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# Development and validation of UPLC method for emtricitabine, tenofovir and efavirenz in pharmaceutical prepartion

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## ABSTRACT

A simple, sensitive and validated UPLC method has been developed to determine Emtricitabine, Tenofovir and Efavirenz simultaneously in synthetic mixture form. Chromatographic separation was achieved on a BEH Phenyl column using a mixture of buffer pH 6.5, Methanol and Acetonitrile in the ratio of 45:27.5:27.5 (v/v) at a wavelength of 260nm. Linearity of the method was found to be in the concentration range of 0.026-0.079μg/ml for Emtricitabine and 0.024-0.088μg/ml for Tenofovir and 0.08-0.24μg/ml Efavirenz with correlation coefficient greater than 0.999. The total eluting time for the three components is less than 1.5 minutes. The method can be used for simultaneous determination of Emtricitabine, Tenofovir and Efavirenz.



## KEYWORDS

UPLC;
Methanol;
Acetonitrile;
Emtricitabine;
Tenofovir;
Efavirenz.

## INTRODUCTION

Tenofovir disproxil fumerate, emtricitabine and efavirenz[1-3] are a novel formulation combining fixed doses of the nucleoside reverse transcriptase inhibitors emtricitabine (200mg) and tenofovir disproxil fumerate fumarate (300mg) with the non-nucleoside reverse transcriptase inhibitor efavirenz (600mg) represents the first once daily, one-tablet antiretroviral regimen[10]. Tenofovir disproxil fumerate fumerate is chemically know as 9-[(R)-2-[[bis [[isopropoxycarbonyl] oxy] methoxy] phosphonyl] methoxy] popyl] adenine Fumarate. Emtricitabine is chemically 5-fluoro-1-(2R, 5S)-[2-hydroxymethyl)-1, 3-oxathiolan-5-ylcytosine5. Efavirenz[4] is (4S)-6-chloro-4- (cyclopropylethynyl)-1, 4-dihydro-4-(trifluoromethyl) 2-H-3, 1-benzoxazin2-one. Literature survey reveals few Chromatographic methods[11-13] for the determination of tenofovir disproxil fumerate, emtricitabine and efavirenz, in biological fluids along with other antiretroviral dugs. So far, only one HPLC procedure has been reported in gradient mode for the estimation of Tenofovir disproxil.

Fumerate, Emtricitabine and Efavirenz from pharmaceutical dosage form[14]. The availability of an HPLC method with high sensitivity and selectivity will be very useful for the determination of tenofovir.

Disproxil fumerate, emtricitabine and efavirenz in pharmaceutical formulations. The aim of the study was to develop a simple, precise and accurate reversed-phase UPLC method in isocratic mode for the estimation of tenofovir disproxil fumerate, emtricitabine and efavirenz in bulk drug samples and in pharmaceutical dosage forms.

We describe in this paper a simple, sensitive and validated UPLC method with total run time less than Five minutes for the simultaneous determination of

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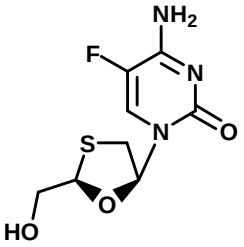

Figure 1 : Emtricitabine

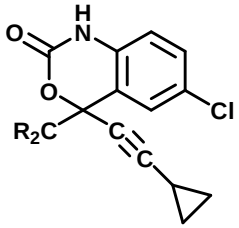

Figure 3 : Efavirenz

emtricitabine, efavirenz and tenofovir disoproxil fumarate the developed method can be applied successfully for quality control and for other analytical purposes.

## MATERIAL AND METHODS

### Chemicals and reagents

Emtricitabine, Efavirenz and Tenofovir disoproxil fumarate reference substances with claimed purity of 99.70%, 99.79% and 99.65% respectively were taken from Precise Pharmaceuticals. Methanol and Acetonitrile (HPLC grade), Triethyl amine and orthophosphoric acid (analytical reagent grade) were purchased from Merck (Mumbai). All excepients used were of pharmaceutical grade. Water for injection was used throughout the experiment. Mobile phase was filtered using 0.2µm cellulose acetate filters made by Millipore (USA) whereas; Syringe filter 0.2µm (cellulose acetate purchased from the local market) were used in the preparation of sample solution.

### Apparatus and chromatographic conditions

UPLC apparatus consisting of Water's UPLC system (Aquity system) equipped with a Binary solvent manager model UPB, UPD PDA detector (set at 260nm), and thermostat column compartment Interface module with Empower-2 UPLC software was used for development and evaluation of this method. Acquity UPLC BEH Phenyl 1.7µm 2.1 × 50mm column was selected. The Solution -A is 0.2% TEA in water pH 6.5 ± 0.05 with Orthophosphoric acid and Solution-B

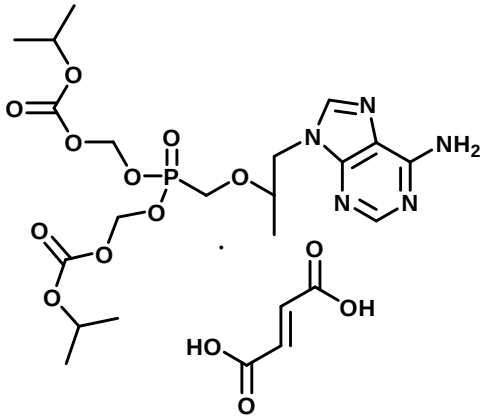

Figure 2 : Tenofovir disoproxil fumarate

Methanol and acetonitrile in the ratio of 50: 50 (v/v). The mobile phase was composed of Solution-A and solution-B in ratio of 45:55(v\v). An external standard method was used and the flow rate was 0.5ml/min.and Column temp is 40°C the UPLC system was operated at room temperature 25°C-20°C. And diluent is mixture of Solution-A and solution-B in ratio of 45:55(v\v).

### Preparation of standard solution

A Stock solution of emtricitabine, efavirenz and tenofovir disoproxil fumarate was prepared at about 0.53mg/ml, 1.6mg/ml and 0.65mg/ml respectively in Diluent. The working standard solution 0.053mg/ml for emtricitabine, 0.16mg/ml for efavirenz and 0.065mg/ml for tenofovir disoproxil fumarate were prepared by diluting the stock solution with mobile phase.

Determination of emtricitabine, efavirenz and tenofovir disoproxil fumarate in their combined dosage forms.

The content of ten tablets where taken and weighed.powder equivalent to emtricitabine 200mg, efavirenz 600mg and tenofovir disoproxil fumarate 300mg in 100ml volumetric flask add 50ml of diluent and flask was sonicated for 10 min. The flask was sonicated and the volume was diluted to the mark with diluent.The above solution was filtered through syringe filter 0.2µm cellulose acetate filters made by Millipore (USA) Appropriate volume of aliquot was diluted with mobile phase to obtain a solution containing 0.053mg/ml of emtricitabine, 0.16mg/ml of efavirenz and 0.065mg/ml of tenofovir disoproxil fumarate.

### Linearity

Linearity of the proposed method was checked by

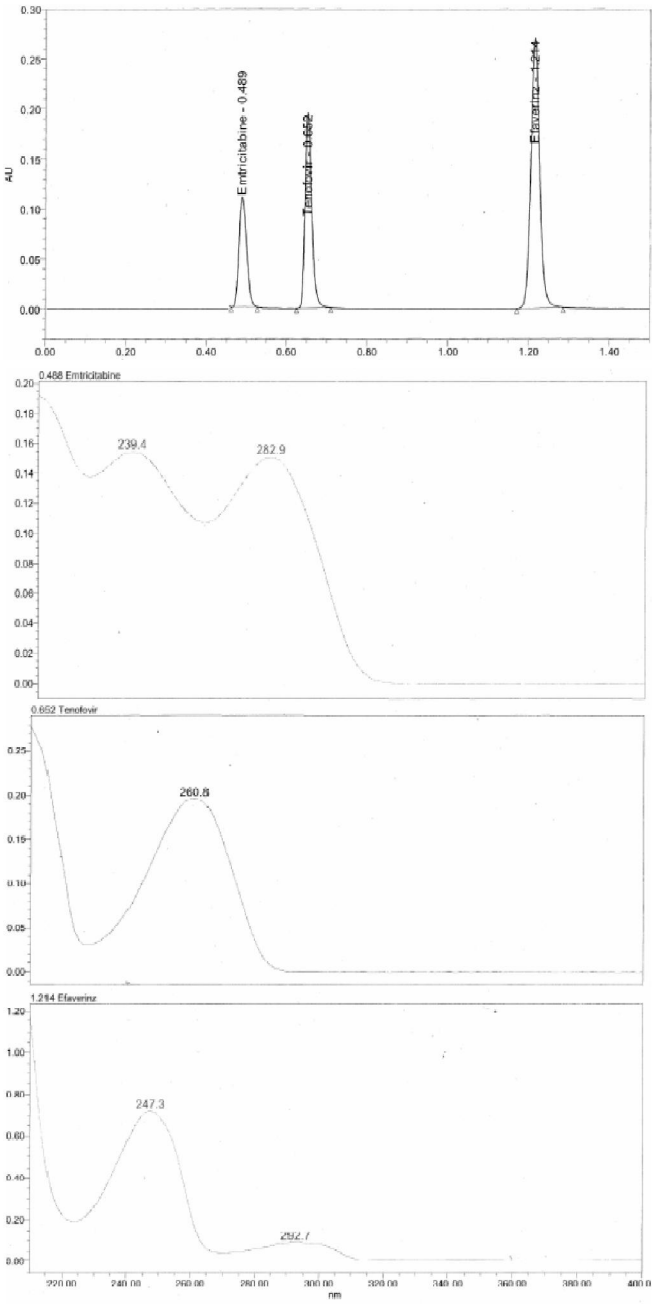


**Figure 4 : Chromatograms and spectra of emtricitabine, tenofovir and efavirenz reference substance**

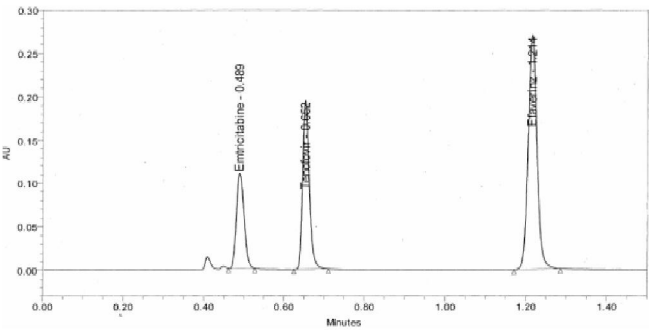


**Figure 5 : Chromatograms of emtricitabine, efavirenz and tenofovir disoproxyl fumarate in sample solution**

**TABLE 1 : Accuracy of the proposed UPLC method**

| Compound | Level (%) | n | Added Conc.µg/ml | Found Conc.µg/ml | % recovery | RSD (%) |
|---|---|---|---|---|---|---|
| Emtricitabine | 50 | 3 | 26 | 26.09 | 100.35 | 0.89 |
| | 100 | 3 | 53 | 52.89 | 99.79 | 1.01 |
| | 150 | 3 | 79 | 79.45 | 100.57 | 0.45 |
| Efavirenz | 50 | 3 | 23.5 | 23.43 | 99.7 | 0.55 |
| | 100 | 3 | 65 | 64.32 | 98.95 | 0.75 |
| | 150 | 3 | 88.5 | 89.1 | 100.68 | 0.58 |
| Tenofovir | 50 | 3 | 80 | 80.1 | 100.13 | 0.72 |
| | 100 | 3 | 160 | 159.78 | 99.86 | 0.37 |
| | 150 | 3 | 240 | 239.5 | 99.79 | 0.64 |

**TABLE 2 : Precision of the proposed UPLC method**

| Compound | conc. µg/ml | n | within a day precision | | Between a day precision | |
|---|---|---|---|---|---|---|
| | | | Mean | RSD (%) | Mean | RSD (%) |
| Emtricitabine | 26 | 5 | 116921 | 0.67 | 110012 | 0.22 |
| | 53 | 5 | 23461 | 0.54 | 23509 | 0.15 |
| | 79 | 5 | 351251 | 0.19 | 350087 | 0.76 |
| Efavirenz | 80 | 5 | 306010 | 0.77 | 306223 | 0.88 |
| | 160 | 5 | 612305 | 0.65 | 623410 | 0.23 |
| | 240 | 5 | 918412 | 0.51 | 928970 | 0.77 |
| Tenofovir disoproxyl fumarate | 23.5 | 5 | 146001 | 0.33 | 145992 | 0.76 |
| | 65 | 5 | 292233 | 0.48 | 300349 | 0.44 |
| | 88.5 | 5 | 438120 | 0.99 | 439001 | 0.15 |

analyzing five solutions in the range of 26-79µg/ml for emtricitabine (26, 39,53,66,79µg /ml), 80-240µg/ml for efavirenz (80, 120, 160, 200, 240µg /ml) and 23.5-88.5µg/ml for tenofovire disoproxil fumarate (23.5, 35.25,65, 76.75, 88.5µg /ml). Each level was made in triplicate.

## Accuracy

Method accuracy was performed by adding known amounts of emtricitabine, efavirenz and tenofovir disoproxil fumarate to the pre analyzed sample and then comparing the added concentration with the found concentration. Three levels of solutions were made which correspond to 50, 100 and 150% of the nominal analytical concentration. Each level was made in triplicate.

## Specificity

Commonly used exceipients (starch, microcrystalline cellulose and magnesium stearate, lactose,) were spiked in to a pre weighed quantity of drugs.The chro-

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**TABLE 3a : Robustness study of emtricitabine**

| Condition | t R (min) | Theoretical Plate | Tailing |
|---|---|---|---|
| Solution A:Solution B (43:57) | 0.381 | 9876 | 1.108 |
| Solution A:Solution B (45:55) | 0.489 | 10679 | 1.190 |
| Solution A:Solution B (47:53) | 0.855 | 10987 | 1.091 |
| Flow Rate (0.6ml/min) | 0.399 | 9765 | 1.389 |
| Flow Rate (0.4ml/min) | 0.611 | 10078 | 1.278 |
| Buffer p H (6.3) | 0.501 | 10210 | 1.211 |
| Buffer p H (2.8) | 0.461 | 11232 | 1.255 |

**TABLE 3b : Robustness study of Efavirenz**

| Condition | t R (min) | Theoretical Plate | Tailing |
|---|---|---|---|
| Solution A:Solution B (43:57) | 1.097 | 9879 | 1.098 |
| Solution A:Solution B (45:55) | 1.214 | 9007 | 0.998 |
| Solution A:Solution B (47:53) | 1.780 | 9987 | 1.178 |
| Flow Rate (0.6ml/min) | 1.017 | 8900 | 1.098 |
| Flow Rate (0.4ml/min) | 1.572 | 9689 | 1.190 |
| Buffer p H (6.3) | 1.309 | 8979 | 1.232 |
| Buffer p H (2.8) | 1.189 | 9077 | 1.178 |

**TABLE 3c : Robustness study of Tenofovir disoproxyl fumarate**

| Condition | t R (min) | Theoretical Plate | Tailing |
|---|---|---|---|
| Solution A:Solution B (43:57) | 0.510 | 7893 | 1.121 |
| Solution A:Solution B (45:55) | 0.652 | 8310 | 1.301 |
| Solution A:Solution B (47:53) | 0.998 | 8103 | 1.480 |
| Flow Rate (0.6ml/min) | 0.54 | 7921 | 1.098 |
| Flow Rate (0.4ml/min) | 0.813 | 8371 | 1.342 |
| Buffer p H (6.3) | 0.551 | 8089 | 1.279 |
| Buffer p H (2.8) | 0.718 | 7907 | 1.361 |

matogram was taken by appropriate dilution and the quantities of drug were determined.

### Robustness

Robustness of the method was performed by intentionally modifying the chromatographic conditions such as composition and flow rate of the mobile phase and pH of the buffer solution. The chromatographic parameters of each analyte such as retention time, tailing factor, resolution and number of theoretical plates were measured at each changed conditions.

### Precision

For evaluating the within-day precision, results of five replicate analyses of three different concentrations of samples were calculated on a single day. The between-day precision was calculated from the same samples analyzed on five different days.

### LOD and LOQ

For calculating the LOD and LOQ values, solutions with known decreased concentrations of analytes were injected into the UPLC system. The limit of detection (LOD) and quantification (LOQ) were then measured by calculating the minimum level at which the analytes can be readily detected (signal to noise ratio of 3:1) and quantified (signal to noise ratio of 10:1) with accuracy, respectively.

## RESULTS

A simple and accurate UPLC method for the simultaneous determination of emtricitabine, efavirenz and tenofovir disoproxyl fumarate in synthetic mixture form. Method development was started with water and Acetonitrile in the ratio of 50:50 (v/v). At this composition although emtricitabine, efavirenz and tenofovir disoproxyl fumarate components were eluted but resolution between emtricitabine and efavirenz is not good and peak shape was not sharp. Mixture of Acetonitrile and Methanol (50:50 v\v) is introduced in the mobile phase and pH of water adjusted to 6.5 with the help of Orthophosphoric acid and Triethyl amine.At the composition of 45:55 v/v (0.2%TEA pH 6.5 with OPA Solution A) and (Acetonitrile and Methanol 50:50 v/v solution B) all three components were eluted with a good resolution and good peak shape. The most appropriate mobile phase composition was Solution A (0.2%TEA in water pH 6.5 by OPA) and Solution B (Acetonitrile and Methanol 50:50 v/v) in the ratio of 45:55 (v/v). Under the Described experimental conditions, sharp peaks that belong to emtricitabine, efavirenz and tenofovir disoproxyl fumarate were obtained at retention times of 0.489, 0.652 and 1.214 minutes respectively as shown in figure 4.

The developed chromatographic method was validated using ICH guidelines[40]. Validation parameters performed include linearity, limit of detection and quantitation, selectivity, robustness, accuracy and repeatability the calibration curve was linear over the concentration range of 26-79µg/ml for emtricitabine, 80-240µg/ml for efavirenz and 23.5-88.5µg/ml for tenofovir

disoproxil fumarate. The correlation coefficient for emtricitabine, efavirenz and tenofovir disoproxyl fumarate was found to be greater than 0.999 which manifests a linear relationship between concentration and the peak area. The linear regression equation for emtricitabine was found to be Y = 53 X + (-338.99) with correlation coefficient equal to 0.999. The linear regression equation for tenofovir was found to be Y = 65 X + (-32.149) with value of correlation coefficient equal to 0.9999. The linear regression equation for efavirenz was found to be Y = 160 X + 552.899 with value of correlation coefficient equal to 0.9999. In this study, the LOD was found to be 2.34µg/ml for Emtrcitabine and 7.2µg/ml for efavirenz and 2.11µg/ml for tenofovir disoproxyl fumarate respectively. The LOQ was found to be 7.8µg/ml for Emtrcitabine and 24µg/ml for efavirenz and 7.05µg/ml for tenofovir disoproxyl fumarate respectively. The recovery and the relative standard deviation for each of the analytes are given in TABLE 1.

The results of within-day and between-day precision are presented in TABLE 2.

Chromatogram of emtricitabine, efavirenz and tenofovir disoproxyl Fumarate in sample in given in figure 5 showing selectivity of the proposed method.

Robustness of the method was performed by intentionally modifying the chromatographic conditions. The results showed that the variance of the conditions had no appreciable effects to that of actual. The results of the robustness study are given in TABLE 3a, 3b and 3c.

## CONCLUSION

A simple and accurate reverse phase UPLC method has been developed for the simultaneous determination of emtricitabine, efavirenz and tenofovir disoproxyl Fumarate. The method was validated by testing its linearity, accuracy, precision, limits of detection and quantitation, selectivity and robustness. The run time of less than five Minutes allows its application for the routine determination of emtricitabine, efavirenz and tenofovir disoproxyl Fumarate.

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