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Chromatographic Separation and in Vitro Dissolution Assessment of Tenofovir disoproxil fumarate, Emtricitabine and Nevirapine in a Fixed Dose Combination of Antiretrovirals

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ABSTRACT

A rapid, economic and robust stability indicating HPLC method was developed and validated to quantify Tenofovir disoproxil Fumarate (TDF), Emtricitabine (EMT) and Nevirapine (NVP) simultaneously at single wavelength (254 nm) in order to assess the in vitro drug release profile from tablet formulations. Chromatographic separation was performed with a gradient elution of samples on a 4.6 mm x 150 mm, 5 µm, Inertsil ODS-2 column with buffered mobile phase containing solvent A (10 Mm ammonium acetate buffer, pH 4.6) and solvent B (acetonitrile) at a flow rate of 1.0 mL/min). In dissolution studies, the sink condition was optimized based on quantitative solubility of TDF, EMT and NVP standards in different dissolution medium as recommended by USP. The proposed HPLC method and dissolution test condition were validated as per ICH guidelines. The results obtained meet the regulatory criteria thereby confirming that the method is suitable for routine quality control analysis and in vitro dissolution studies.

INTRODUCTION

The current guidelines for antiretroviral (ARV) treatment of HIV infection recommend initial treatment with a combination of 3 drugs, 2 of which should be nucleoside / nucleotide reverse transcriptase inhibitors (NRTI/NtRTI) and a third drug that may be a non-nucleoside reverse transcriptase inhibitor (NNRTI) or protease inhibitor (European medical agency report). Tenofovir Disoproxil Fumarate (TDF), Emtricitabine (EMT) and Nevirapine (NVP) (Figure 1) are the antiretroviral drugs that form the first line medicines for the treatment of HIV infected patients. Chemically TDF is bis(isopropyloxy-carbonyloxymethylester of (R)-9-(2-phosphonomethoxy-propyl) adenine with fumaric acid (Indian Pharmacopoeia,2007) and EMT is 4-amino-5-fluoro-1-[(2*S*,5*R*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2- dihydropyrimidin-2-one. Both are nucleoside analogues having potent inhibitory activity against HIV reverse transcriptase (Merck Index, 2006).

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NVP chemically 11-cyclopropyl-4-methyl-5,11-dihydro-6*H*dipyrido[3,2-*b*:2′,3′-*e*][1,4]diazepin-6-one, is a non-nucleoside reverse transcriptase inhibitor (NNRTI) (Montaner et al.,1998; Podzamczer et al., 2002; Staszewski et al.,1999). Several methods were reported for the estimation of TDF, EMT and NVP individually (Cass et al., 2003; Himaja et al., 2014; Ravisankar and Devala Rao, 2013; Sentenac et al., 2003; Wagner et al., 2012) and in combination with other antiretroviral drugs or in biological fluids (Bezy et al., 2005; Notari et al., 2006; Rajani et al., 2013; Rezk et al., 2005). To the best of our knowledge, no analytical method was reported for the simultaneous estimation of TDF, EMT and NVP and to access its application for in vitro dissolution studies of tablet dosage forms in the literatures or pharmacopoeias, which makes it essential to develop such a method for bulk and tablet formulations. In this study, a new fixed oral dose combination containing TDF, EMT (immediate release) and NVP (extended release) has been used. The proposed liquid chromatographic method and dissolution method was validated and applied to the quantification and in vitro dissolution studies of TDF, EMT and NVP in pharmaceutical dosage forms.

MATERIALS AND METHODS

Instrumentation

The chromatographic separations were performed on an Agilent HPLC system with an isocratic pump (G1310A), a DAD detector system (G1314A) and a thermostat column (G1316A) compartment. The data acquisition was processed using chemstation software. Separations were achieved at a temperature of 25°C with a gradient elution of samples on an Inertsil ODS-2 column (4.6 mm I.D 150 mm, 5 µm; Phenomenex, USA) with buffered mobile phase containing solvent A (10 Mm ammonium acetate buffer, pH 4.6 adjusted with glacial acetic acid) and solvent B (acetonitrile) at a flow rate of 1.0 mL/min. The mobile phase was degassed in an ultra sonicator and filtered with a 0.45 µm HV filter by a Millipore vacuum filter system. Peak identity was confirmed by comparing the spectra obtained from PDA detector.
The dissolution tests were performed in an Electro lab TDT-08L with paddle (USP-II) dissolution apparatus (Electrolab, India) at 50 rpm and bath temperature maintained at 37±5 °C. 900 mL of freshly prepared and degassed pH 6.8 phosphate buffers with 6% SLS for NVP and 900 mL of freshly prepared and degassed 0.01N HCl for TDF and EMT were used as dissolution medium. Dissolution samples were collected at different time points using auto sampler. At each time point, a 5 mL sample was collected from each vessel and filtered through a 0.45 µm nylon filter and analyzed using HPLC.

Materials

TDF, EMT and NVP standard and quality control samples were kindly provided by Strides Arcolab, India. Methanol and Acetonitrile was of HPLC grade from Spectrochem, Ammonium Acetate, Glacial Acetic Acid, was of analytical grade from Sdfine chemicals. Concentrated Hydrochloric acid was of analytical grade from Rankem. The common tablet excipients were purchased from local sources. Purified HPLC grade water was obtained by reverse osmosis and filtration through a Milli-Q® system. All the solutions were freshly prepared and analyzed. The nylon filters (0.45 µm) were obtained from Advanced Micro Devices, India.

Preparation of Solutions

For assay method

A primary stock solution of TDF (3 mg/mL), EMT (2 mg/mL), NVP (4 mg/mL), was prepared using diluent (20:30 v/v of 50% acetonitrile and 50% acetic acid). The stock solution was protected from light and used within 24 h. Working standards in diluent were prepared from the above stock solution for the determination of active drugs. Samples in triplicates for each concentration were prepared and peak areas were plotted against respective concentration to obtain the calibration graph.

For in vitro dissolution studies

Standard stock of TDF (0.33 mg/mL) and EMT (0.22 mg/mL) were prepared separately by dissolving about 33 mg (TDF) and 22 mg (EMT) of the reference standard in 100 mL of 0.01N HCl (dissolution media). Standard solution of NVP (0.44 mg/mL) was prepared by dissolving about 44 mg of reference standard in 100 mL of phosphate buffer, pH-6.8 with 6.0 % SLS (dissolution media). In order to assess the linearity of each component, serial dilution of TDF, EMT and NVP standard stocks were made in the range of 20% to 200% of working concentration. A calibration graph was plotted as concentration of drugs versus peak area response. It was found to be linear for all the analytes. The system suitability test was performed from five replicate injections of standard solutions.

Assay procedure for tablets

Twenty tablets of each containing 300 mg of TDF, 200 mg EMT and 400mg NVP were accurately weighed and powdered separately. Tablet powder equivalent to the weight of one tablet was weighed accurately and transferred in to 100 mL volumetric flask and 20 mL of 50% acetonitrile was added and sonicated for 5 min further 60 mL of 50% acetic acid was added and sonicated for 25 mins then volume was made up with 50% acetonitrile. The solution was filtered through 0.45µm nylon filter. Further 5 mL of filtrate was transferred in to 50 mL volumetric flask and made up to volume with 50% acetonitrile. 5 µL of the sample solutions were injected, chromatograms were obtained for the same and amount of drug present was calculated.

In vitro dissolution studies

The tablet formulation contains both immediate release (Emtricitabine and Tenofovir Disoproxil fumarate) and extended release (Nevirapine) drugs. Hence, dissolution studies were carried by following two different methods as per OGD (Office of Generic Drugs) guidelines. The in vitro drug release profile of TDF, EMT and NVP from tablets were developed using USP II (paddle) apparatus at 37 ± 0.5°C at 50 rpm rotation speed. The drug release study of class-I drugs were performed using 0.01N HCl as dissolution medium whereas for class-II drug phosphate buffer with 6% SLS (pH 6.8) was used as dissolution medium. Six tablets were evaluated. At scheduled time intervals, 5 mL of sample was withdrawn and replaced with fresh medium. The solution was filtered through 0.45 µm nylon membrane filter and analyzed by the HPLC method mentioned above.

RESULT AND DISCUSSION

Development and optimization of chromatographic condition

The various chromatographic parameters like mobile phase composition and pH, concentration of buffer solution and percentage of organic modifier, type of column etc. was tried to obtain a good chromatographic resolution. The standard peaks were observed at different wavelength range and a suitable wavelength of 254 nm was selected as the optimum wavelength for HPLC analysis of the labelled drugs as it gives maximum peak symmetry with a flat baseline and minimum signal for blank. The initial chromatographic parameters were designed based on the

compound physico-chemical properties. Various trails have been carried with different stationary phases starting from non-polar to polar using different composition of mobile phases. Various mobile phase buffers with pH in the range of 2.5 to 5 and checked for good resolution and high performance of stationary phase. The best separation was achieved with pH 4.6. Based on trail results good performance and high resolution with symmetric and sharp peaks was achieved using ammonium acetate buffer (pH 4.6) and acetonitrile composition as mobile phase in inertsil ODS-2 column with gradient elution program (Table 1) at a flow rate of 1 mL/min.

Under this chromatographic conditions TDF, EMT and NVP are well resolved and Placebo interference was not observed. Retention times of the drugs obtained under these conditions are 7.8, 4.3 and 6.7 for TDF, EMT and NVP respectively (Figure 2).

Development and optimization of the dissolution test condition

The selection of dissolution medium is absolutely based on the drug solubility and its stability in solid state. For an oral dosage form the dissolution behaviour should be assessed in the pH range of 1.2- 6.8 as per USP. The solubility of the drugs under study was determined using different dissolution media at 37°C. Based on their solubility results, sink conditions were checked. A 0.01 N HCl solution was selected as dissolution media for TDF and EMT release study and a pH 6.8 phosphate buffer with 6 % SLS was selected as dissolution media for NVP release study. Using the selected dissolution media, dissolution testing was performed on tablet (n= 6) using paddle (USP-II) dissolution apparatus with a paddle speed of 50 rpm. The media volume used is 900 mL at a temperature of 37 ± 0.5 °C. Samples were drawn at respective time points using auto sampler. The solution was immediately filtered using a syringe of 0.45 µm nylon filters.

Method validation

The optimized HPLC method for assay and in vitro dissolution studies are validated as per ICH guidelines (ICH 2000, 2005).

System suitability

The RSD values of peak area and retention time for TDF, EMT and NVP in six replicate injections are within 2% indicating the system suitability parameter meets the requirement of method validation. Theoretical plates, tailing factor and resolution were calculated for both assay and dissolution. The results were within the acceptable limits (Table 2).

Linearity

The calibration curves were obtained by plotting the peak area ratios against the drug concentration. The peak area ratios and concentrations were subjected to least square linear regression analysis to obtain the calibration equations and correlation coefficients. The results confirm that there is a good correlation between the peak area ratios and the concentrations of drugs in the range tested for both methods (Table 3).

Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogeneous sample. The precision of analytical method is usually expressed as the standard deviation or relative standard deviation (Coefficient of variation) of series of measurements. The RSD values were found to be within 2 % for both assay and dissolution studies indicating good precision.

Accuracy

Accuracy was examined by calculating the percentage recoveries at different concentration levels by standard addition method (Table 4). The mean percentage recoveries obtained for TDF, EMT, NVP were 101.2%, 100.9%, 101.3% respectively for assay and 99.4% , 100.6%, 101.1 % respectively for dissolution method.

Specificity

The specificity parameter is determined by injecting blank, placebo, standard and sample preparations into the HPLC system and recorded the chromatograms. From the obtained results it was concluded that the peaks of diluent, placebo are not interfering with the standard and sample peak and there was no co-eluting peaks in any of the samples. The peak purities of the compounds were checked by its spectra obtained from PDA detector (Figure 3). Absence of interference was observed. In filter compatibility study dissolution results of filtered sample was found to be within 2.0% range from the unfiltered sample.

Stability

The percentage difference of area response for the peak in standard and sample preparation was within 2.0% from initial area in the specified period of analysis. The results indicate that the standards and samples were stable up to 34 h.

Robustness

The robustness of an analytical method is important as it measures the ability of the developed method to remain unaffected by small but deliberate variations in chromatographic conditions. No significant change was observed in terms of resolution, peak area, RSD, tailing factor and theoretical plates thereby proving the reliability during normal usage.

Application of the proposed methods

The proposed assay method was successfully applied to the analysis of tablet formulation Table 5. The dissolution characteristics of TDF, EMT and NVP are mentioned in Figure 4. By analyzing the in vitro dissolution profiles it was concluded that all the three drugs exhibited good release rate and the dissolution method mentioned above is suitable to simulate the in vitro release of TDF, EMT and NVP from fixed dose tablets.

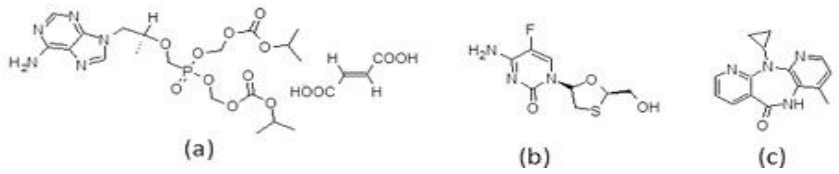

Fig. 1: Chemical structres of (a) tenofovir disoproxil fumarate, (b) emtricitabine and (c) nevirapine.

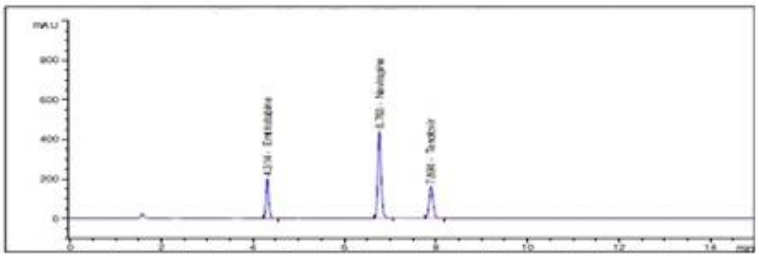

Fig. 2: Resultant HPLC chromatogram following tha analysis of a standard solution of TDF, EMT, and NVP.

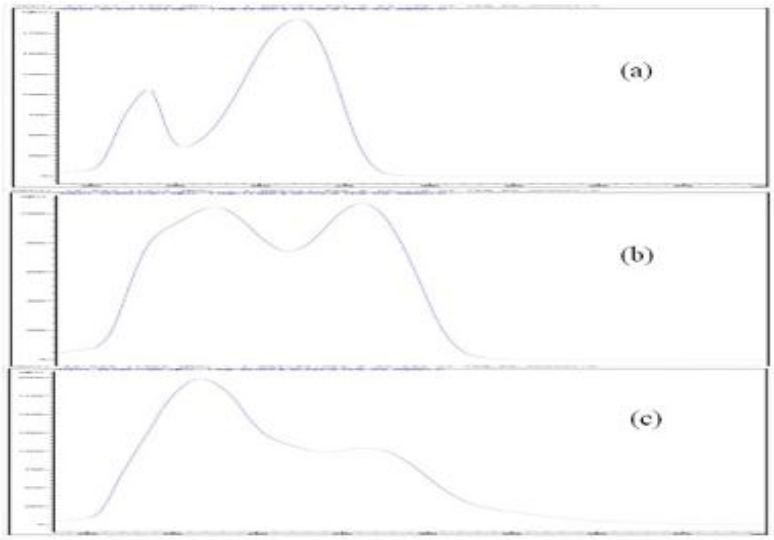

Fig. 3: The spectra of the chromatographic peaks corresponding to (a)TDF, (b)EMT and (c) NVP.

Table 1: Gradient elution program of mobile phase.

Time (min)	Flow (mL min^{-1})	Solvent A (%)	Solvent B (%)
0	1.0	95	5
5	1.0	50	50
10	1.0	80	20
12	1.0	95	5
15	1.0	95	5

Table 2: System suitability results.

Parameters	Assay						Dissolution					
	TDF		EMT		NVP		TDF		EMT		NVP	
	RT (min)	Area response	RT (min)	Area response	RT (min)	Area response	RT (min)	Area response	RT (min)	Area response	RT (min)	Area response
Mean	7.89	955.64	4.31	889.21	6.76	2285.39	4.33	925.74	8.01	2024.30	6.83	3713.85
SD	0.002	0.898	0.002	1.163	0.001	1.893	0.002	0.863	0.002	1.816	0.002	1.976
RSD	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1
Tailing factor	1.165		1.129		1.177		1.012		1.092		1.253	
Theoretical plates	40546		22547		39587		12569		15569		145326	

Table 3: Linearity results.

Parameter	Assay			Dissolution		
	TDF	EMT	NVP	TDF	EMT	NVP
Range (ppm)	73.10-219.31	59.76-179.29	120.10-360.30	66.82-668.2	44.96-449.60	89.03-890.30
Slope	6.389	2.774	9.364	2.774	8.969	8.336
Intercept	3.8932	-2.658	24.1852	-2.658	-3.636	-13.296
Correlation coefficient	1.000	1.000	1.000	1.000	1.000	1.000

Table 4: Recovery analysis results by standard addition method.

Compound	Wt. spiked (mg)		Wt. recovered (mg)		Recovery (%)		Mean RSD (%)	
	Assay	Dissolution	Assay	Dissolution	Assay	Dissolution	Assay	Dissolution
TDF	45.65	149.19	45.75	148.24	100.2	99.4	0.9	1.1
	89.59	298.13	90.75	299.89	101.3	100.5		
	135.50	447.25	138.15	440.17	102.0	98.5		
EMT	30.77	99.89	30.96	100.71	100.6	100.8	0.5	0.6
	60.95	199.60	61.26	201.60	100.5	101.0		
	90.23	299.54	91.61	301.49	101.5	100.7		
NVP	60.90	80.88	61.81	82.02	101.5	101.6	0.3	0.5
	119.82	400.95	121.05	404.21	101.0	100.9		
	180.57	600.56	183.38	605.12	101.6	100.9		

Table 5: Assay results of TDF, EMT and NVP in tablets.

Formulation	Label claim (mg / per tablet)	Amount found (mg) ± SD (n=5)	Recovery (%)	RSD (%)
Tenofovir Disoproxil	300 TDF	300.09 ± 0.05	101.2	0.9
Fumarate, Emtricitabine (IR)[a]	200 EMT	199.85 ± 0.18	100.9	0.5
and Nevirapine (ER)[b] Tablets	400 NVP	400.42 ± 0.21	101.4	0.3

[a] Immediate release., [b] Extended release

CONCLUSION

This is the first report for the simultaneous determination of TDF, EMT and NVP using an HPLC method. The proposed HPLC method has been suitably applied for studying the in vitro dissolution behavior of TDF, EMT and NVP from tablet dosage forms. Both the proposed HPLC and dissolution method were validated as per ICH guidelines and proved to be simple, rapid, reliable and robust and are suitable for routine quality control analysis.

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