Original Research

Simultaneous Determination of Tenofovir Disoproxil Fumarate and Emtricitabine by UV Vierordt's and UFLC Methods

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ABSTRACT

Introduction: Analytical methods like Ultraviolet-visible spectroscopy and High-Performance Liquid Chromatography play a critical role in equivalence and risk assessment and management. Nucleotide reverse transcriptase inhibitors tenofovir disoproxil fumarate (TDF) and Emtricitabine (ETB) is clinically a potent combination against HIV and hepatitis B virus devoid of short-term irritating toxicity.

Objective: The present investigation deals with the development and validation of new UV spectrophotometric Vierordt's and Reverse Phase Ultra-Fast Liquid Chromatography (RP-UFLC) methods for the simultaneous estimation of TDF and ETB in bulk and tablet dosage form as per the International Council for Harmonization (ICH) guidelines.

Methods: Vierordt's method was developed on Shimadzu UV-1800 UV/Visible Spectrophotometer. Reverse phase ultra-fast liquid chromatography was developed on Shimadzu UPLC, X-Bridge C18 column equipped with a photodiode array detector. Mobile phase composed of water: methanol 62:38. The effluent was pass through the column 1.0 ml/min at 30o C and the response was recorded at 254 nm.

Results: Absorption maxima of TDF and ETB were obtained at 260 nm and 281 nm respectively. The RSD of all validation parameters was lesser than 2 % indicated the accuracy of the Vierordt's method. In UFLC, the retention time of ETB and TDB were found to be at 3.371 and 6.850 min respectively. Purity angle and purity threshold of both peaks showed spectral homogeneity over all the peak region indicated its peak purity.

Conclusion: The proposed Vierordt's and RP-UFLC methods found to be sensitive, rapid, accurate and economical and it can be employed for simultaneous estimation of ETB and TDB in bulk drugs and formulations.

Key Words: Vierordt's method, RP-UFLC, Tenofovir disoproxil fumarate, Emtricitabine, UV spectroscopy, Chromatography

INTRODUCTION

Chemically, Tenofovir (TDF) is (R)-(1-(6-amino-9H-purin-9-yl)propan-2-yloxy)methyl phosphonic acid. It is a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase enzyme inhibitors (NtRTIs), which blocks viral production in HIV-infected people.[1] Emitricitabine (ETB) is 4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2-one. It is also a nucleoside reverse transcriptase inhibitor which acts by inhibiting reverse transcriptase enzyme that copies human immunodeficiency virus ribonucleic acid into new viral deoxyribonucleic acid.[2] Combinational antiretroviral therapy of TDF with ETB has significantly greater potency, reduced HIV-related morbidity, mortality, toxicity and offers simplified dosing convenience.[3]

Literature survey revealed that the various analytical methods like high-performance liquid chromatography[4-9] and ultraviolet spectroscopy[10,11] have been reported for simultaneous determination of TDF and ETB however none of these chromatographic methods developed on UFLC which offers better resolution, speed, sensitivity, accuracy compare to conventional liquid chromatography.[12] In the reported high-performance liquid chromatography methods, effluents were not monitored by photodiode-array detector hence the interference of any impurities remained are undetected. Ultra-fast liquid chromatography equipped with photodiode array detector facilitates the accurate detection of co-eluting impurity within the analyte peak by measurement of its peak purity.[13]Vierordt's spectrophotometric method applied for

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simultaneous estimation of drugs that have measurable absorbance at λ_{max} of each other.[14] Vierordt's method has the benefits of being simple, rapid, direct, economic, with minimum data manipulation. It does not require sophisticated techniques or instruments.[15] Therefore, the present investigation deals with development and validation of UV Spectrophotometric Vierordt's and UFLC methods to allow accurate, rapid, sensitive and precise determinations of TDF and ETB in bulk and tablet dosage forms.

MATERIALS AND METHODS

Materials

Pharmaceutical grade TDF and ETB bulk drugs were generously gifted by Hetro Drugs Ltd., Hyderabad, India. HPLC grade methanol and water were purchased from Rankem, Mumbai. Nylon membrane disc filter (0.45μ) and syringe filter procured from by mdi-Cat, Gurgaon. All other chemicals and reagents were of analytical grade. **TENOF EMis the brand of the tablet manufactured by** Hetero Drugs Ltd contains Emtricitabine/Tenofovir disoproxil fumarate 200 mg/300 mg respectively. Tablets were purchased from Nagpur retail pharmacy.

Instruments

Shimadzu prominence ultra-fast liquid chromatography (UFLC) system, Shim-pack GIST-HP C_{18} (150 x 4.6 mm, 3 µm) column manufactured by Shimadzu Pvt. Ltd. from high purity porous spherical silica, Pump LC 20 AD gradient system, connected with SPD-M20A PDA detector empowered with LC solution software. Shimadzu UV-1800 UV/Visible Scanning Spectrophotometer; 115 VAC, bandwidth 1.0 nm, wavelength accuracy ±0.1 nm, detector silicon photodiode. Mettler Toledo XS205D0 analytical balance.

Development and validation of UV spectrophotometric Vierordt's method

Working solutions of standard TDF and ETB were scanned separately in UV range 200–400 nm against water as blank for determining maximum wavelengths (λ_{max}). TDF and ETB displayed λ_{max} at 260 nm and 281 nm, respectively (Fig. 1. a & b). Calibration curves was plotted over a concentration 5,10,15, 20,25 and 30 µg/ml for TDF and 3.5, 7, 14, 21, 28, 35 µg/ml for ETB. The absorbance was measured in triplicate at λ_{max} 260 and 281 nm. Specific absorptivity $A^{1\%}_{1cm}$ of each solution was determined by dividing absorbance of drug with its concentration in gm/100ml. The amounts of the TDF and ETB in laboratory mixture were calculated by using Eq.1 and 2.

$$Cx = \frac{A2\,ay1 - A1\,ay2}{ax2\,ay1 - ax1\,ay2}$$

Eq.1

$$Cy = \frac{A1\,ax2 - A2\,ax1}{ax2\,ay1 - ax1\,ay2}$$

Eq.2

where Cx and Cy are the concentration (µg/ml) of TDF and ETB. A_1 and A_2 are the absorbances of laboratory prepared mixtures (Tablet **TENOF EM**) at 260 nm and 281 nm, respectively. The ax_1 and ax_2 are specific absorptivity's of TDF at 260 nm and 281 nm, respectively. While, ay_1 and ay_2 are specific absorptivity's of ETB at 260 nm and 281 nm, respectively.

All validation procedures of proposed Vierordt's method were performed following the International Conference on Harmonization ICH Q2 (R1) guideline.[16] The per cent mean recovery (±SD) and % relative standard deviations (%RSD) at two concentration levels for TDF (7 and 28 µg/ml) ETB (10 and 25 µg/ml) in their pure state were calculated. The precision of the developed method was ascertained by repeatability and intermediate precision studies. In the intra-day study (repeatability) of the proposed method was performed with a minimum of three replicate measurements. The concentrations of the TDF and ETB were calculated every two hours (0, 3, and 6 h). In inter-day (intermediate precision) studies, the concentrations of both the drugs were calculated on three consecutive days. Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated from linearity regression equations of TDF and ETB.

Development and validation of UFLC Method

Preparation of standard and working solution
Standard TDF (15 mg) and ETB (10 mg) were accurately weighed and transferred into 50 ml volumetric flask. Then, 35 mL of the diluent (water-methanol, 50:50) was added. It was sonicated for 15 min and diluted to up to the mark with the diluent. From the above stock solution, 1 ml of the filtrate was transferred into another 20 mL volumetric flask and volume were made up to the mark with the diluent. The resultant concentration standard TDF (15 µg/ml) and ETB (10 µg/ml) were obtained for the development of the UFLC method.

Preparation of tablet sample solution
Twenty tablets were weighed and powdered finely. Powder quantity equivalent to TDF (15 mg) and ETB (10 mg) were accurately weighed and transferred into 50 ml volumetric flask. It was sonicated for 15 min and diluted to up to the mark with the diluent. From the stock solution, 1 ml of the filtrate was transferred into 20 mL volumetric flask and volume were made up to the mark with the diluent. The working solution pass through the syringe filter.

Chromatographic separation was performed on UFLCC$_{18}$ (150 x 4.6 mm, 3 µm) column equipped with PDA detector. Isocratic elution was carried by mobile phase water: methanol (62:38) at flow rate 1 ml/min; column temperature 35^0 C. Mobile phase was sonicated, degassed and pass through 0.45µ nylon membrane disc filter. The stock solution and working standard prepared in diluents water: methanol (50:50 v/v). Injection volume was 20 µL. Chromatographic peaks of TDF and ETB were integrated to observe its λ_{max} of the UV spectrum by PDA detector. Parameters like peak symmetry, peak purity, tailing factor, R_t and area under curve measured by LC solution software. System suitability parameters were analyzed to evaluate system performance reproducibility. Six replicates of the sample were injected into the column. Parameters such as resolution factor, capacity factor, tailing factor, asymmetry, retention time, number of theoretical plates (N) number of the height equivalent to theoretical plates (HETP), and peak purity was recorded by LC solution software to assess column performance and suitability of the analytical method.

The method validation parameter such as system suitability, specificity, linearity, accuracy, precision and robustness were evaluated as per the ICH guidelines Q2B [16] and USP [17]. Specificity study was performed by analyzing the standard working solution of TDF and ETB spiked with placebo (excipients) and blank (diluents). The linearity of an analytical method was evaluated over the six-concentration range of standard solutions. A minimum of six standard drug concentrations range between 5-30 µg/ml for TDF and 3.5-35 µg/ml for ETB were prepared and the calibration curve was constructed by plotting its peak area versus concentration. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated from the calibration curve by using formulae:

$$LOD = 3.3(SD)/S \text{ and } LOQ = 10(SD)/S,$$

where SD = standard deviation of response (peak area) and S = average of the slope of the linear curve.

The accuracy of the method was determined by recovery studies. The percentage recovery was calculated by preparing standard drug concentrations of TDF (15 µg/ml) and ETB (10 µg/ml) with concentration levels of 50%, 100% and 150%. A known amount of the standard drug was added to the blank sample at each level. The precision was determined to ensure the closeness of the data values to each other for the number of measurements under the same analytical conditions. System precision: The system precision measured by six replicate injections of a homogeneous sample of standard MET (60 µg/ml) and VILDA (40 µg/ml) on the same day and consecutive days. Robustness was determined by small but deliberate change in conditions such as flow rate (1±0.1ml/min), column temperature (35±2°C), and wavelength (λmax257±2 nm).

RESULTS AND DISCUSSION

Optimization of Vierordt's method

The standard solutions of TDF and ETB were scanned separately in the UV range from 400-200 nm and its zero-order spectra were recorded. Maximum absorbance TDF and ETB was obtained at 260 nm and 281 nm for, respectively. The isosbestic point was recorded at 275 nm. The overlain UV absorption spectrum of TDF and ETB in distilled water has shown in (Figure 1).

Calibration curves of TDF and ETB at 260 nm and 281 nm in distilled water showed high linearity. The correlation between sample concentrations and their absorbencies complied with Beer's law as illustrated by high values of regression coefficients ($r^2 \approx 0.999$) and small values of intercepts have shown in (Table 1).

Validation of Vierordt's method

The % RSD values of TDF and ETB was found to be 1.11 and 0.74% for inter-day precision and 0.88 and 0.62 % for intra-day precision respectively. Relative standard deviation was less than 2 %, which indicates that the proposed method is precise. Limit of detection (LOD) for TDF and ETB were found to be 0.521 and 0.236 µg/mL and Limit of Quantitation (LOQ) were found to be 1.873 and 0.847 µg/mL respectively. The results showed that the method was found to be sensitive for the determination of TDF and ETB. Reliability of the proposed method was determined by accuracy by recovery studies. The % mean recovery of TDF and ETB were 99.4–100.4, respectively, indicating the accuracy of the method. The results of recovery studies showed lesser than 2% RSD that indicates the proposed method is highly accurate. The proposed Vierordt's method was successfully applied for simultaneous determination of TDF and ETB in its bulk and tablet dosage form. Vierordt's method has the benefits of being simple, rapid, direct, and economic, with minimum data manipulation and not requiring sophisticated techniques or instruments.[18]

Optimization of the UFLC method

Method development: The RP-UFLC method was optimized for the accurate, precise simultaneous determination of ETB and TDF.RP-UFLC method was developed on Shimadzu UPLC, X-Bridge C$_{18}$ column in mobile phase water: methanol 62:38 v/v. The effluent pass through the column at 1.0 ml/min; 30° C and monitored by PDA detector at 254 nm. During chromatographic trials, broadening of TDF peak was observed as the concentration of methanol increases beyond 35 % v/v as well as TDF peak was shifted towards higher retention time. In the proposed method, the retention time of ETB and TDB were found to be at 3.371 and 6.850 min respectively.

The chromatogram showed in Fig. 2 indicated that both the drugs are well separated with proper symmetry of the peak. Assay of ETB and TDF showed 98.8±0.55 and 99.5±1.25% respectively.

The system suitability parameter of the optimized chromatographic method has shown in Table 2.

Height Equivalence to theoretical plates (HETP) is the measure of zone broadening; in general, the lower the HETP value, narrower is the solute peak. Separation efficiency is enhanced at a lower value of HETP and the higher number of theoretical plates (N). A high Capacity (k') factor value indicates that the sample is highly retained and has spent a significant amount of time interacting with the stationary phase.[19] Proposed method was unaffected by the presence of sample excipients. System suitability parameters indicated the proposed UFLC method has complied as per ICH and USP guidelines.

Validation of optimized UFLC method

The linearity of standard ETB and TDF was studied in the concentration range 3.5-35 µg/ml and 5-30 µg/ml respectively. The regression equation and coefficient of variance (r^2) was found to be y = 222889x + 23810; r^2 = 0.999 for ETB and y = 69511x + 13546; r^2= 0.9997 for TDF. The LOD and LOQ of ETB were found to be 0.040 and 0.125 µg/ml respectively. The LOD and LOQ of TDF were found to be 0.085 and 0.150 µg/ml respectively. Recovery of the spiked drugs was obtained within an acceptable limit at each added concentration. The mean percentage recovery of ETB and TDF was achieved between 99.50±0.25 and 99.44±40 % respectively, indicating that the method was accurate. The system precision was found to be precise for six replicates of standard ETB and TDF. The % RSD of ETB and TDF was 0.06% and 0.08% respectively which was well within the acceptance criteria. In intra-day (n=3) studies, % RSD of ETB and TDF were found to be 0.23% and 0.38% respectively. Inter-day precision (n=3) studies, % RSD of ETB and TDF were found to be 0.820 % and 0.98 % respectively. The results of robustness studies were expressed relative to control. The % RSD caused by deliberate variation in flow rate (1±0.1ml/min), wavelength (257 ±2 nm) and column temperature (35± 2^{0}C) was within the acceptable criteria (≤2%) for both the drug.

CONCLUSION

Vierordt's method was simple, economical and it can be applied for simultaneous determination of TDF and ETB in its bulk or pharmaceutical dosage forms without its prior separation. Statistical analysis of data revealed ultra-high performance method was more accurate and precise relative to Vierordt's method, however, both the method confined to ICH (Q2R1) guidelines. The proposed analytical method can be routinely employed in quality control and research and development activities.

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Table 1: Absorptivity values and regression analysis of Vierordt's method

Drug	Parameters	λ_1 (260 nm)	λ_1 (281 nm)
TDF	Specific absorptivity ($A^{1\%}_{1cm}$)	561.50 ±2.5 (ax$_1$)	83.50 ±1.5 (ax$_2$)
	Linearity range (5-30 µg/ml) Regression Equation,	y =0.536x+0.0187	y =0.0078x+0.0007
	Correlation coefficient	r² = 0.9993	r² = 0.9992
LTB	Specific absorptivity ($A^{1\%}_{1cm}$)	369.00±1.0 (ay$_1$)	530.00±2.0 (ay$_2$)
	Linearity range (5-30 µg/ml) Regression Equation	y =0.0297x+0.0015	y =0.0415x+0.0157
	Correlation coefficient	r² = 0.9995	r² = 0.9994

Mean (n=3), ±SD

Table 2: System suitability parameters for Emtricitabine and Tenofovir disoproxil fumarate

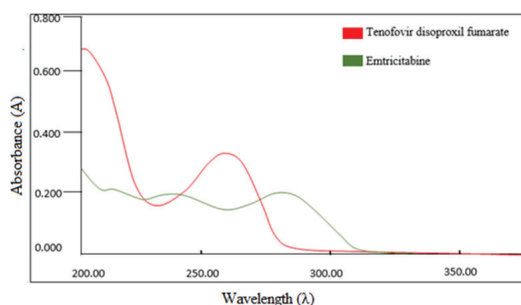| Parameters	ETB			TDF		
	Value	SD	% RSD	Value	SD	% RSD
No. of theoretical plates (N)	28570	56.23	0.221	24768	48.75	0.196
HETP	29.043	0.38	0.525	21.026	0.32	0.485
Tailing factor	1.10	0.041	0.826	1.09	0.032	0.617
Peak purity index		0.0935			0.094236,	
Single point threshold		1.00			1.00	
Resolution factor (Rs)			12.625			
Capacity factor (k')			13.567			

Figure 1: Overlain spectrum of Tenofovir disoproxil fumarate and Emtricitabine.
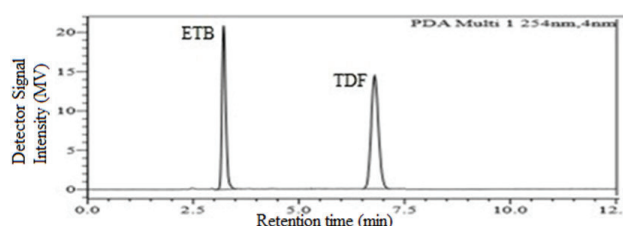

Figure 2: Chromatogram of Emtricitabine and Tenofovir disoproxil fumarate.