ejpmr, 2017,4(7), 605-613

SJIF Impact Factor 4.161

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

A SIMPLE, SELECTIVE, RAPID AND RUGGED METHOD DEVELOPMENT AND VALIDATION OF TENOFOVIR AND RILPIVIRINE IN HUMAN PLASMA USING LIQUID CHROMATOGRAPHY COUPLED WITH TANDEM MASS SPECTROMETRY.

B. M. S. Kumar*[1] and Dr. B. Rajkamal Bigala[2]

[1]Department of Pharmaceutical Chemistry, Mewar University, NH-79, Gangrar, Chittorgarh, Rajastan – 312901.
[2]Mewar University Department of Pharmaceutical Chemistry; Chittorgarh, Rajasthan.

***Corresponding Author: B. M. S. Kumar**

Department of Pharmaceutical Chemistry, Mewar University, NH-79, Gangrar, Chittorgarh, Rajastan – 312901.

Article Received on 10/05/2017 **Article Revised on 30/05/2017** **Article Accepted on 20/06/2017**

ABSTRACT

A simple, selective, rapid and rugged liquid chromatography coupled with Tandem mass spectrometric (LC-MS/MS) method was developed and validated for the quantification of Tenofovir and Rilpivirine in human plasma. The Tenofovir and Rilpivirine were eluted within 4.00 minutes using isocratic mobile phase, the column used was Zorbax 5 µ, C18, 100×4.60 mm having a mobile phase of 5 mM Ammonium acetate, 20.0% and Acetonitrile, 80% (20:80% v/v). The flow rate was 0.70 ml/min at a column temperature of 40 ± 5ºC. Analysis was performed using multiple reaction monitoring (MRM) and ionization was carried out using electrospray positive ionization mode. The retention time for Tenofovir and Rilpivirin are 0.85 min and 2.80 min respectively. The method was validated for linearity, precision, accuracy, specificity, sensitivity, matrix effect, dilution integrity, ruggedness, injection reproducibility and stability. Calibration curve range for Tenofovir and Rilpivirine were 5.000 – 600.000 and 1.000 -203.000 ng/mL respectively. The calibration curves were linear during the course of validation, with correlation coefficients ≥0.9988 and ≥ 0.9992 for Tenofovir and Rilpivirine respectively. The precision and mean accuracy were within the acceptable limits.

KEYWORDS: Tenofovir; Rilpivirine; LC/MS/MS; Validation.

INTRODUCTION

Tenofovir is ({[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy}methyl) phosphonic acid (fig 1) belongs to antiretroviral drugs. It is taken in combination with other antiretroviral drugs for the treatment of HIV-1 infection and also for the treatment of chronic hepatitis B.[1] It is nucleotide reverse transcriptase inhibitor. The reverse transcriptase enzyme is useful for viral production in HIV-infected people. If Tenofovir binds to growing DNA strand, this prevents the formation of the 5' to 3' phosphodiester linkage, which is essential for DNA chain elongation, results in premature termination of DNA transcription. Tenofovir disoproxil fumarate is prodrug of the active ingredient Tenofovir. The oral bioavailability in fasted patients is 25%. When a single oral dose is given in the fasted state, the maximum serum concentration was achieved in 1 hour and Cmax, AUC values are found to be 0.30 ± 0.09µg/mL and 2.29 ± 0.69 µg hr/mL respectively. The oral bioavailability of tenofovir is enhanced by administration with a high-fat meal, but is similar at steady state when administered with or without a typical meal. Tenofovir is excreted by both glomerular filtration and active tubular secretion.[2]

Rilpivirine 4-{[4-({4-[(1E)-2-cyanoeth-1-en-1-yl]-2,6-dimethylphenyl}amino)pyrimidine-2-yl]amino}benzonitrile (Fig 2) is a second-generation non-nucleoside reverse transcriptase inhibitor. When compared to older NNRIT's, it has better therapeutic activity with less side effects. It is a diarylpyrimidine, which binds to reverse transcriptase enzyme which results in termination of HIV-1 replication. No phosphorylation may be required for its activity. Because of its flexibity structure, it can bind to reverse transcriptase enzyme and less likely to develop resistance. Rilpivirine has dose-dependent pharmacokinetics. Rilpivirine is indicated in combination with other agents, for the management of HIV-1 infection in adults.[3] Absorption increases with food. It is metabolized by CYP3A4 in liver. Half life of rilipivirine is 34-55 hours after oral administration and having 99% protein binding capacity. Rilpivirine eexcretes fecally 85% and in urine 6%.[4]

A number of methods have been developed for the determination of Tenofovir and Rilpivirine as a single agent and in combination with other drugs by different analytical techniques such as UV- Spectrophotometry, high performance liquid chromatography (HPLC), liquid

chromatography–tandem mass spectrometry (LC-MS/MS), ultra-high performance liquid chromatography. Djerada Z *et al.*, developed simultaneous method for elvitegravir, raltegravir, maraviroc, etravirine, tenofovir, boceprevir and 10 other antiretroviral agents in human plasma samples with UPLC-MS/MS, this method was applied for therapeutic drug monitoring.[5] Else LJ *et al.*, developed liquid chromatograpy-tandam massspectrometry method for Rilpivirine in human plasma, cervicovaginal fluid, rectal fluid and genital/rectal tissues.[6] Abhijit A. Date *et al.*, developed simple isocratic HPLC – UV method for determination of Rilpivirine from tablets.[7] Podany AT *et al.*, developed LC/MS/MS method using solid-phase extraction for the quantification of Tenofovir in human plasma.[8] Addepalli. V. Raju *et al* developed and validated LC/MS/MS method for the determination of Rilpivirine in Sprague Dawley rat serum and its application to pharmacokinetic study.[9] Ramachandran et al developed a stability indicating RP-HPLC method for the simultaneous estimation of Emtricitabine (EMT) and Tenofovir Desoproxil Fumerate (TDF) in pure and tablet dosage forms.[10] *Rezk NL et all* developed and validated simultaneous method for emtricitabine and tenofovir in human plasma using high-performance liquid chromatography after solid phase extraction.[11] The above methods are time consuming, they require a long time for sample pretreatment for having multiple steps. As per our knowledge the proposed method is the first bioanalytical method for simultaneous estimation of Tenofovir and Rilpivirine by LC-MS/MS in human plasma. liquid-liquid extraction procedure was developed. It requires less time for preparation of sample.The chromatographic conditions were fixed and the results of validation parameters and solution stability studies in advance were established. The method can be applied to pharmacokinetic study of Tenofovir and Rilpivirine in HIV patients.

2. Experimental

2.1. Reagents

Tenofovir, Rilpivirine, Tenofovir-D6 and Rilpivirine-D6 reference standards were purchased from vardha bio Tech, acetonitrile from Merck, acetic acid from Merck, GR grade, Formic acid from Merck, ammonium acetate from Sigma Aldrich, Na_2EDTA from Merck, HPLC grade, tertiary butyl methyl ether from akshaya scientific and HPLC grade water was used.

2.2 Standard solutions

Stock solutions of Tenofovir and Rilpivirine 1.00 mg/mL were prepared in acetonitrile. Intermediate stocks were prepared in acetonitrile: water (50:50% v/v) and the solutions were stored at 5 ± 3ºC. The internal standard solutions of Tenofovir-D6 and Rilpivirine-D6 with concentration of 1.00 mg/ml were prepared in acetonitrile. The internal standard working stock solution was prepared in acetonitrile: water (50:50% v/v) and the solution was stored at 5 ± 3ºC.

2.3 Sample Extraction Method

The internal standards Tenofovir-D6 and Rilpivirine-D6 mixture of 25.0 µL (25.000 ng/mL) was added to polypropylene tubes containing 100.0 µL of plasma sample and to this mixture 100.0 µL of 5.0 mM ammonium acetate in water was added as extraction buffer and vortex for 5.00 sec. To this resulting mixture 1.80 mL of tertiary butyl methyl ether was added for each sample and vortexed for 10.00 min, centrifuged at 4500 rpm, 4°C. The supernatants were evaporated under nitrogen and reconstituted with 100.0 µL of 5.0 mM ammonium acetate: acetonitrile (30:70 v/v; pH: 4.0) solution. The samples were injected onto LC/MS/MS.

2.4 Chromatographic and Mass Spectrometry conditions

The method was developed using Ultra performance liquid chromatography (UPLC) (Waters Corporation; Manchester) coupled with Mass spectrometry (ABSciex, API 4000). The chromatographic peaks were resolved using C18 column, Zorbax C18 5µ 100x4.6mm. The Mass spectrometer was operated using positive Electrospray ionization and quantification was performed using Multi-reaction monitoring (MRM) mode. The MRM transitions for analytes m/z: 288.0/176.0; 367.1/195.1 for Tenofovir and Rilpivirine respectively. The MRM transition for internal standards m/z:294.0/182.0; 373.1/201.1 for Tenofovir-D6 and Rilpivirine-D6 respectively. The source gas and compound parameters were optimized. The isocratic mobile phase consisting of 5.0 mM ammonium format and acetonitrile (20:80 v/v) with flow rate 0.50 mL/min. The auto sampler and column oven temperature was 50 ± 5 and 5 ± 3^{o}C, respectively. The retention time for Tenofovir & Tenofovir D6 found to be 0.85 min and Rilpivirine & Rilpivirine D6 found to be 2.85 min. The total run time for the method was 4.00 mins. The method gave good peak shape, resolution, sensitivity and reproducibility with optimized analytical parameters, optimized analytical parameters were represented in Table 1 & 2.

2.5 Validation

The developed new LC/MS/MS method in human plasma was validated as per US FDA regulatory guidelines.[12]

2.5.1 System suitability

As a first step of method validation, system suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture equivalent to MQC1 concentration of the calibration curve. System suitability was performed at the start of the method validation and on each day as a first experiment.

2.5.2. Carry over Effect

The carryover effect due to the auto sampler was investigated by injecting a sequence of unextracted samples and extracted samples.

2.5.3 Blank matrix specificity

As a first step of method validation, specificity was performed. To test the specificity, twelve (6 normal, 2 (0.5%) Haemolysed, 2 (1.0%) Haemolysed & 2 lipemic) different blank plasma lots were tested. To establish specificity, Six LLOQ standards were extracted. The responses for the blank plasma from the twelve[12] different lots were compared against the LLOQ standard mean area of Tenofovir, Rilpivirine and internal standards. No significant response (≤20% for the analyte response and ≤5% of the internal standard response) was observed at the retention time of the analytes and the internal standards in blank plasma as compared to the LLOQ standard.

2.5.4. Injector Carry over Effect

The injector carryover effect was investigated by injecting a sequence of unextracted and extracted samples.

2.5.5. Linearity

The linearity of the method was determined by using a $1/x^2$ weighted least square regression analysis of standard plots associated with a ten-point standard curve. All the three calibration curves analyzed during the course of validation.

2.5.6. Precision and accuracy

Intra batch and inter batch precision of the method was expressed as coefficient of variation (% CV). It was evaluated by the % CV at different concentration levels corresponding to lower limit of quantification (LLOQ), lower quality control (LQC), Medium quality control- 2 (MQC-2), medium quality control -1 (MQC-1) and higher quality control (HQC) during the course of validation.

Intra and inter batch accuracy was calculated as the absolute value of the ratio of the calculated mean values of the quality control samples to their respective nominal values and expressed as percentage.

2.5.7. Ruggedness

The developed method was evaluated for ruggedness using different column. The precision for the quality control samples at LQC, MQC-2, MQC-1 and HQC concentration levels for different column determined.

2.5.8. Dilution integrity

Dilution Integrity experiment was carried out using the 1.5X of stock prepared in the screened plasma. The samples were diluted two times (1 in 2 dilution) and five times diluted (1 in 4 dilution).

2.5.9. Recovery

The percentage recovery of Tenofovir & Rilpivirine was determined by comparing the mean peak area of Tenofovir & Rilpivirine in extracted LQC, MQC-2, MQC-1 and HQC samples with freshly prepared unextracted LQC, MQC-2, MQC-1 and HQC samples respectively.

For the internal standards, mean peak area of extracted samples was compared to the mean peak area of unextracted samples.

2.5.10. Matrix effect

The matrix effect for the method was assessed by using six lots of screened plasma. The analytes and internal standards were spiked into the extracted plasma blank to obtain the post extracted LQC & HQC samples. The post extracted samples were analysed against fresh calibration curve.

2.5.11. Reinjection reproducibility

The method was established for reinjection reproducibility by injecting the previously passed precision and accuracy batch after a period of 50 hr 30 mins.

2.5.12. Haemolysis Effect

The haemolysis effect (0.5% and 1.0%) of the method was evaluated using 6 replicates of low QC and high QC samples which were spiked in haemolysed plasma. These samples were processed and analysed along with calibration curve prepared by using normal screened biological matrix.

2.5.13. Effect of Potentially Interfering Drugs

The potentially interfering or Co-administration of drugs such as Paracetamol, Ibuprofen and Ondensetron & Diclofenac were evaluated for Tenofovir and Rilipivirine at Cmax concentration level of drugs in K2EDTA plasma. Results were summarized in Table 8.

2.5.14. Stability studies

The stability studies were conducted for Tenofovir and Rilpivirine at various conditions using six replicates of LQC and HQC samples. The stability experiments were conducted in biological matrix as well as in aqueous solution in order to characterize each operation. i.e. short term stock solution stability, long term stock solution stability were performed. The different stability experiments were conducted in plasma was investigated in order to characterize each operation. i.e bench top stability, autosampler stability, post extracted stability at refrigerated temperature, dry extracted stability, stability in dry ice, freeze and thaw stability, long term stability of drug in plasma, whole blood stability of analyte were performed. All the stability study testing conditions were represented in Table 2.

3. RESULTS AND DISCUSSION

System suitability was performed at the start of the method validation and on each day as a first experiment. The % CV of the retention times for Tenofovir and Tenofovir D6 found to be ≤ 0.85; Rilpivirine and Rilpivirine D6 found to be ≤ 2.85. The % CV of the peak area ratio for Tenofovir and Tenofovir D6 found to be ≤

3.52, Rilpivirine and Rilpivirine D6 found to be ≤ 2.85. No significant carry over observed during this experiment.

The twelve human plasma lots investigated were found to be free of significant interference at the retention time of analytes and internal standards (i.e. area of the peak at the retention time of drug in standard blank samples was ≤ 20.00% of the area of the drug in the extracted LLOQ sample; area of the peak at the retention time of ISTD in standard blank samples was ≤ 5.00% of the area of the ISTD in the extracted LLOQ sample). No interference was observed.

Representative chromatograms of Tenofovir and Rilpivirine were represented in Fig 3. Sensitivity of the method was established at LLOQ level and the results were represented for Tenofovir and Rilpivirine in Table 4. The precision and accuracy results for analytes at LLOQ level were shown in Table 5.

The three calibration curves analyzed were found to be linear during the course of validation for the linearity range 5.000 - 600.00 and 1.000-203.00 ng/mL for Tenofovir and Rilpivirine respectively. The overall correlation coefficient (r) was observed to be ≥ 0.9988 and 0.9992 for Tenofovir and Rilpivirine respectively. The overall % mean accuracy for the calibration standards found to be in between 96.34-104.77 and 93.02-102.17% for the Tenofovir and Rilpivirine respectively. The overall precision was ranging from 1.26 – 4.31 and 0.64 – 3.63% for the Tenofovir and Rilpivirine respectively.

The % mean accuracy for Intra batch quality control samples at LQC, MQC-2, MQC-1 and HQC concentration levels for Tenofovir and Rilpivirine were ranged from 97.32% - 101.45% and 102.37– 111.52% respectively. The LLOQ QC samples for Tenofovir and Rilpivirine for Intra batch was ranged from 98.57-101.00% and 103.00-108.00% respectively. The results are summarized in the Table 5.

The % mean accuracy for Inter batch quality control samples at LQC, MQC-2, MQC-1 and HQC concentration levels for Tenofovir and Rilpivirine were ranged from 98.01% - 101.53% and 101.98 – 108.78% respectively. The LLOQ QC samples for Tenofovir and Rilpivirine for Inter batch was 99.82% and 106.04% respectively. The results are summarized in the Table 5.

Intra batch precision of the method was expressed as coefficient of variation (% CV). The % CV at LQC, MQC-2, MQC-1 and HQC concentration levels for Tenofovir and Rilpivirine were ranged from 0.67 – 4.38% and 1.26 – 7.21% respectively. The LLOQ QC samples for Tenofovir and Rilipivirine for Intra batch was ranged from 3.07 – 5.09% and 5.90 – 11.71% respectively. The results are summarized in the Table 5.

Inter batch precision of the method was expressed as coefficient of variation (% CV). The % CV at LQC, MQC-2, MQC-1 and HQC concentration levels for Tenofovir and Rilipivirine ranged from 1.77-2.57% and 2.14 -5.56% respectively. The LLOQ QC samples for Cobicistat and Elvitegravir for Inter batch was ranged 3.92% and 8.42% respectively. The results are summarized in the Table 5.

The % mean recoveries for analyte were determined by measuring the area ratios of the extracted plasma quality control samples against unextracted quality control samples at HQC, MQC-1, MQC-2 and LQC levels. The % mean recovery for Cobicistat at HQC, MQC-1, MQC-2 and LQC levels were found to be 82.11, 79.87, 72.69 and 76.96 respectively. The % mean recovery for Elvitegravir at HQC, MQC-1, MQC-2 and LQC levels were found to be 84.36, 85.75, 75.47 and 77.77 respectively. The % mean recoveries for Internal Standards were determined by measuring the area ratios of internal standards in the extracted samples against unextracted samples respectively. The % mean recoveries for Internal Standards were found to be 83.52 and 85.09 respectively.

The % mean recoveries for analyte were determined by measuring the area ratios of the extracted plasma quality control samples against unextracted quality control samples at HQC, MQC-1, MQC-2 and LQC levels. The % mean recovery for Tenofovir at HQC, MQC-1, MQC-2 and LQC levels were found to be 42.23, 42.65, 41.20 and 42.97 respectively. The % mean recovery for Rilpivirine at HQC, MQC-1, MQC-2 and LQC levels were found to be 72.54, 74.85, 72.08 and 77.58 respectively. The % mean recoveries for Internal Standards were determined by measuring the area ratios of internal standards in the extracted samples against unextracted samples respectively. The % mean recoveries for Internal Standards Tenofovir D6 and Rilipivirine D6 were found to be 44.90 and 74.53 respectively.

The matrix effect of the method was evaluated for Cobicistat and Elvitegravir using quality control samples, LQC and HQC. Matrix effect was assessed by comparing six different lots of post extracted plasma samples area ratio Vs unextracted samples area ratios. No significant matrix effect was found in different sources of human plasma tested for Rilpivirine and Tenofovir; results were represented in Table 6.

The dilution integrity of the method was evaluated by diluting the stock solution to the concentration of 904.400 and 299.900 ng/mL in the screened plasma for Tenofovir and Rilpivirine respectively. The precision and accuracy for dilution integrity standards at 1/2 and 1/4 dilution were determined by analyzing the samples against calibration curve standards. The % mean accuracy for dilution integrity of 1/2 and 1/4 for Tenofovir and Rilpivirine were found to be and 99.99 &

90.36 and 104.76 & 98.26 respectively which is within acceptance limit 85.00 - 115.00%. The precision for dilution integrity of 1/2 and 1/4 for Tenofovir and Rilpivirine were found to be 1.18 & 3.30 and 0.79 & 1.96 respectively.

Ruggedness was performed by using a different column. The % mean accuracy for the quality control samples at LLOQ QC, LQC, MQC-2, MQC-1 and HQC concentration levels for a different column ranged from 92.39 to 104.04 and 99.63 to 104.15 respectively for Tenofovir and Rilpivirine.

The precision for the quality control samples at LLOQ QC, LQC, MQC-2, MQC-1 and HQC concentration levels for the different column ranged from 2.19 to 6.27 and 1.26 to 4.27 for Tenofovir and Rilpivirine respectively.

Reinjection reproducibility was established by re-injecting the accepted precision and accuracy batch after a period of 55 hours 25 minutes. The % mean accuracy of back calculated concentrations for all quality control samples at LQC, MQC-2, MQC-1 and HQC concentration levels were ranged from 93.12 to 103.56 and 97.87 to 106.39 respectively, for Tenofovir and Rilpivirine, which is within acceptance limit 85.00 - 115.00%. The % mean accuracy of back calculated concentrations for all the samples of LLOQ QC was found to be 91.82 and 94.40 respectively, for Tenofovir and Rilpivirine, which is within the acceptance limit of 80.00 - 120.00%. The % CV of back calculated concentrations for all quality control samples of LQC, MQC-2, MQC-1 and HQC concentration levels ranged from 1.59 to 2.96 and 0.83 to 2.90 respectively for Tenofovir and Rilpivirine which are within the acceptance limit of 15.00%. The % CV of back calculated concentrations for all LLOQ QC samples were found to be 7.02 and 3.14 respectively, for Tenofovir and Rilpivirine which is within the acceptance limit of ±20.00%.

The haemolysisy effect of the method was evaluated using 0.5% and 1.0% haemolysed plasma for Tenofovir and Rilpivirine. The precision and accuracy for 0.5% and 1.0% Haemolysed plasma were determined by analyzing the samples against calibration curve standards. The % mean accuracy at 0.5% and 1.0% haemolysed plasma for LQC and HQC found to be 99.77 & 94.53 and 102.28 & 95.42 respectively for Tenofovir and for Rilpivirine the % mean accuracy at 0.5% and 1.0% haemolysed plasma for LQC and HQC found to 96.27 & 105.53 and 97.17 & 104.52 respectively, which is within acceptance limit 85.00 - 115.00%. The precision for 0.5% and 1.0% haemolysed plasma at LQC and HQC found to be 3.65 & 1.36 and 3.33 & 1.67 respectively for Tenofovir, the precision for 0.5% and 1.0% haemolysed plasma at LQC and HQC for Rilpivirine found to be 3.1 & 01.10, 3.56 & 0.6 respectively, which is within acceptance limit 85.00 - 115.00%, results were represented in Table 7.

The method was evaluated for potentially interfering drugs such as Paracetamol, Ibuprofen, Ondensetron and Diclofenac. The interference was determined at low quality control concentration levels of Tenofovir and Rilipivirine. The % nominal for Paracetamol, Ibuprofen, Ondensetron and Diclofenac for Tenofovir at Low quality control samples found to be 100.87, 100.30, 101.56, and 100.47% respectively. The % nominal for Paracetamol, Ibuprofen, Ondensetron and Diclofenac for Rilpivirine at Low quality control samples found to be 95.01, 95.43, 100.51 and 96.31% respectively, which is within the acceptance limit of 85.00 - 115.00%. The results were represented in Table 8.

Short term stock solution stability was determined for Tenofovir and Rilpivirine in diluents 100% acetonitrile and 50 % acetonitrile at concentration equivalent to AQ LQC and AQ HQC (300.000 and 9240.000 ng/mL, 60.000 and 3000.000 ng/mL for Tenofovir and Rilpivirine respectively). Stability was assessed by comparing against the freshly prepared aqueous standards equivalent to AQ LQC and AQ HQC concentration. The duration of stability was found to be 10.0 hr at 2-8ºC. The % mean stability was found to be 100.7 and 97.93 for Tenofovir and Rilpivirine respectively, which is within the acceptance limit of 90.00 - 110.00%.

Long term stock solution stability was determined for the Tenofovir and Rilpivirine at concentration (1000.000 and 1000.000 µg/mL) using aqueous standards equivalent to AQ LQC and AQ HQC concentration of 300.000 and 9240.000 ng/mL, 60.000 and 3000.000 ng/mL for Tenofovir and Rilpivirine respectively. The stability was established after a storage period of 10 days at 2-8 °C. Stability was assessed by comparing against the freshly weighed stock concentration (1000.000 and 1000.000 µg/mL) and prepared aqueous standard equivalent to AQ LQC and AQ HQC concentration. The % mean stability was found to be 99.55 and 101.50 for Tenofovir and Rilpivirine respectively, which is within the acceptance limit of 90.00 - 110.00%.

Stability studies for Tenofovir and Rilpivirine in plasma were established under various conditions using six replicates of LQC and HQC sample. Bench top stability was determined for a period of 15 hours 20 minutes at room temperature. Stability was evaluated by comparing them against freshly spiked calibration standards and quality control samples. The % mean stability of Tenofovir and Rilpivirine for HQC and LQC were found to be 100.80% and 102.59; 97.99 and 99.85% respectively, which is within the acceptance limit of 85.00 - 115.00%. Auto sampler stability of the processed quality control samples was determined for a period of 70 hours 50 minutes by storing them in auto sampler maintained at temperature 5 ± 3°C. Stability was assessed by comparing against the fresh calibration standards and quality control samples. The % mean stability of Tenofovir and Rilpivirine for HQC and LQC

were found to be 99.69 and 99.78%; 100.32 and 100.33% respectively, which is within the acceptance limit of 85.00 - 115.00%. Freeze and thaw stability of the quality control samples was determined after five freeze thaw cycles stored at -80 ± 5 °C. Stability was measured by comparing them against the freshly prepared calibration standards and quality control samples. The % mean stability of Tenofovir and Rilpivirine for HQC and LQC were found to be 99.28 and 101.25%; 99.93 and 99.41 respectively, which is within the acceptance limit of 85.00 - 115.00%. Stability of quality control samples in dry ice was determined for a period of 58 hours 20 minutes. Stability was assessed by comparing them against the fresh calibration standards and quality control samples. The % mean stability of Tenofovir and Rilpivirine for HQC and LQC were found to be 99.61 and 99.84; 98.78 and 94.04 respectively, which is within the acceptance limit of 85.00 - 115.00%. The post extracted Refrigerator Stability was established using quality control samples; it was demonstrated for a period of 30 hours 10 minutes by storing in refrigerator at 2 -8°C, stability was determined by comparing quality control samples against the fresh calibration standards and fresh quality control samples. The % mean stability of Tenofovir and Rilpivirine for HQC and LQC were found to be 98.62 and 99.47%; 98.75 and 108.61 respectively, the results were within the acceptance limit of 85.00 - 115.00%. Dry extract stability was assessed by using quality control samples at 2-8ºC; It was established for a period of 24 hours 18 min. The dry extract stability was evaluated by comparing quality control samples against the fresh quality control samples using calibration standards. The % mean stability of Tenofovir and Rilpivirine for HQC and LQC were found to be 99.01 and 96.93%; 97.78 and 95.93% respectively, which is within the acceptance limit of 85.00 - 115.00%.

Table 1 Optimized mass parameters for Tenofovir and Rilpivirine their corresponding internal standards

Tuning parameters	**Tenofovir**	**Rilpivirine**
Curtain gas (CUR)	25	
Ion spray voltage (IS)	5500	
Nebulizer gas (GS1)	50	
Heater gas (GS2)	40	
Collision gas (CAD)	8	
Declustering potential (DP)	60	55
Entrance potential (EP)	10	12
Collision energy (CE)	25	30
Collision cell exit potential (CXP)	10	15
Dwell time (milli seconds)	100	100
Temp (^{0}C)	500	
Ihe	ON	

Table 2 Chromatography parameters

Mobile Phase	**5.0 mM Ammonium acetate : Acetonitrile (20:80)**
Mobile Phase Flow Rate	0.600mL/min
RT of Tenofovir	0.85 ± 15 sec
RT of Rilpivirine	2.80 ± 15 sec
RT of Internal standard (Tenofovir D6)	0.85 ± 15 sec
RT of Internal standard (Rilpivirine D6)	2.80 ± 15 sec
Total Run Time	4.0 min

Table 3 Stability study conditions and % mean stability results

Stability study	**Condition**	**N**	**% Mean stability**			
			Tenofovir		**Rilpivirine**	
			HQC	**LQC**	**HQC**	**LQC**
Bench top stability	15 hours 20 minutes storage at room temperature	6	100.80	102.59	97.99	99.85
Freeze thaw stability	Five freeze thaw stored at -80±5ºC		99.28	101.25	99.93	99.41
Auto sampler stability	Storage for 70 hours 50 minutes at 5 ±3 ºC		99.69	99.78	100.32	100.33
Dry Ice	58 hours 20 minutes		99.61	99.84	98.18	94.04
Wet extract stability at refrigerated temperature	30 hours 10 minutes storage at 2-8 ºC		98.10	99.23	99.13	97.33
Dry extract stability	Storage at 2-8 ºC for a period of 24 hours 15 minutes		99.01	96.93	97.78	95.93

Table 4 Sensitivity of Tenofovir and Rilpivirine

Tenofovir (Nominal Concentration, ng/mL)	5.00
% CV	3.86
% Mean accuracy	98.54
Rilpivirine (Nominal Concentration, ng/mL)	1.00
% CV	4.95
% Mean accuracy	100.88

Table 5 Intraday and Inter day Precision and Accuracy (completed)

Sample	Intraday (*n*=6)			Inter day (*n*=24)		
	Mean conc. found (ng/mL)	% Mean accuracy	% CV	Mean conc. found (ng/mL)	% Mean accuracy	% CV
Tenofovir						
LLOQ	5.050	101.00	5.09	4.991	99.82	3.92
LQC	14.998	99.99	4.38	15.063	100.42	2.57
MQC 2	101.450	101.45	1.12	101.528	101.53	1.87
MQC 1	214.110	97.32	1.17	215.653	98.02	2.03
HQC	449.888	97.80	1.97	454.707	98.85	1.77
Rilpivirine						
LLOQ	1.071	107.13	7.06	1.060	106.04	8.42
LQC	3.071	102.37	4.93	3.072	102.40	5.56
MQC 1	40.984	102.46	3.32	40.793	101.98	2.84
MQC 2	68.029	111.52	5.16	66.353	108.78	5.16
HQC	157.091	104.73	2.62	157.248	104.83	2.14

Table 6 Matrix effect evaluation for Tenofovir and Rilpivirine

Tenofovir		
Conc. (ng/mL)	**HQC (462.00 ng/mL)**	**LQC (15.00 ng/mL)**
Mean	456.898	15.161
S.D	2.76	0.309
% Nominal	98.9	101.08
% C.V	0.6	2.04
Rilpivirine		
Conc. (ng/mL)	HQC (150.00 ng/mL)	LQC (3.00 ng/mL)
Mean	150.817	2.972
S.D	1.147	0.067
% Nominal	100.55	99.07
% C.V	0.76	2.28

Table 7: haemolysis effect.

Sample	% Haemolysis	Haemolysis Effect		
		Mean conc. found (ng/mL)	% Mean accuracy	% CV
Tenofovir				
LQC	0.5	15.023	100.16	2.7
HQC		455.291	98.55	0.55
LQC	1	15.072	100.48	0.86
HQC		456.107	98.72	0.55
Rilpivirine				
LQC	0.5	2.929	97.63	1.16
HQC		158.774	105.85	0.93
LQC	1	2.966	98.89	3.68
HQC		155.607	103.74	0.92

Table 8: Effect of Co-administered drugs

Drug	Effect of Co-administered drugs		
	Mean conc. found (ng/mL)	% Mean accuracy	% CV
Tenofovir			
Paracetamol	15.129	100.87	1.2
Ibuprofen	15.045	100.3	1.7
Ondensetron	15.234	101.56	1.41
Diclofenac	15.069	100,47	0.82
Rilpivirine			
Paracetamol	2.85	95.01	6.23
Ibuprofen	2.862	95.43	2.91
Ondensetron	3.015	100.51	3.54
Diclofenac	2.889	96.31	3.81

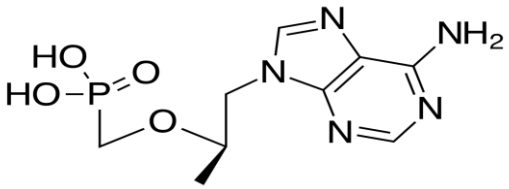

Fig 1. Structure of Tenofovir

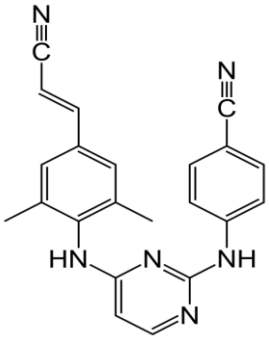

Fig 2. Structure of Rilpivirine

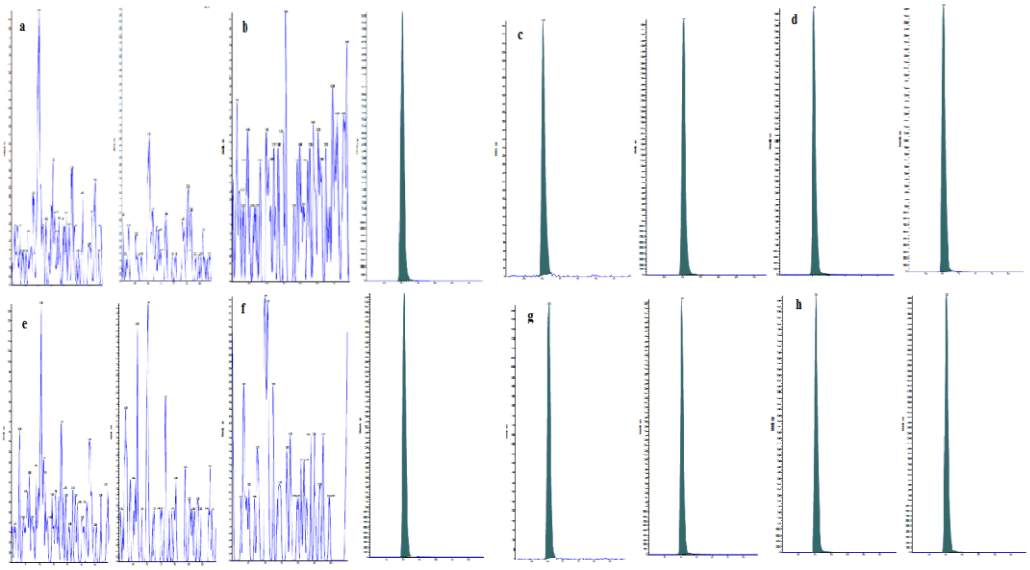

Fig 3. Reprasentative chromatographs of Tenofovir (Blank-a, Blank + Internal standard-b, LLOQ-c, ULOQ-d) and Rilpivirine (Balnk-e, Blank + Internal standard-f, LLOQ-g, ULOQ-h)

CONCLUSION

The experiments performed during the validation, concluded that the method is validated for the simultaneous quantitation of Tenofovir and Rilpivirine in K2EDTA human plasma over the concentration range of 5.000 - 600.00 and 1.000-203.00 ng/mL respectively, using Tenofovir D6 and Rilpivirine D6 as internal standards. The precision and mean accuracy are within the acceptable limits. Consistent recoveries were observed for LQC, MQC-2, MQC-1 and HQC. The method is specific enough in the presence of K2EDTA anticoagulant. The method is precise and accurate enough to dilute samples, if necessary. The different stabilities were established during validation concluded that the intended analytes, Tenofovir and Rilpivirine were stable at different circumstance like bench top stability (15 hours 20 minutes), auto sampler (70 hours 50 minutes), wet extract stability at refrigerator temperature (30 hours 10 minutes), stability in dry ice (58 hr 20 min), dry extract stability at 2 - 8°C (24 hours 15 minutes) and five freeze and thaw cycles at -80 ± 5°C. The Tenofovir and Rilpivirine stock solutions were stable at room temperature for 6 days. Reinjection reproducibility was proved for 55 hrs 25 min. The method was proved to be rugged by different column.

REFERENCES

1. Andrés Duarte-Rojo. Efficacy and safety of tenofovir disoproxil fumarate in patients with chronic hepatitis B. Therap Adv Gastroenterol, 2010; 3(2): 107–119.
2. Kearney BP1, Flaherty JF, Shah J. Tenofovir disoproxil fumarate: clinical pharmacology and pharmacokinetics. Clin Pharmacokinet, 2004; 43(9): 595-612.
3. Sharma M, Saravolatz L. Rilpivirine: a new non-nucleoside reverse transcriptase inhibitor. J Antimicrob Chemother, 2013; 68: 250-6.
4. Mohammed Lamorde, Stephen Walimbwa et al. Steady-state pharmacokinetics of rilpivirine under different meal conditions in HIV-1-infected Ugandan adults. J Antimicrob Chemother, 2015; 70(5): 1482-6.
5. Djerada Z et al. Validation of a fast method for quantitative analysis of elvitegravir, raltegravir, maraviroc, etravirine, tenofovir, boceprevir and 10 other antiretroviral agents in human plasma samples with a new UPLC-MS/MS technology. J Pharm Biomed Anal, 2013; 86: 100-11.
6. Else LJ1, Tjia J, Jackson A, Penchala SD et al. Quantification of rilpivirine in human plasma, cervicovaginal fluid, rectal fluid and genital/rectal mucosal tissues using liquid chromatography-tandem mass spectrometry. Bioanalysis, 2014; 6(14): 1907-1921.
7. Abhijit A. Date,a Annemarie Shibata,b Patrick Bruck, b and Christopher J. Destache. Development and validation of a simple and isocratic reversed-phase HPLC method for the determination of rilpivirine from tablets, nanoparticles and HeLa cell lysates. Biomed Chromatogr, 2015; 29(5): 709–715.
8. Podany AT1,2, Sheldon C2, Grafelman D2, Ohnmacht CM2,3. Assay development for determination of tenofovir in human plasma by solid phase analytical derivatization and LC-MS/MS. Bioanalysis, 2015; 7(24): 3085-95.
9. Addepalli. V. Raju et al.: Development and Validation of a LC-MS/MS Method for the Determination of Rilpivirine in Sprague Dawley Rat Serum and Its Application to Pharmacokinetic Study. Asian Journal of Biomedical and Pharmaceutical Sciences, 2013; 3(21): 23-29.
10. Ramachandran et al. simultanious determination of antiretroviral drugs emtricitabine and tenofovir desoproxil fumerate by a stability indicating rp-hplc method. Int J Pharm Pharm Sci, 2015; 7(9): 390-398.
11. Rezk NL, Crutchley RD, Kashuba AD. Simultaneous quantification of emtricitabine and tenofovir in human plasma using high-performance liquid chromatography after solid phase extraction. J Chromatogr B, 2005; 822: 201–8.
12. FDA. Guidance for industry: Bioanalytical Method Validation. US Department of Health and Human Services, Food and Drug Administration Centre for Drug Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM): Washington, C, May 2001.