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Research Paper

ANALYTICAL METHOD ANDVALIDATIONFOR THE SIMULTANEOUS DETERMINATION OF EMTRICITABINE, TENOFOVIR AND EFAVIRENZBY RP-HPLC METHOD

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A simple and new isocratic RP-HPLC method was developed and validated for the estimation of Emtricitabine, Tenofovir and Efavirenz in pharmaceutical dosage form. The chromatographic separation was performed on Waters Spherisorb column (150×4.6mm, 5µm), mobile phase used for the analysis was prepared by the combination of 65 parts of methanol and 35 parts of 0.1% orthophosphoric acid to prepare 65: 35(v/v) mixture. The run time for the separation was fixed at 10 min and the flow rate was maintained at 0.9 ml/min with the detection wave length of 260 nm. The column temperature was maintained at 25^0C ±5and performed the HPLC analysis. The retention times found to be 1.8 min, 2.6 min and 8.2 min for Emtricitabine, Tenofovir and Efavirenz respectively. Under these optimized conditions the respective drugs were shown symmetrical peaks with low tailing factor and high peak area without interference of any excipients.

Keywords: Emtricitabine, Tenofovir, Efavirenz, High Performance Liquid Chromatography, Validation.

INTRODUCTION

FTC is chemically 4-amino-5-fluoro-1-[(2R,5S)-2-(hy-droxymethyl)-1,3-oxathiolan-5-yl] pyrimi din-2-one. It is nucleoside reverse transcriptase inhibitor. FTC is an analogue of cytidine. It works by inhibiting the reverse transcriptase enzyme which copies the HIV RNA into viral DNA. So that it can help to lower the amount of viral load in patient's body. It leads to increase the immune system cells.[1-2]

TFV is chemically [(2R)-1-(6-aminopurine-9-yl)propan-2-yl] oxymethyl phosphonic acid. It is an acyclic analogue of deoxyadenosine 5'-monophosphate (d-AMP). That lacks a hydroxyl group in the position corresponding to the 3' carbon of the d-AMP, preventing the formation of the 5' to 3' phosphodiester linkage, which is essential for DNA chain elongation. It causes premature termination of DNA transcription, preventing viral replication.[3] EFV is chemically (4S) 6 –_chloro–4 (2–cyclopropylethynyl)–4 (trifluoro methyl) – 1 – H – 3,1 – benzoxazin-2 one.[4-5]

EFV is a non-nucleoside reverse transcriptase inhibitor. It directly binds to the HIV– 1 RT and RNA dependent DNA polymerase, blocking its function to DNA replication. It leads to reduce HIV viral load, retarding damage to the immune system and reduce the risk of developing AIDS. Combination of these three drugs is to target the HIV reverse transcriptase protein in three ways, which reduces the virus capacity to mutate. In combination of three drugs synergic

antiviral effects observed.[6-7]

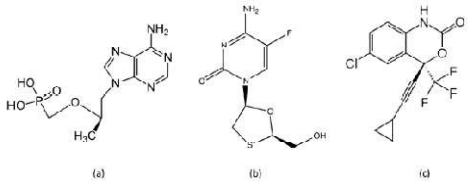

Fig.1: Chemical structure of (a) Tenofovir, (b) Emtricitabine and (c) Efavirenz

EXPERIMENTAL

Chemicals and Reagents:

Methanol (HPLC grade, Merck Ltd), Milli-Q water, Emtricitabine, tenofovir and Efavirenz (Reference standard purchased from Cipla), Ortho phosphoric acid (GR Grade, SD Fine Chem. Ltd).

Instrument:

The chromatographic separation was carried out on an LC – 10 – ATVP HPLC system (Shimadzu class VP).

HPLC separation was tested by using Waters Spherisorb C18 column (250 X 4.6 mm id, ODS 2, 5 µm).[8] The mobile phaseconsists the mixture of 65: 35 % (v/v) methanol and 0.1% ortho phosphoric acid (pH adjusted to 3.0 withacetic acid) operated on isocratic mode and it was filtered through 0.45 micro membrane. The flow rate is 0.9ml/min with detection wavelength of 260 nm at 25ºC and the injection volume is 20 µL.

Drug Standard Stock Solution:

Stock solutions of Emtricitabine, Tenofovir and Efavirenz (5 mg/mL) were prepared separately in a volumetric flask and labeled accordingly

Suitable dilutions of Emtricitabine, Tenofovir and Efavirenz were prepared using 50:50% (v/v) Methanol & Milli-Q water as diluent Solution. A Linear Calibration curve containing 6 non-zero standards were prepared using diluent solution in the concentration range of 7.57 - 50.5 µg/mL, 7.58 - 50.53 µg/mL and 14.93 - 99.5 µg/mL for Emtricitabine, Tenofovir and Efavirenz respectively.

Preparation of sample Solution:

In order to prepare drug sample solutions 20 gm of each drug sample is weighed into a mortar separately and crushed into fine powder with pestle. Now it is transferred into 100 mL of volumetric flasks, diluents are added slowly to make a homogenous solution of drug sample. Now it is passed through 0.45 µm nylon syringe fitter. For the quality control sample preparations, a separate stock was prepared which contains approximately the same concentration of the drug substance and labeled as quality control stock. From the stock, quality control samples were prepared at three concentration levels namely LQC (12.62, 2.63 and 24.88 µg/mL for Emtricitabine, Tenofovir and Efavirenz), MQC (25.25, 25.26 and 49.75 µg/mL), and HQC (37.87, 37.90 and 74.63 µg/mL) so as to obtain low, median and high concentration quality control samples.

Optimized chromatographic conditions:

Chromatographic separations were carried out by using the column Water's Spherisorb 250 ×

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4.6 mm id 5 µm, at room temperature (25±1ºC).Mobile phase is taken as a combination of 65 parts of Methanol and 35 parts of 0.1% orthophosphoric acid (65:35).

The chromatograms were observed, recorded and the peaks were quantified using an automatic integrator. In this method, 0.9 mL/min flow rate was used for the separations with detection at wavelength of 260 nm and the run time fixed at 10 min. The injection volume was 20 µL. The conditions optimized were given in Figure 2.

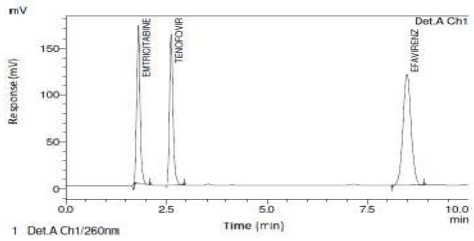

Fig. 2: Chromatogram showing the separation of drugs

RESULTS AND DISCUSSION

Specificity:

Specificity can be determined by comparing the chromatograms obtained from the drugs with the chromatogram obtained from the blank solution. Blank solution was prepared by mixing the excipients in the mobile phase without drug. Drug solutions were prepared individually and the sample containing three drugs was also prepared. Now these mixtures were filtered by passing through 0.45 µmembrane filter before the analysis. In this observation no excipient peaks were obtained near the drug in the study run time. This indicates that the proposed method was specific.

Linearity:

Linearity studies were accomplished for the quantitative determinations of the drugs by the external standard method. Linearity of an analytical method is to verify the test results that are proportional to the concentrations of analyte in a sample in the given range. The column temperature is maintained at 23±1^{0}C throughout the study. The linearity study can be initiated by preparing the serial dilution of the drugs from the corresponding stock, performed the analysis and calibrated the linearity curve of the drugs. From the linear regression equation of the plots, the parameter concentration is evaluated.

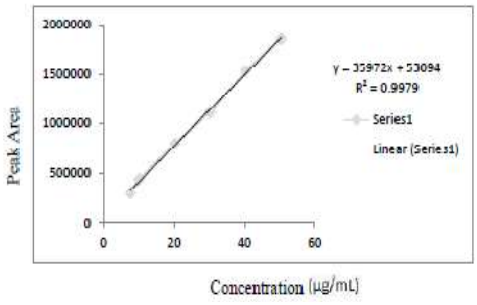

Fig. 3: Linearity curve of Emtricitabine

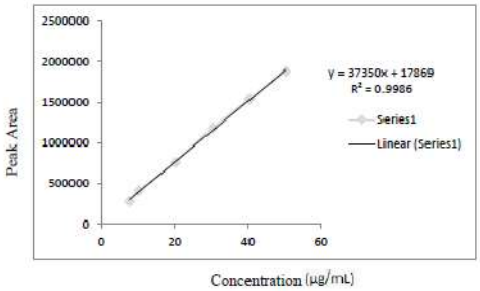

Fig. 4 :Linearity curve of Tenofovir

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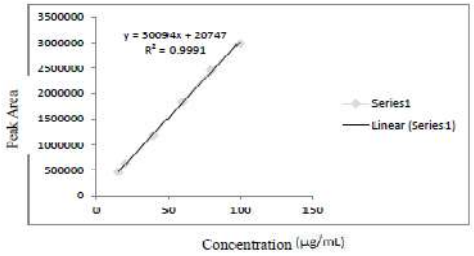

Fig. 5 :Linearity curve of Efavirenz

Accuracy:

Accuracy of the method is the degree of closeness of the measured value with the actual value and it is expressed in terms of % of recovery. An adequate amount of tablet powder of the drugs is weighed accurately, transferred in to a 100 mL of volumetric flask, 30 mL of diluent is added to the flask, mixing is carried out vigoursly and then made up the mark with diluent. Recovery study is carried out by spiking concentration of the pure analyte 80%, 100% and 120% of concentrations and then it is added to predetermined working standard solution of the drug. Performed the analysis for these samples in triplicate at each level as per the proposed method. Standard deviation, relative standard deviation and percentage of recovery values were calculated. By this method percentage of recovery is observed to be ranging from 98.00-102.00% which indicates its accuracy.

Table 1: Actual mobile phase (87.5:32.5) results (Robustness)

	Emtricitabine		Tenofovir		Efavirenz	
Sr. No.	**Retention Time**	**Peak Area**	**Retention Time**	**Peak Area**	**Retention Time**	**Peak Area**
1	1.82	1566570	2.63	1570440	8.43	2510603
2	1.84	1563882	2.65	1540535	8.44	2502803
3	1.83	1535528	2.63	1582210	8.44	2530228
Mean	1.83	1555327	2.64	1564395	8.44	2514545
Std. Dev.	0.01	17199	0.01	21485	0.01	14131
% CV	0.55	1.11	0.44	1.37	0.07	0.56

Table 2: mobile phase variation (30:70) results (Robustness)

	Emtricitabine		Tenofovir		Efavirenz	
Sr. No.	**Retention Time**	**Peak Area**	**Retention Time**	**Peak Area**	**Retention Time**	**Peak Area**
1	1.83	1635512	2.64	1573230	8.41	2428625
2	1.82	1722882	2.65	1540535	8.43	2715803
3	1.83	1535528	2.63	1690210	8.44	2428231
Mean	1.83	1631307	2.64	1601325	8.43	2524220
Std. Dev.	0.01	93748	0.01	78693	0.02	165916
% CV	0.32	5.75	0.38	4.91	0.18	6.57

Robustness:

Statistical analysis showed no significant difference betweenresults obtained employing the analytical conditions establishedfor the method and those obtained in the experimentsin which variations of some parameters were introduced. Thus,the method showed to be robust for changes in mobile phase variations.

CONCLUSION:

This method was validated on the report of precision, accuracy, specificity, linearity. In all the casesthe method was stable with acceptance criteria followed by ICH guidelines. Mobile phase used in this method was very commonly available and it is sufficient for the quantification analysis of Emtricitabine, Tenofovir and Efavirenz either in single dosage or in combined form of formulations in many pharmaceutical laboratories. These drugs were separated in less than 10 min with low tailing factor and good resolution without any interference of excipients. This demonstrates the proposed method was simple, fast, accurate, specific with good retention time, and low cost. Thus the developed method is suitable for the routine quality control analysis of the drugs in bulk and tablet dosage form.

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Conflict of Interest

The authors declare that they have no conflict of interest