INTRODUCTION

Lamivudine, a reverse transcriptase inhibitor, is used in the treatment of HIV-1, HIV-2, and hepatitis B virus infections. It is a synthetic nucleoside analogue and is phosphorylated intracellularly to its active 5'-triphosphate metabolite, lamivudine triphosphate (LTP). This nucleoside analogue is incorporated into viral DNA by HIV reverse transcriptase into a cDNA chain termination 1, 2. Tenofovir disoproxil fumarate is a fumaric acid salt of the fumaric acid ester derivative of tenofovir. Its chemical name is tenofovir disoproxil fumarate and is 9-[R-(2R,5S,8R)-1-{[(isopropoxy)carbonyl]oxy}-3-methoxy(3-hydroxymethyl)propyl]adenine fumarate (1:1). Tenofovir disoproxil fumarate is a salt of an oral prodrug of tenofovir. Tenofovir disoproxil was developed to increase bioavailability because tenofovir was not well absorbed from the intestine. TDF is the first nucleotide analog approved for the treatment of HIV-1 3, 4. Nevirapine falls in the non-nucleoside reverse transcriptase inhibitor (NNRTI) class of antiretrovirals 5. Nevirapine chemically known as 11-cyclopropyl-4-methyl-5,11-dihydro-6H-dipyrido[3,2-b:2′,3′-e][1,4]diazepin-6-one. A literature survey reveals that analytical methods based on HPLC 6-25, HPTLC 26-28, UV Spectrometry 29-32 are available for the determination of these drugs individually and in combination with other drugs in different dosage forms. There is no analytical method reported for the simultaneous determination of Lamivudine, Tenofovir, and Nevirapine in a Combined Dosage Form. The aim of the present work is to develop a simple, precise, accurate, and rapid method for the determination of Lamivudine, Tenofovir DF, and Nevirapine in a combined extended release tablet dosage form.

MATERIALS AND METHODS

Chemicals and Reagents

All the reagents were of ACS or HPLC grade unless stated otherwise. Milli-Q-water was used throughout the experiment. Trifluoro acetic acid (Merck, Mumbai, India) Ammonium di hydrogen phosphate (Merck, Mumbai, India), Di ammonium hydrogen phosphate, Methanol (J.T. Baker, Germany) and acetonitrile (J.T. Baker, Germany), were used. Lamivudine, Tenofovir disoproxil fumarate, Nevirapine standards, related impurity standards and extended release tablet dosage form were obtained from Hetero Labs Ltd (Hyderabad, India). Lamivudine resolution mixture (B) from USP, USA.

Instrumentation

The HPLC system was composed of 2695 water alliance system fitted with 2996 PDA detector with Empower2 software. Analytical column used for this method is Phenomenex Kinetex C18, (100 mm x 4.6 mm) 2.6μm particle size.

Optimization of Chromatographic conditions

The mobile phase was 60:40 acetonitrile:water, pH adjusted to 2.8±0.05 with dilute trifluoro acetic acid. Mobile phase B consisting of mixture of methanol and acetonitrile in the ratio of 10:90 were pumped at a flow rate of 0.8 mL/min in gradient elution mode. Gradient time program as set as T%:B: 0/0, 5/0, 15/10, 45/45, 55/60, 60/70, 65/70, 66/0 and 70/0. Before delivering the mobile phase into the system, it was degassed and filtered through 0.22 μm PVDF filter using vacuum. The injection volume was 20μL and the detection was performed at 260 nm using a photo diode array (PDA) detector. Various compositions of solution A and solution B with different ion-pairing agents were tested for this study.
The typical retention times of Lamivudine, Nevirapine and Tenofovir are 11 minutes, 27 minutes and 38 minutes respectively. The counter ion fumaric acid is also found to be eluting at 2.3 minutes. The criticality of this method are to elute all the active as well as their related impurities with optimum separation and symmetric peak shapes. In this dosage form Tenofovir and Lamivudine are formulated into immediate release (each active ingredient 300mg per tablet) and Nevirapine as extended release (400mg per tablet). Use of tri fouro acetic acid as pH modifier in the preparation of diluents has got important role to achieve sharp peak shapes for Lamivudine related impurities, tenofovir impurities and resolution between lamivudine & diasteriomer of lamivudine as well as complete extraction of nevirapine from extended release part.

**Preparation of mobile phase A**

Transferred about 5.75 g of Ammonium dihydrogen phosphate and 6.6 g of di-ammonium hydrogen phosphate into a beaker containing 1000 mL water and mix. Adjusted the pH of the solution to 2.8±0.05 with 50% Trifluoroacetic acid. Filtered the solution through 0.22 µm membrane filter.

**Preparation of mobile phase B**

Prepared a degassed mixture of methanol and acetonitrile in the ratio of 10:90 (%v/v)

**Preparation of diluents**

Transferred about 1.15 g of Ammonium dihydrogen phosphate and 1.32 g of di-ammonium hydrogen phosphate into a beaker containing 1000 mL water and mix. Adjusted the pH of the solution to 2.0±0.05 with 50% Trifluoroacetic acid. Filtered the solution through 0.22 µm membrane filter.

**Resolution solution preparation**

Accurately weighed and transferred about 5mg of the Lamivudine resolution mixture (contains lamivudine and diastereomer of lamivudine) into a 10ml volumetric flask, added 5 ml of diluent and sonicated to dissolve. Diluted to volume with diluent and mixed.

**Standard solution Preparation**

Accurately weighed and transferred about 50 mg of each Lamivudine and Tenofovir disoproxil fumarate standard and 66mg of Nevirapine standard into a 200 ml volumetric flask, added 120ml of methanol and sonicated to dissolve and diluted to volume with methanol and mixed. Transferred 2.0 ml of above solution into a 200 ml volumetric flask, diluted to volume with diluent and mixed.

**Sample Preparation**

Ten tablets were separately weighed and grounded to fine powder. An amount equivalent to about 200mg of Nevirapine (powder contains 150mg each of lamivudine and tenofovir disoproxil fumarate) was transferred into a 100mL volumetric flask and sonicated in 60ml of diluent to extract all the actives and their related impurities completely. Made up the volume with diluent and mixed. A portion of the above solution was filtered through 0.22µm membrane filter and discarded first few mL of the filtrate.

**Table 1. System suitability Data**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution (R) between Lamivudine Diasteriomer and Lamivudine</td>
<td>2.4</td>
</tr>
<tr>
<td>Theoretical Plates (T)</td>
<td>Lamivudine: 5021 Tenofovir :12941 Nevirapine: 12062</td>
</tr>
<tr>
<td>% RSD</td>
<td>Lamivudine: 0.9 Tenofovir: 1.1 Nevirapine: 0.8</td>
</tr>
</tbody>
</table>

**Table 2. Forced degradation data**

<table>
<thead>
<tr>
<th>Stress condition</th>
<th>% Degradation observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamivudine</td>
<td>Nevirapine</td>
</tr>
<tr>
<td>Acid stress condition</td>
<td>0.3</td>
</tr>
<tr>
<td>Base stress condition</td>
<td>0.2</td>
</tr>
<tr>
<td>Oxidative stress condition</td>
<td>66.2</td>
</tr>
</tbody>
</table>

**Table 3. Linearity data**

**Table 4. Precision and Intermediate Precision data**

<table>
<thead>
<tr>
<th>Impurity Name</th>
<th>Relative retention time</th>
<th>Correlation coefficient (r)</th>
<th>Limit of Quantification (LOQ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamivudine carboxylic acid impurity</td>
<td>0.92 w.r.t lamivudine</td>
<td>0.998</td>
<td>0.033</td>
</tr>
<tr>
<td>Tenofovir mono ester impurity</td>
<td>0.40 w.r.t tenofovir</td>
<td>0.999</td>
<td>0.018</td>
</tr>
<tr>
<td>Tenofovir Dimer impurity</td>
<td>1.13 w.r.t tenofovir</td>
<td>0.997</td>
<td>0.031</td>
</tr>
<tr>
<td>Nevirapine related compound A</td>
<td>1.10 w.r.t nevirapine</td>
<td>0.999</td>
<td>0.021</td>
</tr>
<tr>
<td>Nevirapine related compound B</td>
<td>0.90 w.r.t nevirapine</td>
<td>0.999</td>
<td>0.023</td>
</tr>
</tbody>
</table>

**Table 5. Accuracy data**

<table>
<thead>
<tr>
<th>Impurity Name</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05% Level</td>
</tr>
<tr>
<td>Lamivudine carboxylic acid impurity</td>
<td>95.3</td>
</tr>
<tr>
<td>Tenofovir mono ester impurity</td>
<td>97.6</td>
</tr>
<tr>
<td>Tenofovir Dimer impurity</td>
<td>96.1</td>
</tr>
<tr>
<td>Nevirapine related compound A</td>
<td>97.3</td>
</tr>
<tr>
<td>Nevirapine related compound B</td>
<td>98.1</td>
</tr>
</tbody>
</table>

**Table 6. Robustness data**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Deliberate change</th>
<th>Resolution</th>
<th>Minimum theoretical plates</th>
<th>Maximum tailing factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate (0.8mL/min)</td>
<td>0.7mL/min</td>
<td>2.3</td>
<td>8380</td>
<td>14063</td>
</tr>
<tr>
<td></td>
<td>0.9mL/min</td>
<td>1.9</td>
<td>8302</td>
<td>13980</td>
</tr>
<tr>
<td>Temperature (35°C)</td>
<td>30°C</td>
<td>1.8</td>
<td>8255</td>
<td>14110</td>
</tr>
<tr>
<td></td>
<td>40°C</td>
<td>2.0</td>
<td>8510</td>
<td>14032</td>
</tr>
<tr>
<td>pH of buffer (2.8)</td>
<td>2.7</td>
<td>2.1</td>
<td>8798</td>
<td>14008</td>
</tr>
<tr>
<td></td>
<td>2.9</td>
<td>2.0</td>
<td>8610</td>
<td>14180</td>
</tr>
</tbody>
</table>

N= six sample preparations
¥ = Average of three determinations
Figure 1: Chemical structure of Lamivudine

Figure 2: Chemical structure of Tenofovir disoproxil fumarate

Figure 3: Chemical structure of Nevirapine

Figure 4: Typical Chromatogram of Blank

Figure 5: Typical Chromatogram of Resolution solution

Figure 6: Typical Chromatogram of Standard solution

Figure 7: Typical Chromatogram of Spiked sample solution

Figure 8: Typical Chromatogram of Acid stress condition

Figure 9: Typical Chromatogram of Base stress condition

Figure 10: Typical Chromatogram of peroxide stress condition
RESULTS AND DISCUSSION

Optimum separation between lamivudine diasteromer & lamivudine, all potential degradation impurities of tenofovir and nevirapine was achieved with the optimized conditions. Above proposed method was validated as per ICH guidelines and current industrial practices, results are presented.

Method validation

The aim of method validation was to confirm that the present method was suitable for its intended purpose as described in ICH guidelines Q2(R1).31 The described method has been extensively validated in terms of specificity, precision, linearity, accuracy, limit of detection (LOD) and quantification (LOQ), and robustness. The precision was expressed with respect to the intra- and inter-day variation in the expected drug concentrations. The accuracy was expressed in terms of percent recovery of the known amount of impurities added to the sample preparation.

System suitability

System suitability tests are an integral part of a liquid chromatographic method, and they were used to verify that the proposed method was able to produce good resolution between the peaks of interest with high reproducibility. The system suitability was determined by injecting resolution solution and six replicate injections from freshly prepared standard solutions and analyzing each solute for their peak area, theoretical plates (N), resolution (R) and tailing factors (T). System suitability requirements for the proposed method are (i) the resolution (R) between Lamivudine dia stereomer and Lamivudine should not be less than 2.0, from resolution solution (ii) the theoretical pates (T) should not be less than 3000 for all peaks from standard solution, (iii) the % of RSD for peak areas of Lamivudine, Tenofovir DF and Nevirapine peaks from replicate injections of standard solutions is not more than 5.0. The results of the system suitability test in comparison with the required limits are shown in Table 1. According to the results presented, the proposed method fulfills these requirements within the accepted limits.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Specificity was tested by injecting the sample by spiking with appropriate levels of impurities and demonstrating the separation of these impurities individually and/or from other components in the sample matrix. Moreover, identification of each impurity was confirmed with relative retention times as compared with those of pure standards. Results are presented in Table 1.

Forced degradation studies

Forced degradation studies were performed to provide an indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted to stress conditions like acid hydrolysis (using 1 N HCl), base hydrolysis (using 1 N NaOH), and oxidative degradation (using 3.0% H2O2) to evaluate the ability of the proposed method to separate degradation products from each other and active ingredients as well. To check and ensure the homogeneity (peak purity) of all peaks in the stressed sample solutions, photo diode array detector was employed. In forced degradation it was observed that tenofovir is susceptible for degradation in acid and base stress conditions, where as lamivudine susceptible for peroxide stress condition and nevirapine is found to be stable in all the three stress conditions. Results are tabulated in Table 2.

Linearity

The linearity of the method was tested in order to demonstrate proportional relationship of response versus analyte concentration over the working range. It is usual practice to perform linearity experiments over a wide range of analyte. This gives confidence that the response and concentration are proportional and consequently ensures that calculations can be performed using a single reference standard/working standard, rather than the equation of a calibration line. The linearity of detector response to different concentrations of impurities was studied by preparing a series of solutions using Lamivudine, Tenofovir disoproxil fumarate and their related substances at five different concentration levels ranging from 0.05% to 0.50% of test concentration of respective active substance. The data were subjected to statistical analysis using a linear-regression model; the regression equations and coefficients (r2) are given in Table 3. The results have indicated good linearity.

Limit of detection and quantification

The Limit of detection (LOD) and Limit of quantification (LOQ) are established represent the concentration of the analyte that would yield signal-to-noise ratios of 3 for LOD and 10 for LOQ respectively. LOD and LOQ were determined by measuring the magnitude of analytical background by injecting blank samples and calculating the signal-to-noise ratio for each compound by injecting a series of solutions until the S/N ratio 3 for LOD and 10 for LOQ. The results are given in Table 3.

Precision

Six sample solutions were prepared using single sample Lot of tablet dosage form by spiking with 0.2% w/w of each impurity and the precision of the method was tested. The % RSD indicate that proposed method has got acceptable level of repeatability. The results are given in Table 4.

Ruggedness (Intermediate precision)

Ruggedness is the intraday variation obtained at different concentration levels, and is expressed in terms of RSD calculated for each day. The RSD values were found to be below 3.8% (for all impurities). The intermediate precision is the interday variations calculated for six sample preparations in each set expressed in terms of % RSD values. Results indicate the proposed method has got a good intermediate precision. The ruggedness of the method was determined by analyzing the same samples in triplicate for 2 days by another instrument by a different analyst with different lots of reagents and columns. The results are given in Table 4.

Accuracy

Accuracy of the proposed method was established by recovery experiments. This study was employed by spiking of known amounts of related compounds into the sample solution ranging from 0.05%-0.3% for test concentration, in triplicate and injected into the chromatographic system. The resulting mixtures were analyzed as described in proposed method. Results obtained from recovery studies are given in Table 5.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate, variations in method parameters, and provides an indication of its reliability during normal usage. In the present study, an experimental design was planned for robustness testing varying some conditions, e.g. Flow rate, column temperature, pH of the buffer and filter variability. The results are shown in Table 6. Method was found sensitive to pH of the buffer...
solution as well as diluent. Based on the results we recommend a tighter limit for pH variation i.e. ±0.1 pH units. It can be seen that, with every employed condition, there were no dramatic changes in the chromatographic behavior of impurities. All parameters have been observed within the limits required for system suitability tests.

**Stability of Analytical solutions**

The stability of the resolution, standard and sample solutions are tested at regular intervals. The stability of solutions was determined by comparing results with freshly prepared standard solutions. The differences in values were within 0.05% for known and unknown impurities and 0.2% for total impurities up to 48hrs.

**CONCLUSION**

The validated stability-indicating HPLC method has proved to be simple, accurate, precise and reliable. The proposed method provides a good resolution between all the impurities and potential degradation impurities. The developed method reported herein was validated by evaluation of the validation parameters as described in ICH guidelines. System suitability, specificity, linearity, LOD, LOQ values, precision, accuracy and robustness of the proposed technique were obtained during the validation studies. The developed method is also stability-indicating and can be used for the routine analysis of combined extended release tablet dosage form of lamivudine, tenofovir disoproxil fumarate and nevirapine also to check the purity and shelf life stability in extended release tablet dosage forms.

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**REFERENCES**

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