UV Spectrophotometric Method Development and Validation for the Simultaneous Estimation of Efavirenz, Emtricitabine and Tenofovir Disoproxil Fumarate in Marketed Formulation

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INTRODUCTION

In 2008, around 33.4 million people were infected with HIV and there were around 2 million deaths in the same year [1]. Atripla, a combination of a fixed dose of tenofovir, emtricitabine, and efavirenz was approved for the treatment of this disease by the Food and Drug Administration (FDA) on July 12, 2006. In the United States, Atripla was the first fixed dose formulation available to combine two distinct groups of antiviral drugs into a single tablet. Also available are several generic Atripla drugs, such as Viraday from Cipla Ltd. and Vonavir from Emcure Ltd. Efavirenz (EFV, brand names Sustiva and Stocrin) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) and is used as part of highly active anti-retroviral therapy (HAART) for the treatment of a human immune deficiency virus (HIV) type 1. Efavirenz is chemically described as (S)-6-chloro-(cyclopropylmethylnyl)-1, 4-dihydro-4-(trifluoromethyl)-2H-3, 1-benzoxazin-2-one. Its empirical formula is C14H9ClF3NO2. Efavirenz is a white to slightly pink crystalline powder with a molecular mass of 315.68 g/mol. It is practically insoluble in water (<10 μg/mL) [2]. Emtricitabine (ETB) is a nucleoside reverse transcriptase inhibitor (NRTIs). Chemically it is 5-fluoro-1-(2R, 5S)-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine (Figure 1). FTC is the (-) enantiomer of this analog of cytidine which differs from other cytidine analogs, in that it has fluorine in 5th position. FTC is an antiviral agent used for the prevention of perinatal HIV-1 reverse transcriptase [3]. It is also active against Hepatitis B virus [4, 5]. Tenofovir disoproxil fumarate (a produg of tenofovir), marketed by Gilead Sciences under the trade name Viread, belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors [6] (nRTIs), which block reverse transcriptase, an enzyme crucial to viral production in HIVinfected people. In vivo tenofovir disoproxil fumarate is converted to tenofovir, an acyclic nucleoside phosphorylase (nucleotide) analog of adenosine 5’-monophosphate. IUPAC: is([(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy)methyl) phosphonic acid. Tenofovir belongs to a class of...
antiretroviral drugs known as nucleotide [7] analogue reverse transcriptase inhibitors (NtRTIs), which block reverse transcriptase, an enzyme crucial to viral production in HIV-infected people. Tenofovir is currently in late stage clinical trials for the treatment of hepatitis B. Tenofovir disoproxil fumarate is an acyclic nucleoside phosphonatediester analog of adenosine monophosphate. Tenofovir inhibits the activity of HIV reverse transcriptase by competing with the natural substrate deoxyadenosine 5’-triphosphate and, after incorporation into DNA, by DNA chain termination. Specifically, the drugs are analogues of the naturally occurring deoxynucleotides needed to synthesize the viral DNA and they compete with the natural deoxynucleotides [8] for incorporation into the growing viral DNA chain.

Various spectrophotometric, [9-12] HPLC, [13-22] HPTLC [23] methods are reported in the literature for the estimation of EFV individually and in combination with other drugs. However, no spectrophotometric method has yet been reported for simultaneous estimation of EFV, EMT, and TDF in pharmaceutical dosage forms. The methods mentioned in the literature, especially the chromatographic techniques, are time-consuming, costly, and require expertise. A simple and accurate UV spectrophotometric method developed can be highly useful for the routine analysis of synthetic mixture formulations. Hence, an attempt has been made to develop and validate in accordance with ICH guidelines [24-25].

**EXPERIMENTAL WORK**

**Reagents and chemicals**
Reference standard of EFV, EMT and TDF was a generous gift from Pharmaceutical Company. Methanol, HCl was procured from Rankem, RFCL Limited, New Delhi, India. NaOH was procured from Hi-media laboratory Pvt. Ltd. All solvents and reagents were of analytical grade. All the solutions were protected for light and were analyzed on the day of preparations. Triple distilled water was generated in the house. Distilled water was obtained by Mili Q apparatus by Millipore (Millford, USA) for whole experimental work.

**Instrument**
In UV-spectrophotometric method, Labindia model-3000+ series were used, which is a wavelength accuracy ±1 nm, with 1cm quartz cells.

**Method development**

**Linearity range and calibration graph**

**Preparation of Standard Stock Solution (Stock-A)**
Standard stock solutions were prepared by dissolving separately 100 mg of each drug in 50mL 0.1 N NaOH in 100 ml volumetric flask. The flask was sonicated for about 10 min to solubilize the drug and the volume was made up to the mark 100ml with 0.1 N NaOH to get a concentration of 1000 µg/ml (Stock-A) for both drugs.

**Preparation of Sub Stock Solution (Stock-B)**
Aliquots of 2.5 ml withdrawn with help of pipette from standard stock solution A of EFV, EMT and TDF and transferred into 25 ml volumetric flask separately and diluted up to 25 ml with 0.1 N NaOH that gave concentration of 100 µg/ml (Stock-B).

**Preparation of Working Standard Solution**

1) 0.1 ml, 0.2 ml, 0.3 ml, 0.4 ml and 0.5 ml from sub stock solution (Stock-B) were taken separately in 10 ml volumetric flask and volume was made up to 10 ml with 0.1 N NaOH. This gave the solutions of 10µg/ml, 20µg/ml, 30µg/ml, 40µg/ml and 50µg/ml respectively for EFV.

2) Aliquots of 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml and 2.5 ml withdrawn with help of pipette from standard stock solution (Stock-B) separately in 10 ml
volumetric flask and volume was made up to 10ml with 0.1 0.1 N NaOH. This gave the solutions of 5μg/ml, 10μg/ml, 15μg/ml, 20μg/ml and 25μg/ml respectively for EMT.

3) Aliquots of 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml and 2.5 ml withdrawn with help of pipette from standard stock solution (Stock-B) separately in 10 ml volumetric flask and volume was made up to 10ml with 0.1 0.1 N NaOH. This gave the solutions of 5μg/ml, 10μg/ml, 15μg/ml, 20μg/ml and 25μg/ml respectively for TDF.

**Selection of wavelength for linearity**

Solutions of 10 μg/ml of EFV, 10μg/ml EMT and 10μg/ml TDF were prepared separately. The solutions were scanned in the spectrum mode from 200 nm to 400 nm. The maximum absorbance of EFV, EMT and TDF was observed at 240.0 nm, 256.0nm and 316.0 nm, respectively. EFV showed linearity in the concentration range of 10-50 μg/ml and EMT and TDF showed linearity 5-25μg/ml at their respective maxima. Calibration curve was plotted, absorbance versus concentration.

**Study of Overlay Spectra**

Working standard solution from the standard stock solution prepared in concentration 10μg/ml of EFV, 10μg/ml of EMT and 10μg/ml of TDF were scanned in the spectrum mode over the range of 200-400 nm against 0.1 N NaOH as blank and the overlain spectra of the two were recorded. EFV showed an absorbance peak at 240.0 nm, whereas EMT at 256.0 nm
and TDF at 316 nm. The overlain spectra also showed isoabsorptive points at 250.00 nm. Due to difference in absorbance maxima and having no interference with each other so both drug can be simultaneously estimated by simultaneous equation method.

Simultaneous equation method is based on the absorption of drugs (X, Y, and Z) at the wavelength maximum of the other. Three wavelengths selected for the method are 240.0 nm, 256nm and 316nm that are $\lambda_{\text{max}}$ of EFV, EMT and TDF respectively. The absorbances were measured at the selected wavelengths and absorptivities (A₁% 1cm) for both the drugs at both wavelengths were determined as mean of five independent determinations. Concentrations in the sample were obtained by using following equations.

$$
CX = \frac{(A_1(ay2az3 - az2ay3) - ay1(A2az3 - az2A3) + az1(A2ay3 - ay2A3))}{ax1(ay2az3 - az2ay3) - ay1(ax2az3 - az2ax3) + az1(ax2ay3 - ay2ax3)}
$$

$$
CY = \frac{(ax1(A2az3 - az2A3) - A1(ax2az3 - az2ax3) + az1(ax2A3 - A2ax3))}{ax1(ay2az3 - az2ay3) - ay1(ax2az3 - az2ax3) + az1(ax2ay3 - ay2ax3)}
$$

$$
CZ = \frac{(ax1(ay2A3 - A2ay3) - ay1(ax2A3 - A2ax3) + A1(ax2ay3 - ay2ax3))}{ax1(ay2az3 - az2ay3) - ay1(ax2az3 - az2ax3) + az1(ax2ay3 - ay2ax3)}
$$

Where, A₁, A₂ and A₃ are absorbances of mixture at 240.0 nm, 256nm and 316nm respectively, ax1, ax2 and ax3 are the absorptive of EFV at 240, 256 and 316 nm respectively, ay1, ay2 and ay3 are the absorptive of EMT at 256, 240 and 316 nm respectively, az1, az2 and az3 are the absorptive of TDF at 316, 256 and 240 nm respectively.

Validation of simultaneous equation method

Linearity

Linearity of both drugs was established by response ratios of drugs. Response ratio of drug is calculated by dividing the absorbance with respective concentration. Then a graph was plotted between concentration and response ratio table 1.

Accuracy

The accuracy of the proposed methods was assessed by recovery studies at three different levels i.e. 80%, 100%, 120%. The recovery studies were carried out by adding known amount of standard solution of EFV, EMT and TDF to pre-analysed Capsule solutions. The resulting solutions were then re-analysed by proposed methods. Whole analysis procedure was repeated to find out the recovery of the added drug.

Mixed Standard Study

Capsules of EFV, EMT and TDF combination are available in 600:200: 240mg. Mixed standard are prepared in the ratio of 60:20:24 from standard sub stock solution of 100μg/ml in 3 replicate of 5 concentrations. Solutions containing known concentration of two drugs are considered as laboratory samples (mix standards) to check the results of developed method.
sample. This recovery analysis was repeated at 3 replicate of 5 concentrations levels table 2.

**Precision**

Precision of the methods was studied at three level as at repeatability, intermediate precision (Day to Day and analyst to analyst) and reproducibility. Repeatability was performed by analyzing same concentration of drugs for five times. Day to day was performed by analyzing 5 different concentration of the drug for three days in a week table 3.

**Analysis of tablet Formulation**

Twenty tablets were taken and determine the average weight, tablets ground to a fine powder; amount equal to 60mg of EFV (20mg EMT and 24 mg TDF) was taken in 10 ml volumetric flask. Then 5ml of 0.1 N NaOH was added and the flask was sonicated for about 10 min to solubilize the drug present in capsule powder and the volume was made up to the mark with 0.1 0.1 N NaOH. After sonication filtration was done through Whatman filter paper No. 41. Filtrate was collected and further diluted with 0.1 N NaOH to get the final concentrations of all three drugs in the working range. The absorbance of final dilutions were observed at selected wavelengths and the concentrations were obtained from simultaneous equation method. The procedure was repeated for five times table 4.

**DISCUSSION**

The linearity of analytical method was carried out to check its ability to elicit test results that are proportional to the concentration of analyte in sample within a given range. Different levels of standard solutions were prepared and estimate into the UV and the results was recorded. The validity and reliability of proposed methods were assessed by recovery studies. The recovery of added standards (80%, 100% and 120%) was found at three replicate and three concentrations level. The value of % means just close to 100, SD and % RSD are less than 2 indicate the

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Table-1: Results of linearity of Efavirenz (EFV), Emtricitabine (EMT) and Tenofovir disoproxil fumarate (TDF)

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>EFV</th>
<th>EMT</th>
<th>TDF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (μg/ml)</td>
<td>10-50</td>
<td>5-25</td>
<td>5-25</td>
</tr>
<tr>
<td>Correlation Coefficient (r²)*</td>
<td>0.999</td>
<td>0.999</td>
<td>0.999</td>
</tr>
<tr>
<td>Slope (m)*</td>
<td>0.011</td>
<td>0.023</td>
<td>0.016</td>
</tr>
<tr>
<td>Intercept (c)*</td>
<td>0.005</td>
<td>0.003</td>
<td>0.002</td>
</tr>
</tbody>
</table>

*Value of three replicate

Table-2: Results of recovery study

<table>
<thead>
<tr>
<th>% Level</th>
<th>% MEAN±SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EFV</td>
</tr>
<tr>
<td>80%</td>
<td>99.54±0.314</td>
</tr>
<tr>
<td>100%</td>
<td>99.38±0.203</td>
</tr>
<tr>
<td>120%</td>
<td>99.35±0.543</td>
</tr>
</tbody>
</table>

* Value of three replicate and five concentrations.

Table-3: Results of precision

<table>
<thead>
<tr>
<th>Parameter</th>
<th>% MEAN±SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EFV</td>
</tr>
<tr>
<td>Repeatability</td>
<td>99.61±0.076</td>
</tr>
<tr>
<td>Intermediate precision</td>
<td></td>
</tr>
<tr>
<td>Day to day precision</td>
<td>99.44±0.124</td>
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<tr>
<td>Analyst-to-Analyst</td>
<td>99.70±0.079</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>99.63±0.086</td>
</tr>
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</table>

* Value of five replicate and five concentrations

Table-4: Assay of tablets formulation

<table>
<thead>
<tr>
<th>% Conc. Found</th>
<th>EFV</th>
<th>EMT</th>
<th>TDF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate 1</td>
<td>99.79</td>
<td>99.5</td>
<td>99.48</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>99.65</td>
<td>99.27</td>
<td>99.49</td>
</tr>
<tr>
<td>Average</td>
<td>99.72</td>
<td>99.38</td>
<td>99.48</td>
</tr>
<tr>
<td>S. D.</td>
<td>0.099</td>
<td>0.163</td>
<td>0.007</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.099</td>
<td>0.164</td>
<td>0.007</td>
</tr>
</tbody>
</table>

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accuracy of method. Precision was determined by repeatability and Intermediate precision of drug. Repeatability result indicates the precision under the same operating condition over short interval time. The intermediate precision study is expressed within laboratory variation on different days and analyst by analyst variation by different analyst. The value of SD and %RSD are less then 2 indicate the precision of method. The results of the analysis of tablets formulation were reported. The assay value of drugs was close to 100, SD and % RSD are less than 2 indicate the no interference of excipient in the estimation of drugs.

CONCLUSION
The simultaneous equation method (Vierordt’s method) has been successfully applied for simultaneous determination of EFV, EMT, and TDF in combined sample solution, and they were found to be accurate, simple, rapid, and precise. Once the equations were constructed, analysis required only measuring the absorbance values of the sample solution at the selected wavelengths followed by few simple calculations. The proposed method was completely validated showing satisfactory data for all the method validation parameters tested. Simultaneous equation method comparably noted to be very efficient in every aspect. Unlike HPLC, by using simultaneous equation method (UV) the data’s can be generated applying simple calculations. So these methods can be easily and conveniently adopted for routine quality control analysis of these cited drugs.

REFERENCES

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