoproxil fumarate.

MATERIALS AND METHODS

Chemicals and reagents

Dolutegravir, emtricitabine, tenofovir disoproxil fumarate standards, standard materials of degradation impurities, process impurities and chemical reagents were obtained from, Hetero labs limited, Hyderabad, India. Ammonium acetate (Merck grade), Orthophosphoric acid (Merck grade), Formic acid (Merck grade), Methanol (Rankem HPLC grade) and Acetonitrile (Rankem

HPLC grade) were purchased from Merck chemicals, India and Rankem Chemicals India. High-quality pure water was prepared in-house by use of a Millipore (Billerica, MA, USA) Milli-Q water-purification system.

Instrumentation

System used for method development and validation study was Water HPLC equipped with 2998 photodiode array detector and acquity QDA mass detector. Same Method was also developed on Waters Acquity UPLC-H class equipped with 2998 photo diode array detector. The output signal was monitored and processed using Empower 3 software (Waters Corporation, Milford, MA, USA).

HPLC Conditions

The chromatographic separation was accomplished on a new - shell Bi-phenyl polar stationary phase, Phenomenex kinetex Bi-phenyl 250x4.6mm, 5 μ m. Mobile phase A was ammonium acetate (10mM) adjusted pH 3.0 with 50% formic acid solution and Mobile phase B was Acetonitrile, ammonium acetate (10mM) adjusted pH 3.0 with 50% formic acid solution, and Methanol in the ratio of 70:15:15%v/v/v. The gradient programme T (min) =% mobile phase B: 0=5, 7=5, 20=25, 40=35, 65=55, 80=70, 90=75, 105=75, 110=5 and 120=5 with flow rate 0.6 mL/min. The injection volume was 10 μ L and detection wavelength was set at 260nm. The column temperature was maintained at 30°C. Sample cooler was maintained at 5°C.

UPLC Conditions

The chromatographic separation was accomplished by a same stationary phase used for HPLC. A Core-shell polar stationary phase Phenomenex kinetex Bi-phenyl 100x2.1mm, 1.7 μ m column. Mobile phase A was ammonium acetate (10mM) adjusted pH 3.0 with 50% formic acid solution and Mobile phase B was Acetonitrile, ammonium acetate (10mM) adjusted pH 3.0 with 50% formic acid solution, and Methanol in the ratio of 70:15:15%v/v/v. The gradient programme T (min) =% mobile phase B: 0=3, 5.2=25, 13.2=35, 23.2=50, 29.2=70, 33.2=75, 39.2=75, 41.2=3 and 45=3 with flow rate 0.3mL/min. The injection volume was 1 μ L and detection wavelength was set at 260nm. The column temperature was maintained at 35°C. Sample cooler was maintained at 5°C.

LC-QDa Conditions

This experiment was performed on Waters HPLC coupled with a PDA detector followed by a QDA mass detector. Phenomenex kinetex Bi-phenyl 250x4.6mm, 5 μ m column. Mobile phase A was ammonium acetate (10mM) adjusted pH 3.0 with 50% formic acid solution and Mobile phase B was Acetonitrile, ammonium acetate (10mM) adjusted pH 3.0 with 50% formic acid solution, and Methanol in the ratio of 70:15:15%v/v/v. The gradient programme T (min) =% mobile phase B: 0=5, 7=5, 20=25, 40=35, 65=55, 80=70, 90=75, 105=75, 110=5 and 120=5 with flow rate 0.6 mL/min. The injection volume was 10 μ L and detection wavelength was set at 260nm. The column temperature was maintained at 30°C. Sample cooler was maintained at 5°C. Wavelength scanning was from 200nm to 400nm for detector1 (PDA detector). Ionization mode was ESI+, probe temperature was 450°C, capillary voltage was 0.8kv, acquisition type was full scan m/z 100 to 1250 (centroid) and cone voltage was 15 volts for detector2(QDA detector).

Preparation of solutions

Preparation of Standard solution

A standard solution containing 2.5µg/mL of dolutegravir, 10µg/mL of emtricitabine and 15µg/mL of tenofovir disoproxil fumarate was prepared in 50:50%v/v of 0.1% orthophosphoric acid solution and Methanol used for calculation of known and unknown impurities.

Preparation of sample solution

A test solution containing of 500µg/mL of dolutegravir, 2000µg/mL of emtricitabine and 3000µg/mL of tenofovir disoproxil fumarate was prepared by taking tablet powder equivalent to 50mg of dolutegravir dissolved in 100 mL of 50:50%v/v of 0.1% orthophosphoric acid solution and Methanol, sonicated sample solution for 45 minutes. Centrifuged the sample solution at 5000 rpm for 5 minutes. Filtered the sample solution through 0.45µm PVDF (Millipore) filter. The concentration (50µg/mL of Dolutegravir, 200µg/mL of Emtricitabine and 300µg/mL) of above sample solution was diluted to 10 folds and determined for assay.

Preparation of spiked sample solution

A sample solution containing 500µg/mL of dolutegravir, 2000µg/mL of emtricitabine and 3000µg/mL of tenofovir disoproxil fumarate was spiked with all impurities at 0.5% level was used as spiked sample.

Method validation

The described method has been validated for the potential impurities of dolutegravir, emtricitabine and tenofovir disoproxil fumarate using FDA and ICH guidelines. The key analytical parameters that are required for validation are precision, accuracy, linearity, LOD, LOQ and ruggedness.

Specificity

The ability of the method can be defined by specificity, that should separate active compounds, placebo peaks if any, process impurities, potential impurities, unknown degradation impurities formed during degradation studies and also from stability studies. It was confirmed by injecting individual impurities at specification level and also from spiked sample spiked with all potential impurities at specification level.

Forced degradation studies

To check and ensure the homogeneity (peak purity) of dolutegravir, emtricitabine and tenofovir disoproxil peak in the stressed sample solutions, photo diode array detector was employed. Separate stressed conditions were applied for both tablets and active pharmaceutical ingredient (API). Stress studies were performed on equivalent quantity of dolutegravir, emtricitabine and tenofovir disoproxil fumarate tablets by subjecting with acid (0.1M HCl, room temperature, 1h), base (0.1 M NaOH, room temperature, 1h), oxidative (1 % H₂O₂, room temperature, 1h), thermal (80 °C, 24 hrs), and photolytic (overall illumination not less than 1.2 million lux hours, and integrated near ultraviolet energy not less than 200 W h/m²) and the active pharmaceutical ingredient (API) with a initial concentration of 500 µg mL⁻¹ of dolutegravir, 2000. µg mL⁻¹ of emtricitabine and 3000 µg mL⁻¹ of tenofovir disoproxil fumarate by subjecting (0.1M HCl 80°C, 1hr), (0.1M NaOH, 80°C,1hr) and (1%H₂O₂,80°C,1hr) for dolutegravir sodium, (0.1M HCl RT,

1hr), (0.1M NaOH, RT,1hr) and (1%H₂O₂,RT,1hr) for both emtricitabine and tenofovir disoproxil fumarate to evaluate the proposed method to separate dolutegravir, emtricitabine and tenofovir disoproxil from its degradation products. To ensure the peak homogeneity of above degradation samples, samples were diluted to 10 folds and assay was also calculated for the same samples against reference standards. Chromatograms for stressed samples were illustrated in Fig.5 (a-f). Homogeneity of dolutegravir, emtricitabine and tenofovir disoproxil peaks in stressed samples were ensured by peak purity. Photo diode array was employed throughout the analysis to assess the peak purity. QDa mass detector was used to determine the mass of degradation products.

Precision

System precision for the proposed method was confirmed by injecting a standard solution containing dolutegravir, emtricitabine and tenofovir disoproxil fumarate at 0.5% of specification level (500µg/mL of dolutegravir, 2000µg/mL of emtricitabine and 3000µg/mL of tenofovir disoproxil fumarate) six times into the chromatographic system and reported % RSD. A spiked sample preparation spiked with all potential impurities at 0.5% specification level (500µg/mL of dolutegravir, 2000µg/mL of emtricitabine and 3000µg/mL of tenofovir disoproxil fumarate) was injected six times to perform the method precision and reported %RSD for all impurities. Intermediate precision was also demonstrated by preparing standard solution preparation and spiked sample preparation same as above on different day, different analyst, different instrument and reported %RSD.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ of the method was determined by preparing a series of solutions containing dolutegravir, emtricitabine and tenofovir disoproxil fumarate and all potential impurities at a signal to noise ratio of 3:1 and 10:1 respectively. Precision at LOQ for all potential impurities as well as three active components was also established using above sample solutions and reported %RSD for all peaks.

Linearity

The linearity of detector response for dolutegravir, emtricitabine, tenofovir disoproxil fumarate and their impurities was studied by preparing a series of solutions using dolutegravir, emtricitabine, tenofovir disoproxil fumarate and their impurities at five different concentrations levels ranging from 0.05% to 150% of test concentration (500µg/mL/2000µg/mL/3000µg/mL). The correlation coefficients, slopes, R² value and y-intercepts of the calibration curves were determined.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the true value and the value found. The study was carried out by spiking known impurities in triplicate at %0.05%, 100% and 150% of the analyte concentration (500µg/mL for dolutegravir, 2000µg/mL for emtricitabine and 3000µg/mL for tenofovir disoproxil fumarate) and reported % recovery and %RSD at each level.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, deliberate variations in method conditions, it provides an indication of the reliability of

the method during normal use. To determine robustness, the experimental conditions were deliberately changed and resolution of dolutegravir, emtricitabine, tenofovir disoproxil fumarate and its impurities was evaluated. The normal mobile phase flow rate was 0.6 mL min⁻¹ for HPLC and 0.3 mL/min for UPLC. To study the effect of the flow rate on resolution, the flow rate was changed to 0.5 and 0.7 mL min⁻¹ for HPLC and 0.27 and 0.33 mL/min for UPLC. Remaining parameters same for both HPLC and UPLC. The effect of the column temperature was studied at 30 and 35 °C. The effect of pH of buffer was studied at varying ± 0.2 pH units (at 2.8 and 3.2 buffer pH).

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

The main objective of this chromatographic method is to achieve the separation of impurities (key RAW materials, intermediates, bi-products from the synthesis of dolutegravir, emtricitabine and tenofovir disoproxil fumarate, stability impurities, and degradation products) and the main three components in a single HPLC and UPLC method, it should be a stability indicating, free of interference from excipients, robust and enough for routine use in quality control laboratory [38].

Selection of wavelength is critical and usually a compromise for different compounds with different absorption maxima. Dolutegravir, emtricitabine and tenofovir disoproxil fumarate and its impurities exhibit different maximum. But based on the response of dolutegravir, emtricitabine and tenofovir disoproxil fumarate, impurities and also considering the lowest label claim of the drug (dolutegravir, emtricitabine and tenofovir disoproxil fumarate tablets 50mg/200mg/300mg) 260nm was selected as detection wavelength for the method. This wavelength was sufficiently selective and absorption was sufficient for all impurities.

The structural review and also based on the literature reveals the polar nature of emtricitabine and its impurities and non polar nature of Tenofovir disoproxil fumarate, dolutegravir and its impurities. Dolutegravir, emtricitabine and tenofovir disoproxil fumarate and their impurities have different affinities towards stationary phase due to different molecular structures and mass. Different screening experiments were conducted using C8, C18, CN, Phenyl, new core-shell C8, C18 and Bi-phenyl stationary phases manufactured by different vendors for selection of column. Better resolution, peak tailing and column performance was yielded finally on a new Core-Shell polar Phenomenex Kinetex Bi-phenyl column. For extended column life and repeatability, column temperature was maintained at 30°C. Same conditions were also achieved in UPLC by using same stationary phase column (Phenomenex Kinetex Bi-phenyl column). Effect of various temperatures on resolution was studied at 30°C and 35°C.

The effect of buffer pH on retention times of dolutegravir, emtricitabine, tenofovir disoproxil fumarate and its impurities were studied between pH 2.8 to pH 3.2 keeping chromatographic parameters unchanged. A robust pH which did not have impact in resolution was fixed at pH 3.0 for both HPLC and UPLC.

Different flow rates between 0.5 mL/min to 0.7 mL/min were tested for all three active peaks and impurities for HPLC and between 0.2 mL/min to 0.4 mL/min for UPLC. Finally flow rate were selected as 0.6 mL/min for HPLC and 0.3 mL/min for UPLC based on the column back pressure, resolution between active as well as all impurities and run time.

The blend solution containing dolutegravir 500 µg/mL, 2.5 µg/mL each of the impurity, emtricitabine 2000 µg/mL, 10 µg/mL each of the impurity and tenofovir disoproxil fumarate 3000 µg/mL, 15 µg/mL was prepared in the diluents was used for optimization of chromatographic conditions. Different buffers such as potassium phosphate, sodium phosphate, sodium perchlorate and ammonium acetate were used to evaluate for spiked sample and overall chromatographic performance. Finally a mass compatible ammonium acetate was found to be suitable for better separation of all impurities. Ammonium acetate buffer ranging from 10 mM to 50 mM were studied and observed that change in buffer concentration did not offer significant changes in elution pattern as well as resolution. From the preparation of spiked sample it is clear that, resolution is critical between three active components and its all impurities. Hence different columns with different stationary phases like C8, C18, phenyl, Cyno, New core shell technology with C8, C18 and bi-phenyl were used to optimize and finally resolution between three active components and all impurities were found to be good in a new core-shell Kinetex Bi-phenyl with 250 x 4.6 mm, 5 µm column. The pH values optimized were 2.5, 3.0, 3.5, 5.0, and 7.0. Finally the best results were obtained at pH 3.0 ± 0.05 by adjusting with diluted formic acid. Choice of mobile phase and its pH is justified by the excellent symmetry of peaks and adequate retention times of three active components, known impurities and their degradants. After a series of experiments, the method has been finalized on Kinetex Bi-phenyl with 250 x 4.6 mm, 5 µm column using Mobile phase A with 0.01 M Ammonium acetate, adjusted pH 3.0 with diluted formic acid solution and Mobile phase B with Acetonitrile, 0.01 M Ammonium acetate, adjusted pH 3.0 with diluted formic acid solution, methanol and acetonitrile in the ratio of 70:15:15 %v/v/v. The HPLC gradient programme T (min) = % mobile phase B: 0=5, 7=5, 20=25, 40=35, 65=55, 80=70, 90=75, 105=75, 110=5 and 120=5 with flow rate 0.6 mL/min. Different column oven temperatures were tried at 30°C, 35°C, and 40°C for better peak shape, base line and resolution. Better base line and resolution between impurities was observed at a column oven temperature 30°C. After finalizing the HPLC conditions same method was transferred to UPLC with same mobile phase A, Mobile phase B and with same stationary phase of Kinetex Bi-phenyl with 100 x 2.1 mm, 1.7 µm. The UPLC gradient programme T (min) = % mobile phase B: 0=3, 5.2=25, 13.2=35, 23.2=50, 29.2=70, 33.2=75, 39.2=75, 41.2=3 and 45=3 with flow rate 0.3 mL/min. Comparative chromatographic conditions for both HPLC and UPLC were represented in Table 8. Interference with the excipients (placebo) was also checked and no interference was observed at the retention times of all impurities and dolutegravir, emtricitabine and tenofovir disoproxil fumarate. Several preliminary chromatographic runs were performed to investigate the suitability for drug content estimation and cost because of the increasing importance of rapid economic analysis in pharmaceutical analysis.

System suitability parameters were evaluated for dolutegravir, emtricitabine and tenofovir disoproxil fumarate and their all impurities in the final method. The USP tailing factor for all impurities and Dolutegravir, Emtricitabine and Tenofovir disoproxil fumarate was found to be less than 1.5. The USP resolution (Rs) of dolutegravir, emtricitabine and tenofovir disoproxil fumarate and the potential impurities was greater than 2.0 between all impurities. A chromatogram obtained from dolutegravir, emtricitabine and tenofovir disoproxil fumarate spiked with their fourteen impurities at 0.5% level by HPLC and UPLC were shown in Fig. 3 and 4. The system suitability results were presented in Table 1.

Table 1: System suitability results

Compound	RT (%RSD) ^a	RT (in min) ^a	RRT ^b (n=6)	Peak area (%RSD) ^a (n=6)	USP tailing ^a (n=6)	Resolution ^c
ECT RC-02	0.1	8.06	0.45	0.6	1.0	---
ECT RC-03	0.0	9.44	0.53	0.3	1.1	2.0
Emtricitabine	0.0	18.10	---	0.3	1.1	35.8
ECT RC-04	0.0	20.45	1.14	0.7	1.0	10.7
TNF RC-01	0.0	23.35	0.44	1.1	1.1	12.8
DOL RC-04	0.0	35.72	0.56	0.8	1.0	45.5
TNF RC-02	0.0	41.28	0.78	1.5	1.0	11.5
TNF RC-03	0.0	42.85	0.82	0.6	1.0	2.5
DOL RC-02	0.0	51.79	0.83	0.5	0.9	23.8
Tenofovir disoproxil	0.0	53.03	---	0.5	2.6	3.6
TNF RC-04	0.0	54.93	1.05	0.9	0.9	3.9
DOL RC-01	0.0	58.45	0.93	1.6	0.9	9.0
TNF RC-05	0.0	60.54	1.14	0.6	1.5	3.5
DOL RC-03	0.0	61.83	0.97	0.6	0.9	5.8
Dolutegravir	0.0	63.34	---	0.5	0.9	4.8
TNF RC-06	0.0	82.21	1.55	0.8	1.0	56.0
ECT RC-01	0.0	82.97	4.61	1.0	1.0	3.1

^a Mean (n= 6).^b Relative retention times(RRT)were calculated against the retention time (RT) of Emtricitabine, Tenofovir disoproxil and Dolutegravir.^c Resolutions were calculated between two adjacent components.

Table 2: Summary of forced degradation data

Parameter	% of impurity (Dolutegravir)							
	DOL RC-01		DOL RC-02		DOL RC-03		DOL RC-04	
	DS	DP	DS	DP	DS	DP	DS	DP
Acid (0.1M HCl 80°C, 1hr)	ND	ND	0.39	ND	0.06	0.07	ND	ND
Base (0.1M NaOH, 80°C,1hr)	ND	ND	0.03	ND	0.07	0.07	ND	ND
Peroxide (1%H ₂ O ₂ ,80°C,1hr)	ND	ND	0.02	ND	0.07	0.06	ND	ND
Thermal 80°C/24hrs	NA	ND	NA	ND	NA	ND	NA	ND
Photolytic	NA	ND	NA	0.03	NA	0.08	NA	ND
Parameter	% of impurity (Emtricitabine)							
	ECT RC-01		ECT RC-02		ECT RC-03		ECT RC-04	
	DS	DP	DS	DP	DS	DP	DS	DP
Acid (0.1M HCl 80°C, 1hr)	ND	ND	0.16	0.20	0.12	0.16	10.7	0.20
Base (0.1M NaOH, 80°C,1hr)	ND	ND	ND	0.15	ND	0.15	14.3	0.10
Peroxide (1%H ₂ O ₂ ,80°C,1hr)	ND	ND	ND	4.95	ND	0.15	0.22	0.09
Thermal 80°C/24hrs	NA	0.20	NA	0.27	NA	ND	NA	1.88
Photolytic	NA	ND	NA	0.28	NA	0.16	NA	0.10

DS=Drug substance, DP= Drug product

Parameter	% of impurity (Tenofovir dsoproxili)											
	TNF RC-01		TNF RC-02		TNF RC-03		TNF RC-04		TNF RC-05		TNF RC-06	
	DS	DP	DS	DP	DS	DP	DS	DP	DS	DP	DS	DP
Acid (5N,60°C, 10min)	1.65	1.60	ND	ND	0.03	0.04	ND	0.01	0.05	0.06	0.09	0.07
Base (5N,60°C, 10min)	10.2	1.54	ND	ND	ND	ND	ND	ND	0.04	0.07	0.07	0.08
Peroxide (10%,60°C, 10min)	0.48	1.50	ND	ND	0.03	0.03	ND	0.01	0.06	0.06	0.09	0.07
Water (10%,60°C, 10min)	NA	6.5	NA	ND	NA	ND	NA	0.03	NA	1.15	NA	0.65
Thermal 105°C/24hrs	NA	1.52	NA	ND	NA	0.03	NA	ND	NA	0.08	NA	0.08

Table 3: Summary of % Assay and mass balance

Parameter	%Assay			Mass balance		
	DOL [@]	ECT [#]	TNF ^{\$}	DOL [@]	ECT [#]	TNF ^{\$}
Acid	98.7	97.8	97.5	98.2	97.3	98.5
Base	99.3	98.6	86.3	99.0	98.4	97.8
Peroxide	101.2	94.1	92.8	98.9	97.0	98.8
Thermal	99.6	97.2	96.4	98.8	98.6	98.1
Photolytic	99.9	98.8	98.2	99.6	99.0	99.2

[@] Dolutegravir, [#] Emtricitabine and ^{\$} Tenofovir disoproxil

Table 4: Summary of mass results

Sample type	Degradation condition	Name of degradation product	Relative Retention time (RRT)	Mass of degradation product (m/z)
Drug product	Acid and Base	DP1	0.64 ^a	418.21
		DP2	0.65 ^a	418.18
	Thermal	DP3	0.31 ^a	416.04
		DP4	0.60 ^a	663.17
		DP5	0.61 ^a	490.14
		DP6	0.67 ^a	819.21
		DP7	1.27 ^a	606.14
Drug substance (Dolutegravir)	Acid	DP8	0.69 ^b	438.18
		DP9	0.71 ^b	438.18

^a Relative retention time with respect to Tenofovir disoproxil and impurities belongs to Tenofovir disoproxil^b Relative retention time with respect to Dolutegravir and impurities belongs to Dolutegravir

Table 5 (a): LOD, LOQ, Regression and precision data for Dolutegravir

Compound/impurity	Dolutegravir	DOL RC01	DOL RC02	DOL RC03	DOL RC04
LOD ($\mu\text{g/mL}^{-1}$)	0.0125	0.0110	0.0113	0.014	0.010
LOQ ($\mu\text{g/mL}^{-1}$)	0.0412	0.039	0.0425	0.0386	0.033
Precision at LOQ (%RSD)	6.6	6.8	4.3	3.5	7.3
Signal/Noise ratio (at LOQ)	11	15	15	13	9
Regression equation (Y)	59163.26	37671.13	49386.54	55453.96	39123.32
Slope(b)					
Correlation coefficient	0.99974	0.99912	0.99979	0.99965	0.99852
R ² value	0.9995	0.9994	0.9996	0.9993	0.999
Precision (%RSD)	0.5	0.98	0.47	0.70	1.82
Intermediate precision (%RSD)	1.35	1.30	0.95	0.54	1.32

Table 5 (b): LOD, LOQ, Regression and precision data for Emtricitabine

Compound/impurity	Emtricitabine	ECT RC-01	ECT RC-02	ECT RC-03	ECT RC-04
LOD ($\mu\text{g/mL}^{-1}$)	0.016	0.013	0.015	0.013	0.015
LOQ ($\mu\text{g/mL}^{-1}$)	0.052	0.043	0.050	0.035	0.047
Precision at LOQ (%RSD)	7.2	4.2	5.3	5.1	6.3
Signal/Noise ratio (at LOQ)	14	15	12	10	13
Regression equation (Y)	59163.26	13863.47	18571.05	28148.03	27164.71
Slope(b)					
Correlation coefficient	0.99974	0.99905	0.99964	0.9986	0.99966
R ² value	0.9995	0.998	0.9993	0.999	0.9998
Precision (%RSD)	0.5	1.25	0.66	0.94	0.69
Intermediate precision (%RSD)	0.85	2.05	0.88	1.38	2.53

Table 5 (c): LOD, LOQ, Regression and precision data for Tenofovir disoproxil

Compound/impurity	Tenofovir disoproxil	TNF RC-01	TNF RC-02	TNF RC-03	TNF RC-04	TNF RC-05	TNF RC-06
LOD ($\mu\text{g/mL}^{-1}$)	0.253	0.148	0.135	0.128	0.140	0.243	0.142
LOQ ($\mu\text{g/mL}^{-1}$)	0.758	0.463	0.422	0.41	0.386	0.732	0.430
Precision at LOQ (%RSD)	5.6	2.8	4.2	4.8	5.3	2.6	4.3
Signal/Noise ratio (at LOQ)	14	17	15	13	10	12	15
Regression equation (Y)	26619.73	19290.67	13213.74	20300.61	29075.07	11819.13	30556.97
Slope(b)							
Correlation coefficient	0.99974	0.99967	0.99834	0.99981	0.99991	0.99891	0.99954
R ² value	0.9997	0.99967	0.9996	0.999	0.999	0.9978	0.9991
Precision (%RSD)	0.5	1.05	1.84	0.65	1.15	0.56	0.76
Intermediate precision (%RSD)	1.35	1.72	1.22	0.35	2.08	0.84	1.93

Table 6: Summary of recovery data

Amount spiked ^a	% Recovery ^b				
	Dolutegravir				
	Dolutegravir	DOL RC01	DOL RC02	DOL RC03	DOL RC04
LOQ	101.2	100.3	105.3	100.6	95.3
(%RSD)	1.08	2.15	0.49	3.5	2.81
100%	100.3	101.8	100.7	102.8	100.8
(%RSD)	0.92	1.53	0.46	1.22	1.62
150%	102.4	100.6	100.4	100.1	98.6
(%RSD)	1.36	1.06	1.08	1.05	1.95
Amount spiked ^a	% Recovery ^b				
	Emtricitabine				
	Emtricitabine	ECT RC-01	ECT RC-02	ECT RC-03	ECT RC-04
LOQ	98.6	95.3	94.9	99.3	102.8
(%RSD)	1.86	2.3	0.68	1.43	2.92
100%	100.8	98.5	100.2	102.5	100.3
(%RSD)	1.33	3.42	1.18	0.93	1.05
150%	101.9	96.8	99.5	101.2	100.5
(%RSD)	1.14	2.06	1.54	1.3	1.22

Amount spiked ^a	% Recovery ^b						
	Tenofovir disoproxil						
	Tenofovir disoproxil	TNF RC-01	TNF RC-02	TNF RC-03	TNF RC-04	TNF RC-05	TNF RC-06
LOQ	98.3	99.7	92.9	94.2	100.9	93.2	98.5
(%RSD)	2.55	1.56	2.33	3.42	1.2	0.87	1.22
100%	100.6	106.4	97.3	97.3	103.5	104	104.6
(%RSD)	1.53	2.32	1.36	2.24	1.86	0.87	1.24
150%	98.1	98.8	98.1	98.6	99.8	98.1	101.7
(%RSD)	1.26	2.24	2.04	2.28	1.35	0.47	1.55

Table 7a: Robustness data by HPLC

Compound name/Impurity Name	HPLC							
	pH 2.8	pH 3.0	pH 3.2	0.5 mL/min	0.6 mL/min	0.7 mL/min	30°C	35°C
ECT RC-02	---	---	---	---	---	---	---	---
ECT RC-03	5.0	2.0	2.0	2.0	2.0	1.9	2.0	1.9
Emtricitabine	30.4	35.8	35.0	32.9	35.8	37.5	35.8	37.0
ECT RC-04	10.9	10.7	10.6	10.5	10.7	10.6	10.7	10.4
TNF RC-01	12.9	12.8	12.7	10.8	12.8	14.3	12.8	14.0
DOL RC-04	45.3	45.5	44.5	47.1	45.5	42.9	45.5	43.0
TNF RC-02	9.9	11.5	11.3	11	11.5	11.6	11.5	12.7
TNF RC-03	2.3	2.5	2.4	2.4	2.5	2.4	2.5	3.0
DOL RC-02	22.9	23.8	23.4	22.7	23.8	23.8	23.8	22.5
Tenofovir disoproxil	2.0	3.6	3.5	3.6	3.6	3.6	3.6	4.2
TNF RC-04	3.6	3.9	3.8	3.8	3.9	3.9	3.9	3.9
DOL RC-01	10.4	9.0	8.9	9.2	9.0	8.7	9.0	8.6
TNF RC-05	4.5	3.5	3.5	2	3.5	5.6	3.5	4.3
DOL RC-03	5.4	5.8	6.0	7.9	5.8	4.6	5.8	4.3
Dolutegravir	4.3	4.8	4.2	4.3	4.8	4.4	4.8	4.4
TNF RC-06	55.1	56.0	55.1	52.6	56.0	53.6	56.0	58.3
ECT RC-01	3.2	3.1	3.2	4.3	3.1	1.9	3.1	1.9

^aamount of all impurities spiked individually from LOQ to 150% specification limit^bMean, %RSD for three determinations

Table 7b: Robustness data by UPLC

Compound name/Impurity Name	UPLC							
	pH 2.8	pH 3.0	pH 3.2	0.27 mL/min	0.3 mL/min	0.33 mL/min	30°C	35°C
ECT RC-02	---	---	---	---	---	---	---	---
ECT RC-03	6.4	2.4	2.0	2.5	2.4	2.4	2.4	2.4
Emtricitabine	16.0	21.5	24.4	21.9	21.5	21.6	21.5	21.5
ECT RC-04	9.2	8.3	8.2	8.2	8.3	8.4	8.3	8.3
TNF RC-01	16.7	15.9	15.9	14.7	15.9	16.8	15.9	16.1
DOL RC-04	33.7	25.1	34.1	21.9	25.1	24.5	25.1	33.4
TNF RC-02	9.3	16.6	10.2	11.3	16.6	16.2	16.6	11.4
TNF RC-03	1.9	2.0	1.9	2.0	2.0	2.0	2.0	2.1
DOL RC-02	18.2	19.3	18.5	18.3	19.3	18.8	19.3	18.3
Tenofovir disoproxil	1.9	2.0	2.4	2.0	2.0	2.0	2.0	2.0
TNF RC-04	3.1	3.1	3.3	3.1	3.1	3.2	3.1	3.2
DOL RC-01	9.7	7.2	6.5	6.9	7.2	7.3	7.2	6.9
TNF RC-05	5.5	6.5	6.7	6.3	6.5	6.7	6.5	6.7
DOL RC-03	2.9	2.1	2.0	2.4	2.1	1.9	2.1	1.9
Dolutegravir	3.4	3.5	3.5	3.4	3.5	3.5	3.5	3.5
TNF RC-06	57.1	56.4	55.0	54.4	56.4	58.6	56.4	55.1
ECT RC-01	4.3	4.5	4.6	3.7	4.5	5.5	4.5	5.2

Table 8: Comparative chromatographic conditions for HPLC and UPLC

Chromatographic conditions	HPLC	UPLC
Column	Kinetex Bi-phenyl 250x4.6mm,5 μ	Kinetex Bi-phenyl 100x2.1mm,1.7 μ
Flow	0.6ml/min	0.3mL/min
Injection volume	10 μ L	1 μ L
Detector wavelength	260nm	260nm
Column oven temperature	30°C	30°C
Sample cooler temperature	5°C	5°C
Runtime	120min	45min
Mobile phase A	10mM Ammonium acetate buffer pH 3.0	10mM Ammonium acetate buffer pH 3.0
Mobile phase B	Acetonitrile, 10mM Ammonium acetate buffer pH 3.0 and methanol in the ratio of 70:15:15%v/v/v	Acetonitrile, 10mM Ammonium acetate buffer pH 3.0 and methanol in the ratio of 70:15:15%v/v/v

Figure 1 (A): (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

Figure 1 (B): 4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one

Figure 1 (C): Bis{[(isopropoxycarbonyl)oxy]methyl}({[(2R)-1-(6-amino-9H-purin-9-yl)-2-propenyl]oxy}methyl)phosphonate

Figure 1 (A): Dolutegravir, (B): Emtricitabine, (C): Tenofovir disoproxil fumarate

Figure 2 (a): (4R,12aS)-N-(2,4-difluorobenzyl)-7-methoxy-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

Figure 2 (b): N-(2,4-Difluorobenzyl)-9-hydroxy-2-(4-hydroxybutan-2-yl)-1,8-dioxo-1,8-dihydro-2H-pyrido[1,2-a]pyrazine-7-carboxamide

Figure 2 (c): (4R,12aS)-N-(4-Fluorobenzyl)-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

Figure 2 (d): (4R,12aS)-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-carboxylic acid

Figure 2 (e): (2R, 5S)-5-(4-amino-5-fluoro-2-oxo-2H-pyrimidin-1-yl)-[1,3]oxathiolane-2-carboxylic acid(1R,2S,5R)-methyl-ester

Figure 2 (f): 5-Fluoro-1-[(2R,5S)-2-(hydroxymethyl)-3-oxo-1,3-oxathiolan-5-yl]cytosine

Figure 2 (g): (5S)-5-(4-amino-5-fluoro-2-oxopyrimidin-1(2H)-yl)-1,3-oxathiolane-2-carboxylic acid

Figure 2 (h): (2r,5s)-5-fluoro-1-(2-(hydroxymethyl)-1,3-oxthiolan-5-yl)pyrimidine-2,4(1h,3h)-dione

Figure 2 (i): (((R)-1-(6-Amino-9H-purin-9-yl)propan-2-yloxy)Methyl(hydroxy)phosphoryloxy)Methyl isopropyl carbonate

Figure 2 (j): 5-[[[(1R)-2-(6-Amino-9H-purin-9-yl)-1-methylethoxy]methyl]-2,4,6,8-tetraoxa-5-phosphanonanedioc Acid (1-Methylethyl,methyl) Ester 5-Oxide

Figure 2 (k): [[2-(6-Amino-9H-purin-9-yl)-1-methyl ethoxy]methyl]-phosphonic acid iso propoxy carbonyl oxymethyl ester isopropyl ester

Figure 2 (l): 5-[[[(1R)-2-(6-Amino-9H-purin-9-yl)-1-methylethoxy]methyl]-2,4,6,8-tetraoxa-5-phosphanonanedioc Acid (1-methylethyl,propyl) Ester 5-Oxide

Figure 2 (n): 5-[[[(1R)-2-[6-[[[[9-(2R)-2,11-Dimethyl-5-[[[(1-methylethoxy)carbonyl]oxy]methoxy]-5-oxido-9-oxo-3,6,8,10-tetraoxa-5-phosphadodec-1-yl]-9H-purin-6-yl]amino]methyl]amino]-9H-purin-9-yl]-1-methylethoxy]methyl]-2,4,6,8-tetraoxa-5-phosphanonedioic Acid 1,9-Bis(1-1-methylethyl) Ester 5-Oxide

Figure 2 (a): DOLRC-01, (b): DOLRC-02, (c): DOLRC-03, (d): DOLRC-04, (e): ECTRC-01, (f): ECTRC-02, (g): ECTRC-03, (h): ECTRC-04, (i): TNFRC-01 (Mono-POC PMPA), (j): TNFRC-02(MOC-POC PMPA), (k): TNFRC-03 (iPr-POC PMPA), (l): TNFRC-04 (n-POC-POC PMPA), (m): TNFRC-05 (Tenofovir mixed dimer), (n): TNFRC-06 (Tenofovir dimer)

Figure 4: Typical chromatogram of spiked sample (UPLC)

Figure 5 (b)

Figure 5 (d)

Figure 5 (e)

Figure 5 (f)

Figure 5 (a): Chromatogram of sample under acid stressed condition, (b): Chromatogram of sample under base stressed condition, (c): Chromatogram of sample under oxidative stressed condition, (d): Chromatogram of sample under thermal stressed condition, (e): Chromatogram of sample under photolytic stressed condition, (f): Chromatogram of Dolutegravir API under acid stressed condition

Figure 6 (a)

Figure 6 (b)

Figure 6 (c)

Figure 6 (d)

Figure 6 (e)

Figure 6 (f)

Figure 6 (g)

Figure 6 (a): Mass Spectrum of unknown degradants of Dolutegravir API under acid stress condition, (b): Mass Spectrum of unknown degradation product formed in acid and base stress conditions, (c): Mass Spectrum of unknown degradation product formed in thermal stress conditions, (d): Mass Spectrum of unknown degradation product formed in thermal stress conditions, (e): Mass Spectrum of unknown degradation product formed in thermal stress conditions, (f): Mass Spectrum of unknown degradation product formed in thermal stress conditions, (g): Mass Spectrum of unknown degradation product formed in thermal stress conditions

Forced Degradation

Degradation was not observed for dolutegravir in finished product due to conditions employed were not sufficient to degrade dolutegravir. ECT RC02 was observed in oxidative and thermal stress conditions, ECT 04 was formed in thermal stress condition. TNF RC01 found to form all stress conditions. TNF RC02, TNF RC05 and TNF RC07 were found to form in thermal stress conditions. Separate conditions were employed for dolutegravir alone. Two major degradation products were observed for dolutegravir in acid stress condition (Figure 5(a-f)). Mass balance, % assay and % of impurities formed were reported in Table 2 and 3. The major unknown degradation products formed in the above stress conditions were identified by PDA detector followed by a QDA mass detector with same method. Which revealed protonated molecular ion peaks [$M=H$ at m/z DP1-418.21 and DP2-418.18 for Sample preparation in acid and base hydrolysis, at m/z DP3-416.04, DP4-663.17, DP5-490.14, DP6-819.21 and DP7-606.14 for thermal degradation sample and at m/z DP8-438.18 and DP9-438.18 for Dolutegravir API in acid hydrolysis condition. Mass of unknown degradation peaks were illustrated (Figure 6(a-g)).

Degradation condition, relative retention times and mass of unknown degradation products formed were reported in Table 4. Assay of the stressed samples was performed by comparison with reference standards. Assay was also performed for dolutegravir, emtricitabine and tenofovir disoproxil spiked with all impurities at the specification level (0.5 %).

Method Validation

Precision

The repeatability of the method was demonstrated by six fold analysis of sample solution containing 500 $\mu\text{g mL}^{-1}$ of dolutegravir, 2000 $\mu\text{g mL}^{-1}$ of emtricitabine and 3000 $\mu\text{g mL}^{-1}$ of tenofovir disoproxil fumarate tablets spiked with 0.5% of all impurities for HPLC and UPLC (Figure 3 and 4). The %RSD for the area of each peak was calculated and found to be within 1.9 %. The intermediate precision of the method was also evaluated using different analyst and different instrument, and performing the analysis on different days. The %RSD for the area of each peak was also calculated and found to be within 2.53%. The results were presented in Table 5(a-c). These results confirmed the method was highly precise.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ of the method were determined by injecting standard solutions of progressively decreasing concentration under the chromatographic conditions described above. The LOD was defined as the concentration for which the signal-to-noise ratio was 3:1 and the LOQ was defined as the concentration for which the signal-to-noise ratio was 10:1. The determined limit of detection and limit of quantification values for dolutegravir, emtricitabine, tenofovir disoproxil and their impurities were reported in Table 5(a-c).

Linearity

The linearity of detector response for dolutegravir, emtricitabine, tenofovir disoproxil fumarate and their all fourteen impurities were studied by preparing a series of solutions using dolutegravir, emtricitabine, tenofovir disoproxil fumarate and their all fourteen impurities at five different concentrations levels ranging from 0.05% to 150% of test concentration (500 $\mu\text{g/mL}$ /2000 $\mu\text{g/mL}$ /3000 $\mu\text{g/mL}$). The correlation

coefficients, slopes, R^2 value and y -intercepts of the calibration curves were determined. The result showed that an excellent correlation existed between the peak areas and concentration of the analyte (Table 5(a-c)).

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the true value and the value found. The study was carried out by spiking known impurities in triplicate at %0.05%, 100% and 150% of the analyte concentration (500 $\mu\text{g/mL}$ for dolutegravir, 2000 $\mu\text{g/mL}$ for emtricitabine and 3000 $\mu\text{g/mL}$ for tenofovir disoproxil fumarate). The % recoveries of all these impurities were found to be in-between the predefined acceptance criterion of 85.0-115.0%. The % recovery of dolutegravir, emtricitabine and tenofovir disoproxil fumarate was ranging from 98.5 to 101% w/w in its assay method (Table 6).

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, deliberate variations in method condition and it provides an indication of the reliability of the method during normal use. To determine robustness, the experimental conditions were deliberately changed and resolution of dolutegravir, emtricitabine, tenofovir disoproxil fumarate and its impurities was evaluated. The normal mobile phase flow rate was 0.6 mL/min for HPLC and 0.3 mL/min for UPLC. To study the effect of the flow rate on resolution, the flow rate was changed to 0.5 and 0.7 mL min^{-1} for HPLC and 0.27 and 0.33 mL/min for UPLC. Remaining parameters were same for both HPLC and UPLC. The effect of the column temperature was studied at 30 and 35 °C. The effect of pH of buffer was studied at varying ± 0.2 pH units (at 2.8 and 3.2 buffer pH). The resolution between three active ingredients and impurities in different robustness conditions by HPLC and UPLC were reported in table 7(a-b).

Solution stability

Solution stability was carried out by leaving spiked sample solutions in HPLC/UPLC sample cooler at 5°C for 48 h. At room temperature dolutegravir and emtricitabine were found to stable, tenofovir disoproxil fumarate was found to degrade. Hence stability was carried out at 5°C. Content of all impurities was determined for every 12 h interval up to the study period. No significant changes were observed in the content of these impurities during the solution stability experiments. The results confirmed that the solutions were stable for up to 48 h during the determination of related compounds.

CONCLUSION

A simple and selective stability-indicating, mass compatible, gradient RP-HPLC/UPLC/QDA method has been developed for the quantitative determination and identification of impurities for dolutegravir, emtricitabine, tenofovir disoproxil fumarate tablets. This method is capable of separating all impurities with good resolution within 120 min for HPLC and 45 min for UPLC. This method exhibited excellent performance in terms of sensitivity and speed. Forced degradation studies were conducted, and the major degradants were identified using a new QDA mass detector with the same method. A systematic mechanism for the formation of degradation impurities was proposed based on the known reactivity of the drug through hydrolysis and thermal. The developed method was validated as per the ICH guidelines and found to be specific, precise, accurate and linear. Thus, the method can be used for quality control analysis of pharmaceutical dosage form.

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