Development and Validation of Analytical Method for Determination of Dolutegravir Sodium, Lamivudine and Tenofovir Disoproxil Fumarate Using Reverse Phase High Performance Liquid Chromatography

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ABSTRACT

A simple reliable analytical method was developed to determine dolutegravir sodium, lamivudine and tenofovir disoproxil fumarate in pharmaceutical effluents which are releasing into domestic water bodies by using reverse phase High Performance Liquid Chromatography (HPLC) with ultraviolet absorption detection. The proposed method was quite reproducible and sensitive enough to detect the compounds at less than 10 ppm level, which can replace the troublesome non-reproducible conventional analytical methods like UV-Visible spectrophotometric analysis or titrimetric analysis. A Reversed-Phase HPLC method was developed and validated for the estimation of dolutegravir sodium, lamivudine and tenofovir disoproxil fumarate in effluents or pharmaceutical industry washouts. The separation was achieved on C18 symmetry C-18 column (250 × 4.6 mm i.d., 5.0 μm) using sodium dihydrogen phosphate with SDS as ion pair reagent having a pH of 2.0 as mobile phase A and acetonitrile and methanol as mobile phase B in gradient mode as mobile phase and at a flow rate of 1.0 ml/min. Detection was carried out using a UV detector at 260 nm. The total chromatographic analysis time per sample was about 30.0 min with dolutegravir sodium, lamivudine and tenofovir disoproxil fumarate eluting at retention time of about 5.2 min for lamivudine, 11.0 min for dolutegravir sodium and 13.0 min for tenofovir disoproxil fumarate. The method was validated for accuracy, precision, specificity, linearity and sensitivity. Validation studies demonstrated that this HPLC method is accurate, specific, rapid, reliable and reproducible. Linearity was observed for dolutegravir sodium, lamivudine, and tenofovir disoproxil fumarate in the concentration range of 0.05-7.5 μg/ml (R2 > 0.95), the Limit of Detection (LOD) and Limit of Quantitation (LOQ) was found to be for 0.017 μg/ml and 0.053 μg/ml respectively for dolutegravir sodium, 0.016 μg/ml and 0.048 μg/ml for lamivudine and 0.018 μg/ml and 0.054 μg/ml for tenofovir disoproxil fumarate, the method was validated as per ICH guidelines. The RSD for intra-day and inter-day precision were found to be less than 5%. The percentage recovery was in good agreement and the method is simple, specific, precise, and accurate for the determination of dolutegravir sodium, lamivudine and tenofovir disoproxil fumarate in the pharmaceutical industry washouts.

Keywords: Dolutegravir sodium, Lamivudine, Tenofovir disoproxil fumarate, Liquid chromatography

INTRODUCTION

The production of pharmaceuticals [1-3] in large quantities has been in progress for many years, however it was only in the recent times that scientific studies on the same has been initiated and examining the associated effluent and waste generation and their respective impact. Many surveys and studies have confirmed the presence of pharmaceuticals in effluents, municipal wastewaters, surface waters, ground water and to a lesser extent, drinking water. The ongoing global process of urbanization and population growth has increased the demand for clean water, leading to an increase in the volume of effluent to be treated.

Similarly, there is a growing demand for new products in several categories such as antibiotics, anti-retroviral, anti-cancer, and all other therapeutic categories, which leads to an increase in new emerging contaminants released into the environment, typically at levels in the nano-gram to low microgram per liter range, often without any knowledge of potential related risks to humans and damage to ecosystems. Anti-retroviral and antibiotics were recently ranked as a major risk group because of their high toxicity to algae and bacteria, even at low concentrations.
These risks include an increase in the occurrence of fatal cases of hospital-borne infections with such pathogens that develop resistance towards antibiotics [1-3].

A preliminary survey [4-8] of the databases of the environmental agency in the licensing process for the pharmaceutical industry, showed inconsistencies in data on the monitoring of solid waste production as well as noncompliance with the requirements to meet effluent discharge regulations. Inspections in the industries found that waste management is still in its infancy and that the large diversity in production results in the generation of a highly fluctuating effluent composition. This greatly impairs the efficiency of current treatment systems as well as the analytical techniques, which are employing for their estimation in the pharmaceutical washouts or effluents.

Since very limited scientific work is available for the estimation of various drug substances in the literature, the author has selected the molecule dolutegravir sodium, Lamivudine (LAM) and Tenofovir Disoproxil Fumarate (TDF) in a combination belongs to antiretroviral and developed a simple, rapid and accurate reverse phase liquid chromatographic method for the estimation of dolutegravir sodium, LAM and TDF by reverse phase liquid chromatographic method, which can be employed for the estimation of the selected drug substance in various pharmaceutical washouts or effluents either single or for estimation of three compounds, which will reduce the analysis time at the laboratory.

Dolutegravir is chemically(RS)(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-carboxamide3 (C_{20}H_{9}F_{2}N_{4}O_{6}) and molecular weight 419.38 g/mol. It is slightly soluble in water and methanol. Dolutegravir [9-11] is an FDA approved drug for the treatment of Human Immune Virus (HIV) infection. Dolutegravir is an integrase inhibitor. DTG is an Integrase Strand Transfer Inhibitor (INSTI) that does not require ritonavir for cytochrome P450 3A4 inhibition [12], and preferentially blocks the strand transfer step of integration of the viral genome into the host cell’s DNA, which is a two-step process mediated by the viral integrase enzyme.

Tenofovir [1,2,7,8,12] is chemically [(2R)-1-(6-aminopurin-9-yl)propan-2-yl]oxymethylphosphonic acid, a nucleotide analog of adenosine monophosphate. Its molecular formula is C_{22}H_{20}N_{2}O_{4}P and molecular weight is 635.52 g/mol TDF is an oral prodrug of tenofovir. TDF, a Nucleotide Reverse-transcriptase Inhibitor (NRTI) blocks the enzyme reverse transcriptase, an essential enzyme that is required for the replication of viral DNA [5].

LAM [1-3,13-15] chemically-(2R-cis)-4-amino-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-2(1H)pyrimidinone1, is a synthetic nucleoside analogue with potent activity against HIV and Hepatitis B Viruses (HBV) through inhibition of reverse transcriptase activity. It has a molecular formula of C_{21}H_{19}N_{2}O_{4}S and molecular weight of 229.3 g/mol, soluble in water, sparingly soluble in methanol and practically insoluble in acetone.

Literature review revealed that there is no HPLC method for the determination of Dolutegravir Sodium, LAM and TDF in pharmaceutical effluents at part per million (ppm) to nano level and only one spectrophotometric [14-16], method is available, which is not a reproducible analytical method and thee limitation for this method is it can’t detect the drug substances below mg/ml concentration [9,16-19]. Similarly, various journals have been referred for the procedure for method development [20-27]. In the present work, a simple, rapid and accurate reverse phase liquid chromatographic method for the estimation of Dolutegravir Sodium, Lamivudine, and Tenofovir Disoproxil Fumarate by reverse phase liquid chromatographic method has been developed for the determination of Dolutegravir Sodium, Lamivudine, and Tenofovir Disoproxil Fumarate and validated as per ICH guidelines [14].
MATERIALS AND METHODS

Reagents and chemicals
Sodium dihydrogen phosphate, sodium dodecyl sulphate, acetonitrile (HPLC grade), ortho phosphoric acid, were obtained from Merck (India). All chemicals were of an analytical grade and used as received.

Instrumentation
Chromatographic separation was achieved by using a Waters 2489 UV 2695 pump; Waters 2998 PDA 2695 pump Software Empower 2 photodiode array detector was used.

Buffer preparation
Buffer was prepared by dissolving 2.72 g of sodium dihydrogen phosphate and 200 mg of sodium dodecyl sulphate into a 1000 ml of purified water and mixed. Adjusted pH to 2.0 (± 0.05) with dilute ortho phosphoric acid solution. Filter the solution through 0.45 μm membrane filter.

Mobile phase preparation
Mobile phase (A): Use filtered and degassed buffer as mobile phase A. Mobile phase (B): Prepare a filtered and degassed mixture of Buffer and acetonitrile in the ratio of 200:800 v/v respectively.

Diluent preparation
Mobile phase-B is used as diluent.

Preparation of swab blank
Transfer 10 ml of diluent into a clean test tube. Place one cleaned Swab in the test tube and sonicates for 5 min. Filter through 0.45 μm Polyvinylidene Difluoride (PVDF) filter.

Standard preparation (Dolutegravir Sodium, LAM, and TDF)
Accurately weigh and transfer about 2.72 g of Dolutegravir Sodium in 1000 ml of purified water and mix. Adjust pH to 3.0 (± 0.05) with dilute ortho phosphoric acid solution. Filter the solution through 0.45 μm membrane filter. Prepare a filtered and degassed mixture of Buffer and acetonitrile in the ratio of 500:500 v/v respectively.

All peaks are not separated and Dolutegravir Sodium, LAM and TDF peak was eluted closely. For next trial the mobile phase composition was changed slightly. The mobile phase composition was Buffer and acetonitrile in gradient mode and observed peaks are little separated but the peak shape was little broad.

Again the mobile phase was modified by adding ion pair reagent and pH was changed to pH 2.0 (adjusted by using dilute ortho phosphoric acid): Acetonitrile in gradient mode at flow rate 1.0 ml/min. UV detection was performed at 260 nm. The retention time of Dolutegravir Sodium, LAM and TDF was about 5 min for lamivudine, 11.0 min dolutegravir and 13.0 for tenofovir was observed and the peak shape was good.

The chromatogram of Dolutegravir Sodium, LAM and TDF with all components using the proposed method. System suitability results of the method are presented in Table 1. Dolutegravir sodium, Lamivudine and TDF show significant UV absorbance at Wavelength 260 nm. Hence this wavelength has been chosen for detection in analysis of residue in Dolutegravir Sodium, LAM and TDF.

RESULTS AND DISCUSSION

Method development
To develop a suitable and robust LC method for the determination of Dolutegravir Sodium, LAM and TDF, different mobile phases were employed to achieve the best separation and resolution [20-25]. The method development was started with Hypersil BDS (Make: Thermo Fisher; 150 × 4.6 mm I.D; Particle size 3 μm) column was used for analysis at ambient column temperature. The mobile phase was pumped through the column at a flow rate of 1.0 ml/min. The sample injection volume was 20 μl. The photodiode array detector was set to a wavelength of 260 nm for the detection and chromatographic runtime was 30 min.
Method validation

System suitability

To demonstrate system suitability, the standard solution prepared as per method and injected six replicate injections into the HPLC system as per methodology [26,27]. The system suitability parameters were evaluated from the standard solution and found to be within the acceptance criteria. The %RSD for Dolutegravir Sodium, LAM and TDF peak areas from six replicate injections of standard solution was found to be within the limits. The results are summarized in Figures 1-3 and Table 1.

Table 1: Summary of system suitability

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of the compound</th>
<th>Tailing factor</th>
<th>Theoretical plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lamivudine</td>
<td>0.9</td>
<td>6767</td>
</tr>
<tr>
<td>2</td>
<td>Dolutegravir</td>
<td>1.0</td>
<td>26002</td>
</tr>
<tr>
<td>3</td>
<td>Tenofovir</td>
<td>1.1</td>
<td>36436</td>
</tr>
</tbody>
</table>

Figure 1: Representative Chromatogram of Blank

Figure 2: Representative Chromatogram of Sensitivity Solution

Figure 3: Representative Chromatogram of Standard
Specificity

Blank interference

A study was conducted to demonstrate the noninterference of swab, prepared triplicate swab blanks of each plate (Stainless steel plate,) and injected into the HPLC system as per the proposed test method. Evaluated the interference of swab blanks at the retention time of Dolutegravir Sodium, LAM and TDF peak and found no peaks at the retention time of Dolutegravir Sodium, LAM and TDF peak. The results are Figure 4.

![Swab Blank](image)

Figure 4: A typical HPLC Chromatogram showing the no interference of diluent

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

A study was conducted to establish the LOD and LOQ of Dolutegravir Sodium, LAM and TDF based on slope method. Prepared a series of solutions from 1 to 150% of standard concentration of Dolutegravir Sodium, LAM, and TDF. These solutions were injected into the HPLC system as per methodology.

Plotted a graph by taking concentration on X-axis and area on Y-axis, calculated the standard error and slope of the calibration curve. The predicted LOQ concentration and LOD concentration are calculated by using formula given below. The results are summarized in the Table 2.

\[ \text{LOQ} = \frac{10 \times \sigma}{S}, \quad \text{LOD} = \frac{3.3 \times \sigma}{S} \]

\(\sigma=\text{Standard Error of the calibration curve; } S=\text{Slope of the calibration curve.}\)

LOQ precision

Precision at LOQ [26,27] concentration was established for dolutegravir sodium, LAM and TDF. Six samples were prepared by diluting standard stock solution to obtain the LOQ concentration and injected in to HPLC system as per methodology. Calculated the %RSD for dolutegravir sodium, LAM and TDF in ppm. The results were found to be within the acceptance criteria and the data are summarized in Table 2-4 and Figure 5.

Linearity

Linearity [22-27] is carried out under LOD-LOQ establishment experiment, the same linearity establishment data can be used to deduce the linearity from LOQ level to 150% specification level. A graph was plotted to concentration in ppm on X-axis versus response on Y-axis. Calculated % y-intercept and correlation coefficient. The results and the linearity graph are presented in Figures 6-9.

### Table 2: Precision at LOD and LOQ for dolutegravir

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>LOD concentration (ppm)</th>
<th>LOQ concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Area Response</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1700</td>
<td>4801</td>
</tr>
<tr>
<td>2</td>
<td>2001</td>
<td>4849</td>
</tr>
<tr>
<td>3</td>
<td>1600</td>
<td>4838</td>
</tr>
<tr>
<td>4</td>
<td>1500</td>
<td>4950</td>
</tr>
<tr>
<td>5</td>
<td>1652</td>
<td>5450</td>
</tr>
<tr>
<td>6</td>
<td>2500</td>
<td>4690</td>
</tr>
<tr>
<td>Average</td>
<td>1825</td>
<td>4930</td>
</tr>
<tr>
<td>% RSD</td>
<td>18.56</td>
<td>5.4</td>
</tr>
</tbody>
</table>
Table 3: Precision at LOD and LOQ for lamivudine

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>LOD concentration (ppm)</th>
<th>LOQ concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Area response</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>987</td>
<td>1983</td>
</tr>
<tr>
<td>2</td>
<td>1005</td>
<td>2003</td>
</tr>
<tr>
<td>3</td>
<td>850</td>
<td>2084</td>
</tr>
<tr>
<td>4</td>
<td>910</td>
<td>1831</td>
</tr>
<tr>
<td>5</td>
<td>581</td>
<td>1916</td>
</tr>
<tr>
<td>6</td>
<td>1100</td>
<td>1938</td>
</tr>
<tr>
<td>Average</td>
<td>905</td>
<td>1959</td>
</tr>
<tr>
<td>% RSD</td>
<td>18.21</td>
<td>4.4</td>
</tr>
</tbody>
</table>

Table 4: Precision at LOD and LOQ for tenofovir disoproxil fumarate

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>LOD concentration (ppm)</th>
<th>LOQ concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>815</td>
<td>1546</td>
</tr>
<tr>
<td>2</td>
<td>1125</td>
<td>1543</td>
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<tr>
<td>3</td>
<td>912</td>
<td>1463</td>
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<tr>
<td>4</td>
<td>564</td>
<td>1638</td>
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<tr>
<td>5</td>
<td>671</td>
<td>1472</td>
</tr>
<tr>
<td>6</td>
<td>919</td>
<td>1438</td>
</tr>
<tr>
<td>Average</td>
<td>834</td>
<td>1517</td>
</tr>
<tr>
<td>% RSD</td>
<td>21.76</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Figure 5: Representative chromatogram for LOQ level

![Representative chromatogram for LOQ level](image)

Figure 6: The results and the linearity graph

![The results and the linearity graph](image)
Figure 7: The results and the linearity graph

<table>
<thead>
<tr>
<th>Name of the level</th>
<th>% level</th>
<th>in %</th>
<th>in ppm</th>
<th>Area Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level-1</td>
<td>100</td>
<td>0.035</td>
<td>0.500</td>
<td>20798</td>
</tr>
<tr>
<td>Level-2</td>
<td>100</td>
<td>0.063</td>
<td>5.000</td>
<td>592009</td>
</tr>
<tr>
<td>Level-3</td>
<td>100</td>
<td>0.025</td>
<td>8.000</td>
<td>399416</td>
</tr>
<tr>
<td>Level-4</td>
<td>100</td>
<td>0.075</td>
<td>7.500</td>
<td>399416</td>
</tr>
<tr>
<td>Level-5</td>
<td>100</td>
<td>0.075</td>
<td>7.500</td>
<td>399416</td>
</tr>
<tr>
<td>Level-6</td>
<td>100</td>
<td>0.075</td>
<td>7.500</td>
<td>399416</td>
</tr>
</tbody>
</table>

Slope 2307136
Intercept -13885
Res sum of squ 24553
CC (r) 0.9904
Rsq (r2) 0.9810
LOD 0.616
LOQ 0.648

Figure 8: The results and the linearity graph

<table>
<thead>
<tr>
<th>Name of the level</th>
<th>% level</th>
<th>in %</th>
<th>in ppm</th>
<th>Area Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level-1</td>
<td>100</td>
<td>0.001</td>
<td>0.050</td>
<td>1517</td>
</tr>
<tr>
<td>Level-2</td>
<td>10</td>
<td>0.095</td>
<td>0.500</td>
<td>16272</td>
</tr>
<tr>
<td>Level-3</td>
<td>50</td>
<td>0.250</td>
<td>2.500</td>
<td>99400</td>
</tr>
<tr>
<td>Level-4</td>
<td>100</td>
<td>0.035</td>
<td>5.000</td>
<td>164766</td>
</tr>
<tr>
<td>Level-5</td>
<td>125</td>
<td>0.052</td>
<td>6.250</td>
<td>235359</td>
</tr>
<tr>
<td>Level-6</td>
<td>150</td>
<td>0.075</td>
<td>7.500</td>
<td>324371</td>
</tr>
</tbody>
</table>

Slope 4116894
Intercept -110443
Res sum of squ 22513
CC (r) 0.9631
Rsq (r2) 0.9763
LOD 0.048
LOQ 0.054

Figure 9: Representative chromatogram for Linearity
Method precision (Repeatability)

A study of repeatability [26,27] of Dolutegravir Sodium, LAM and TDF from the surfaces was conducted in six preparations by spiking 1 ml of dolutegravir sodium, LAM and TDF sample stock solution over 10 × 10 sq. cm stainless steel plate. The sample was dried, by blowing warm air. The stainless steel surface was swabbed horizontal, vertical and diagonal. The liquid absorbed by swabs was squeezed out into test tube having 10 ml of diluent, mixed, sonicated for 5 min and swabs were discarded. The solution injected into HPLC system as per methodology. The results are summarized in Table 5.

Table 5: Method precision (Repeatability)

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Dolutegravir</th>
<th>Lamivudine</th>
<th>Tenofovir</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>99.8</td>
<td>99.1</td>
<td>89.9</td>
</tr>
<tr>
<td>2</td>
<td>100.2</td>
<td>98.2</td>
<td>99.9</td>
</tr>
<tr>
<td>3</td>
<td>99.6</td>
<td>95.8</td>
<td>94.8</td>
</tr>
<tr>
<td>4</td>
<td>100.4</td>
<td>89.9</td>
<td>97.4</td>
</tr>
<tr>
<td>5</td>
<td>99.0</td>
<td>96.7</td>
<td>95.7</td>
</tr>
<tr>
<td>6</td>
<td>99.4</td>
<td>97.8</td>
<td>93.8</td>
</tr>
<tr>
<td>Average</td>
<td>99.73</td>
<td>96.25</td>
<td>95.25</td>
</tr>
<tr>
<td>%RSD (Limit NMT 10.0)</td>
<td>0.52</td>
<td>3.45</td>
<td>3.56</td>
</tr>
</tbody>
</table>

Intermediate precision

Perform the intermediate precision [26,27] of same sample by different analyst, different instrument, and different column and on different day. Prepared six samples by spiking 1 ml of dolutegravir sodium, LAM and TDF sample stock solution over 10 × 10 sq. cm stainless steel plate. The sample was dried, by blowing warm air. The stainless-steel surface was swabbed horizontal, vertical and diagonal. The liquid absorbed by swabs was squeezed out into test tube having 10 ml of diluent, mixed, sonicated for 5 min and swabs were discarded. The solution injected into HPLC system as per methodology. The results are summarized in Table 6 and Figure 10.

Table 6: Intermediate precision

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Dolutegravir</th>
<th>Lamivudine</th>
<th>Tenofovir</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>93.1</td>
<td>99.7</td>
<td>92.7</td>
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<tr>
<td>2</td>
<td>95.6</td>
<td>91.8</td>
<td>93.7</td>
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<td>3</td>
<td>98.7</td>
<td>97.4</td>
<td>89.4</td>
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<tr>
<td>4</td>
<td>88.9</td>
<td>98.5</td>
<td>94.8</td>
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<tr>
<td>5</td>
<td>84.7</td>
<td>97.5</td>
<td>96.7</td>
</tr>
<tr>
<td>6</td>
<td>91.7</td>
<td>92.7</td>
<td>97.1</td>
</tr>
<tr>
<td>Average</td>
<td>92.12</td>
<td>96.27</td>
<td>94.07</td>
</tr>
<tr>
<td>%RSD (Limit NMT 10.0)</td>
<td>5.36</td>
<td>3.36</td>
<td>3.02</td>
</tr>
</tbody>
</table>

Figure 10: Representative chromatogram for method precision
Accuracy
A study of recovery [20-25] of Dolutegravir Sodium, LAM and TDF from the surfaces was conducted in triplicate preparations by spiking 1 ml of Dolutegravir Sodium, LAM and TDF to obtain about 2.5 ppm (for 50% recovery), about 5 ppm (for 100% recovery) and about 7.5 ppm (for 200% recovery) from sample stock solution over 10 × 10 stainless steel plate. The sample was dried, by blowing warm air. The sample swabbed horizontal, vertical and diagonal. The liquid absorbed by swabs was squeezed out into test tube having 10 ml of diluent, mixed, sonicated for 5 min and swabs were discarded. The solution injected into HPLC system as per methodology. The results are summarized in Tables 7a-7c and Figure 11.

Table 7a: Recovery on stainless steel plate for Dolutegravir sodium

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>% Recovery 50%</th>
<th>% Recovery 100%</th>
<th>% Recovery 200%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>99.7</td>
<td>97.5</td>
<td>89.7</td>
</tr>
<tr>
<td>2</td>
<td>98.0</td>
<td>98.1</td>
<td>94.7</td>
</tr>
<tr>
<td>3</td>
<td>98.7</td>
<td>96.7</td>
<td>97.1</td>
</tr>
<tr>
<td>Average (NLT 80.0%)</td>
<td>98.80</td>
<td>97.43</td>
<td>93.83</td>
</tr>
<tr>
<td>% RSD (NMT 10)</td>
<td>0.86</td>
<td>0.72</td>
<td>4.02</td>
</tr>
<tr>
<td>Overall average</td>
<td>96.7</td>
<td>Recovery factor</td>
<td></td>
</tr>
</tbody>
</table>

Table 7b: Recovery on stainless steel plate for Lamivudine

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>% Recovery 50%</th>
<th>% Recovery 100%</th>
<th>% Recovery 200%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94.8</td>
<td>96.7</td>
<td>94.6</td>
</tr>
<tr>
<td>2</td>
<td>96.7</td>
<td>98.5</td>
<td>96.7</td>
</tr>
<tr>
<td>3</td>
<td>98.0</td>
<td>93.1</td>
<td>89.7</td>
</tr>
<tr>
<td>Average (NLT 80.0%)</td>
<td>96.50</td>
<td>96.10</td>
<td>93.67</td>
</tr>
<tr>
<td>% RSD (NMT 10)</td>
<td>1.67</td>
<td>2.86</td>
<td>3.84</td>
</tr>
<tr>
<td>Overall average</td>
<td>95.4</td>
<td>Recovery factor</td>
<td></td>
</tr>
</tbody>
</table>

Table 7c: Recovery on stainless steel plate for Doltegravir

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>% Recovery 50%</th>
<th>% Recovery 100%</th>
<th>% Recovery 200%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>87.5</td>
<td>86.7</td>
<td>89.4</td>
</tr>
<tr>
<td>2</td>
<td>89.7</td>
<td>85.7</td>
<td>86.7</td>
</tr>
<tr>
<td>3</td>
<td>88.6</td>
<td>83.8</td>
<td>85.9</td>
</tr>
<tr>
<td>Average (NLT 80.0%)</td>
<td>88.60</td>
<td>85.40</td>
<td>87.33</td>
</tr>
<tr>
<td>% RSD (NMT 10)</td>
<td>1.24</td>
<td>1.72</td>
<td>2.10</td>
</tr>
<tr>
<td>Overall average</td>
<td>87.1</td>
<td>Recovery factor</td>
<td></td>
</tr>
</tbody>
</table>

Figure 11: Representative chromatogram for recovery at 100% level
Solution stability

A study to establish bench top stability [20-25] of Dolutegravir Sodium, LAM and TDF in sample solution and standard solution was conducted at initial, 1 day and 2 days. The % recovery of Dolutegravir Sodium, LAM and TDF in sample solution and standard solution was estimated against freshly prepared standard at each time. The difference in %RSD and sample solutions from initial to 1 day and 2 days was calculated against freshly prepared standard at each time and results are summarized in Table 8a-8c.

Table 8a: Bench Top Stability of standard and sample solution for dolutegravir

<table>
<thead>
<tr>
<th>Time in days</th>
<th>% Assay of standard preparation</th>
<th>Difference from initial (NMT 2.0)</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>99.1</td>
<td>-</td>
<td>99.8</td>
<td>100.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>99.8</td>
<td>0.7</td>
<td>98.9</td>
<td>99.5</td>
<td>0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>2</td>
<td>99.4</td>
<td>0.3</td>
<td>99.4</td>
<td>98.9</td>
<td>0.4</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Table 8b: Bench Top Stability of standard and sample solution for lamivudine

<table>
<thead>
<tr>
<th>Time in days</th>
<th>% Assay of standard preparation</th>
<th>Difference from initial (NMT 2.0)</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>99.8</td>
<td>-</td>
<td>99.1</td>
<td>98.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>99.6</td>
<td>0.2</td>
<td>98.2</td>
<td>99.7</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>99.4</td>
<td>0.5</td>
<td>99.7</td>
<td>99.4</td>
<td>0.8</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Table 8c: Bench top stability of standard and sample solution for tenofovir

<table>
<thead>
<tr>
<th>Time in days</th>
<th>% Assay of standard preparation</th>
<th>Difference from initial (NMT 2.0)</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>89.8</td>
<td>-</td>
<td>89.9</td>
<td>99.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>89.4</td>
<td>0.4</td>
<td>90.8</td>
<td>99.5</td>
<td>0.9</td>
<td>0.4</td>
</tr>
<tr>
<td>2</td>
<td>88.8</td>
<td>1</td>
<td>90.2</td>
<td>98.9</td>
<td>0.3</td>
<td>1</td>
</tr>
</tbody>
</table>

Bench top stability of mobile phase

A study to establish the bench top stability of mobile phase was conducted at initial, day 3. The mobile phase was prepared as per the test method, analyzed and kept on bench top in well closed condition. Standard solution prepared as per test method and injected into HPLC system with the mobile phase kept on bench top at day 3. The System suitability parameters found to be within the limits. The results are summarized in Table 9.

Table 9: Bench top stability of mobile phase

<table>
<thead>
<tr>
<th>System Suitability Parameters</th>
<th>Initial</th>
<th>Day-3</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>The USP Tailing factor for dolutegravir sodium, lamivudine, and</td>
<td>0.9</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td>tenofovir disoproxil fumarate from standard solution.</td>
<td>1.1</td>
<td>1.1</td>
<td>NMT 2.0</td>
</tr>
<tr>
<td>The USP plate count for dolutegravir sodium, lamivudine, and</td>
<td>8072</td>
<td>23957</td>
<td>32493</td>
</tr>
<tr>
<td>tenofovir disoproxil fumarate peak from standard solution.</td>
<td>32493</td>
<td>53</td>
<td>24444</td>
</tr>
<tr>
<td>% RSD for the peak areas of dolutegravir sodium, lamivudine, and</td>
<td>0.8</td>
<td>1.2</td>
<td>0.5</td>
</tr>
<tr>
<td>tenofovir disoproxil fumarate peak areas from six replicate</td>
<td>0.5</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td>injections of standard solution.</td>
<td></td>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NMT 5.0</td>
</tr>
</tbody>
</table>

Robustness

Similarly, robustness [24-28] also evaluated and found that the method is robust enough for various robustness parameters such as flow variation, column temperature variation and mobile phase composition variation. All the system suitability criteria is meeting in all the robust parameters, this indicates that the proposed analytical method is robust enough for the estimation of dolutegravir sodium, LAM and TDF by using the analytical method.

CONCLUSION

A simple, economic, accurate and precise HPLC method was successfully developed. In this method, it was carried out by using symmetry C18, (250 × 4.6 mm) with 5 µm particle size. Injection volume of 20 µl is injected and eluted with the mobile phase A as buffer of NH₄PO₄, pH 2.0 with dilute ortho phosphoric acid and buffer and acetonitrile as mobile phase B over gradient program, which is pumped at a flow rate of 1.0 ml/min. Detection, was carried out at 260 nm.
All the 3 compounds are well resolved from each peak and there is no interference from blank. The results obtained were accurate and reproducible. The method developed was statistically validated in terms of Selectivity, accuracy, linearity, precision, robustness, stability of solution and mobile phase stability.

For Selectivity, the chromatograms were recorded for standard and sample solutions of dolutegravir sodium, LAM and TDF. Selectivity studies reveal that the peak is well separated from each other. Therefore, the method is selective for the determination of dolutegravir sodium, LAM and TDF.

The LOD and LOQ was found to be for 0.017 µg/ml and 0.053 µg/ml respectively for dolutegravir sodium, 0.016 µg/ml and 0.048 µg/ml for LAM and 0.018 µg/ml and 0.054 µg/ml for tenofovir. The linearity results for dolutegravir sodium, LAM and TDF in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.95. Calibration curve was plotted and correlation co-efficient for dolutegravir sodium, LAM and TDF found to be more than 0.95.

The accuracy studies were shown as % recovery of dolutegravir sodium, LAM and TDF at 50%, 100% and 200%. The limit of % recovered shown is not less than 80% and the results obtained were found to be within the limits. Hence the method was found to be accurate. The accuracy studies showed % recovery of the dolutegravir sodium, LAM and TDF in the range 88-100% respectively.

For Precision studies 6 replicate injections were performed. %RSD was determined from the peak areas of dolutegravir sodium, LAM and TDF. The acceptance limit should be not more than 10%.

Hence, the chromatographic method developed for dolutegravir sodium, LAM and TDF are rapid, simple, sensitive, precise and accurate. Therefore, the proposed method can be successfully applied for the routine analysis of the active pharmaceutical ingredients for assurance of its presence in pharmaceutical effluents.

REFERENCES
