Simultaneous estimation of Tenofovirdisoproxilfumarate, Efavirenz and Lamivudine in fixed dose combination tablets by ultraviolet spectrophotometry in multicomponent mode and its application in rat plasma

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ABSTRACT

A simple and sensitive UV spectrophotmetric method using multi-component mode was developed for the simultaneous estimation of tenofovirdisoproxilfumarate, efavirenz and lamivudine in fixed dose combination tablets using a mixture of phosphate buffer pH 5 and methanol (50:50 %v/v) as solvent system. The detection was carried out at 247.0, 271.8 and 260.4 nm for efavirenz, tenofovirdisoproxilfumarate and lamivudine respectively. Linearity was observed over concentration range of 0.4-24, 0.2-12 and 0.2-12 µg/mL for efavirenz, tenofovirdisoproxilfumarate and lamivudine respectively and the coefficient of determination was found to be 0.999 for all three drugs. The developed method was validated as per ICH guidelines and all the parameters are within the limits. The limit of quantitation values was found to be 0.192µg/ml, 0.198µg/ml and 0.385µg/ml for efavirenz, tenofovirdisoproxilfumarate and lamivudine respectively and reflects the sensitivity of the method. The developed method was optimised and applied in spiked rat plasma.

INTRODUCTION

Nucleoside reverse transcriptase inhibitors (NRTIs) were the first class of drugs that were introduced as antiretroviral agents for the treatment of human immunodeficiency virus (HIV) infection. Tenofovir disoproxil fumarate (TDF) is an antiretroviral agent belonging to this class used in the management of HIV infection in adults. It is an orally bioavailable prodrug of tenofovir and the first nucleotide analogue approved for HIV-1 treatment. Chemically it is bis (isopropyloxy carbonyloxy methyl ester of (R)-9-(2-phasphonomethoxy propyl) adenine with fumaric acid. EPIVIR is a brand name for lamivudine (LAM), is also a nucleoside reverse transcriptase inhibitor class antiretroviral agent. It is a synthetic nucleoside analogue with activity against HIV-1 and hepatitis B virus. The chemical name of lamivudine is (2R, cis)-4-amino-1-(2-hydroxymethyl-1, 3-oxathiolan-5-yl)- (1H) -pyramidine-2-one. Lamivudine is the (-) enantiomer of a dideoxy analogue of cytidine and also referred to as (-)2',3'-dideoxy,3'-thiacytidine. Efavirenz (EFV) belongs to the class of non-nucleoside reverse transcriptase inhibitors (NNRTI). It is a synthetic purine derivative used for the treatment of HIV-1. Chemically it is (S)-6-chloro-4-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one. [1-2]

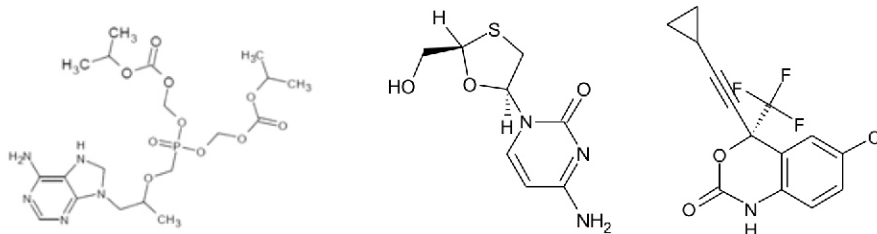Structures of TDF, LAM and EFV are shown in fig. 1.

Fig. 1 : Structure of tenofovir disoproxil fumarate, lamivudine and efavirenz

Taking two or more drugs at a time is called combination therapy.Some of the diseases treated with combination therapy include tuberculosis, leprosy, cancer, malaria, and HIV/AIDS. One major benefit of combination therapy is that they reduce development of drug resistance, since a pathogen or tumor have less resistance to multiple drugs simultaneously. The multi-component mode is the one in which the concentration of each constituent component is determined by using absorption spectrum of the mixed sample with pure standards or standards made up of multiple constituent components. Mixed samples with up to 8 constituent components can be determined quantitatively. In addition to using pure samples of each constituent component as the standard samples, a mixed sample in which the concentration of each constituent component is known may also be used. The effects of interference among the various constituent components can be minimized by using a mixed sample as the standard sample. The analytical wavelengths used in this method are the absorption maximum of the individual constituent components.

Most of the methods available for simultaneous estimation of these three drugs are HPLC and spectroscopy methods. Literature review reveals that only few RP-HPLC methods[3-8] andUV spectrophotometric methods [9-15] were developed for the simultaneous estimation of TDF, EFV and LAM in bulk and tablet dosage form. Existing UV methods were developed using derivative technique and simultaneous equation methods. Sspectroscopictechniques are simple, faster and relatively less expensive. In the present study a UV spectrophotometric method was developed for simultaneous estimation of Tenofovirdisoproxilfumarate, Efavirenz, andLamivudine in pharmaceutical formulations and plasma samples using multi component mode.

MATERIALS AND METHODS

Materials and Equipments- Chemicals used for the development of method are methanol, sodium hydroxide, potassium dihydrogen orthophosphate, water and disodium EDTA. All the chemicals used are of HPLC grade and are procured from SDFCL, Mumbai.

TDF, LAM and EFV standards are Gift Sample from Aurobindo pharmaceuticals Pvt. Ltd, Hyderabad.

Double beam UV-Visible spectrophotometer (SCHIMADZU-1800) with UV probe 4.0 software was used for all spectrophotometric measurements.

METHOD DEVELOPMENT AND OPTIMIZATION

Selection of solvent

Solvent selection is the first step involved in the method development. Solvent is selected based on the solubility of the drugs. Five solvents were selected and five trails were done using water, methanol, buffer pH 5.0, methanol: water (50: 50) and methanol: phosphate buffer pH 5.0 (50: 50).

Trials were performed by with 10μg/mL concentration of all the three drugs separately in selected solvents and the absorption spectra were recorded and the suitable solvent was selected based on the absorbance observed for all the three drugs without interference. The overlay absorption spectra of three drugs in different solvents are shown in the figures 2, 3 and 4.

PREPARATION OF STANDARD AND SAMPLE SOLUTIONS

Preparation of standard solutions

Mixed standard solution containing 1000μg/mL of TDF and LAM and 2000μg/mL of EFV was prepared in methanol and phosphate buffer pH 5.0 (50:50). From this required working standard solutions were prepared by further dilution with the same solvent.

Determination of absorption maximum: 10μg/mL standard solutions of TDF, EFV & LAM were prepared separately and were scanned in the range of 200-400 nm to determine the absorption maximum for their quantitative determination. The overlay absorption spectrum is given in fig. 5.
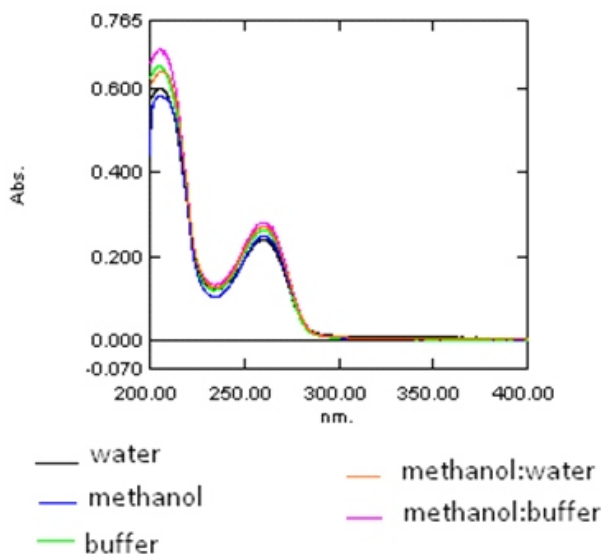

Fig. 2 : Overlay absorption spectrum of tenofovirdisoproxilfumarate in different solvents.
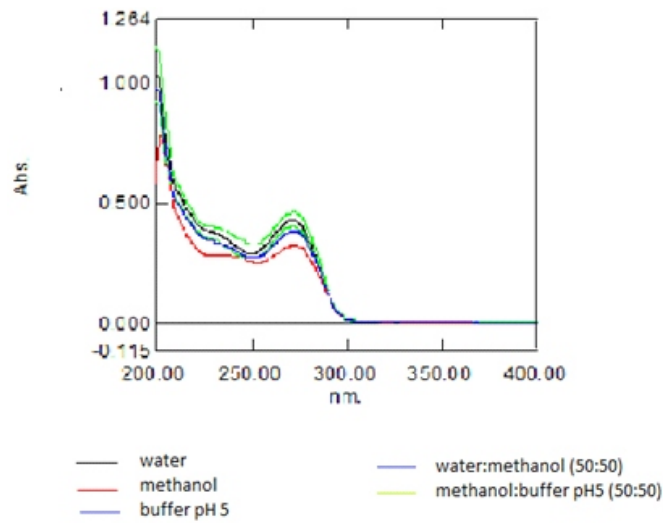
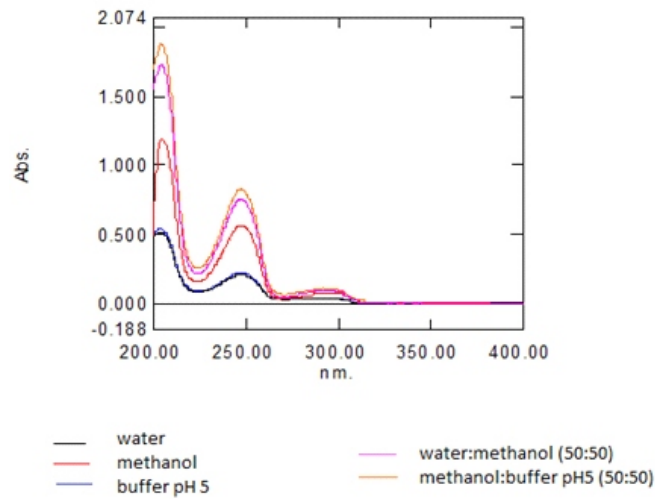**Fig. 3 :** Overlay absorption spectrum of lamivudine in different solvents

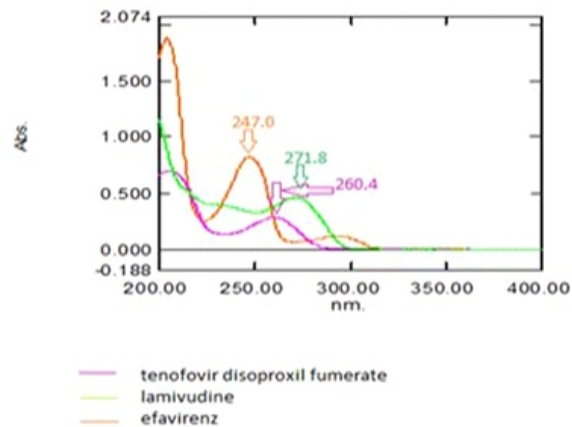**Fig. 4 :** Overlay absorption spectrum of efavirenz in different solvents

Fig. 5 : Overlayabsorption spectrum of TDF, LAM and EFV inmethanol: phosphate buffer pH 5.0 (50:50)

Table 1 : Optimized UV conditions and Spectral data

Method		Multi component method	
Solvent (diluent)		Methanol : Phosphate buffer pH 5 (50:50)	
Wavelength range		200-400nm	
Drug	Efavirenz	Tenofovirdisoproxilfumerate	Lamivudine
Wavelength for quantitation	247.0 nm	260.4 nm	271.8nm
Concentration range	0.4-24 µg/mL	0.2-12 µg/mL	0.2-12µg/mL
Regression equation	y=0.070x + 0.008	y=0.084x + 0.006	y=0.062x + 0.005
Slope	0.070	0.084	0.062
Intercept	0.008	0.006	0.005
Correlation Coefficient (r2)	0.999	0.999	0.999
Sandell's sensitivity (µg/cm2/0.001 absorbance units)	0.01266	0.01031	0.01370
Molar absorptivity (litre/moles/cm)	2.24×10^4	5.42×10^4	1.45×10^4
Limit of detection	0.127 µg/mL	0.063 µg/mL	0.065 µg/mL
Limit of quantification	0.385 µg/mL	0.192 µg/mL	0.198 µg/mL

Selection of analytical concentration ranges

From the working mixed standard stock solution , appropriate aliquots like 0.02, 0.1, 0.3, 0.6, 0.9, 1.2ml solutions were transferred and diluted with the diluent (methanol: phosphate buffer pH 5.0 50:50) to get calibration standards in the concentrations ranging from 0.2-12µg/ml of TDF & LAM and 0.4-24 µg/ml of EFV.

The absorbance of these solutions was measured at 247.0 nm, 260.4 nm and 271.8 nm against solvent.

METHOD VALIDATION

The method developed was validated according to the ICH Guidelines Q2 (R1): Validation of Analytical Procedures: Text and Methodology, for different validation parameters Linearity, Accuracy, Precision and Limit of detection and Limit of quantitation. [16]

Linearity

Linearity of the method was proved by taking standard solutions of pure drugs in the analytical concentration ranges0.2-12µg/ml of TDF & LAM and 0.4-24 g/ml of EFV. The absorbance of the solutions was measured at 247.0 nm, 260.4 nm and 271.8 nm against solvent blank. Calibration curves were constructed and linearity was verified by regression analysis. Linear regression equation and correlation coefficient for all the three drugs were reported.The sensitivity of proposed method for measurement of Tenofovirdisoproxilfumarate, Efavirenz and Lamivudine was estimated in terms of LOD & LOQ. They were determined using visual evaluation method and standard deviation method according to the International Conference of Harmonization (ICH) guidelines for the validation of analytical

procedure. The precision of an analytical method is the degree of agreement among individual test results, when the method is applied repeatedly to multiple samplings of homogeneous sample. It provides an indication of random errors in results and expressed as relative standard deviation (%RSD). Precision of the method is reported as repeatability, intraday and inter day precision.

Accuracy for drug substance was determined on samples of standarddrug solutions at varying concentration levels in the range of 80%-120% (4.8µg/mL, 6 µg/mL and 7.2 µg/mL for TDF & LAM and 9.6 µg/mL, 12 µg/mL and 14.4 µg/mL for EFV) by analyzing three replicates of each sample as a batch in a single assay.

Recovery studies (drug product)

The accuracy of the method for analysis of drug product was determined by taking 15 tablets. Tablets were weighed, powdered and the powder equivalent to 100 mg of TDF and LAM and 200 mg of EFV was transferred and the estimation was carried out. Recovery studies were carried out by adding known amount of standard drug (1.6µg/mL, 2µg/mL and 2.4µg/mL of TDF & LAM and 3.2µg/mL, 4µg/mL and 4.8 µg/mL for EFV) to the sample solution (2µg/mL of TDF & LAM and 4µg/mL of EFV).The % recovery was calculated and reported.

ANALYSIS OF FIXED DOSE COMBINATION TABLETS

Twenty tablets of fixed dose combination tablets (Tenolam E manufactured by Hetero Labs Ltd., Hyderabad containing 300mg, 300mg of TDF and LAM and 600mg of EFV) were weighed and finely powdered. The powder equivalent to 100mg was weighed and transferred to 100mL volumetric flask

Table 2 : Assay data

Brand name	Drugs	Label claim(mg)	Assay (%)
TANOLAME	TDF	300	92.493
	LAM	300	91.66
	EFV	600	92.24

Table 3 : Recovery studies data for drug product in rat plasma

Drug	Concentration of Sample + Standard (ng/ml) A	Total concentration (ng/ml)	Absorbance*	Concentration recovered (µg/ml) B	% Recovery a/b*100	Mean
TDF	100−100	200	0.038	196.03	98.01	
	100−400	500	0.145	479.47	95.89	97.11
	100−800	900	0.295	876.82	97.43	
LAM	100−100	200	0.033	191.87	95.94	
	100−400	500	0.122	481.30	96.26	96.34
	100−800	900	0.242	871.55	96.84	
EFV	200−200	400	0.041	378.02	94.51	
	200−800	1000	0.173	958.24	95.82	94.54
	200+1600	1800	0.337	1679.12	93.28	

* Average of 3 determinations

containing 25mL of solvent and sonicated for 30mins. The volume was made up to mark with methanol to obtain a solution of 1000µg/mL of TDF & LAM and 2000µg/mL of EFV. The solution was centrifuged at 2500rpm for 15 mins. This solution was further diluted to prepare 6µg/mL of TDF & LAM and 12µg/mL of EFV. The absorbance was measured at 247.0, 260.4 and 271.8nm against solvent blank and amount of drugs present in the formulation was calculated.

APPLICATION OF THE METHOD IN SPIKED RAT PLASMA

The developed method was optimised and validated for its application in rat plasma. [17]

Extraction of plasma

Blood samples were collected by retro-orbital puncture into vials containing disodium EDTA. Plasma was separated from blood samples by centrifugation at 1400 rpm for 7 minutes. Separated plasmalayer is collected and stored at -20 ^{0}C for further use.

Selection of extraction process

Liquid-liquid extraction process was selected for the extraction of drug from the spiked rat plasma solutions. Methanol was selected as the solvent for the extraction of drug spiked in plasma.The volume of the solvent used in extraction process should be optimized for the better extraction of the sample from the plasma. Two trails were done to optimize the volume of the solvent. In the first trial 300µL of the methanol was used and in the second trial 500µL for extraction of drugs from plasma. The absorbance of the extracted samples was measured at 247.0 nm, 260.4 nm and 271.8 nm and absorption spectrum was recorded and checked foe efficiency of extraction process.

Plasma treatment procedure

100µL of plasma sample was spiked with appropriate volume of the mixed standard stock solution and 500µL of methanol.The mixture was vortexed for 1 minute and centrifuged at 3000 rpm for 12minutes at 4 ^{0}C.The organic layer was separated & filtered through a 0.2µm cellulose acetate filter.The organic layer was

evaporated on a vacuum evaporator. The residue was reconstituted in 10mL of solvent and used for absorbance measurements.

The method was validated by reporting linearity and recovery studies in plasma samples. Linearity was determined by preparing series of calibration standards in plasma from the working mixed standard solution.Appropriate aliquots like 0.01, 0.02, 0.04, 0.06, 0.08 and 0.1ml solutions were pipette in to eppendorf tubes containing 100µL of plasma. 500µL of methanol was added and the extraction was carried out as mentioned above. After obtaining the residue, the residue was reconstituted with solvent to 10mL to obtain working standard concentration ranging from 100-1000ng/ml of TDF & LAM and 200-2000ng/ml of EFV. The absorbance measurements were taken at 247.0nm, 260.4nm and 271.8nm against treated blank plasma.

RESULTS AND DISCUSSION

New UV spectrophotometry method was developed and validated by Shimadzu UV-1800 instrument using multi-component mode.All the three drugs are soluble in water, methanol, phosphate buffer pH 5.0, methanol: water (50:50) and methanol: phosphate buffer pH 5.0.The overlay absorption spectra of individual drugs in all the solvents revealed that TDF and LAM has shown almost same absorbance in all water, methanol, phosphate buffer pH 5.0, methanol: water (50:50) and methanol: phosphate buffer pH 5.0 (50: 50). The maximum absorbance of all the three drugs was found in methanol: phosphate buffer pH 5.0 (50:50). So this was selected as solvent for further analysis. Overlay absorption spectrum of TDF, LAM and EFVin different solvents are shown in fig 4, 5 and 6.From the overlay absorption spectrum of TDF, LAM and EFV shown in fig 7, the absorption maximum of EFV, TDF and LAM were found to be 247.0, 260.4 and 271.8 nm respectively.In the concentration range of 0.2-12 µg/ml of TDF & LAM and 0.4-24µg/ml of EFV, there was a linear relationship between concentration and absorbance values and selected as Beers law range for proving linearity and quantitative determination.

Validation parameters

The linearity of the method was established by performing linear regression analysis for the calibration curve constructed between concentration and absorbance. Linearity was obtained in the concentration range of 0.2-12µg/ml of TDF & LAM and 0.4-24 g/ml of EFV. Overlayabsorption spectrum of mixed standard solutions for linearity is shown in fig. 8 and standard calibration curve data of TDF, LAM and EFV for above mentioned concentration ranges are given in table 1.The limit of detectionand limit of quantitation were calculated using visual evaluation and standard deviation methods. The optimised UV method conditions and spectral data are given in table 1.

The precision of the method was determined at three levels, repeatability, intra-day and inter-day precision. Precision of the assay procedure was assessed calculating the intra-and-inter day variation for each concentration. Therepeatability, intra-day variability and inter-day variability of the assay values are showing %RSD less than 2.5.

Accuracy of the method for drug substance and drug product was determined by reporting the deviation from true value and percent recovery values. The method was applied successfully for the determination of three drugs in the marketed formulation and the results obtained were in good agreement with the corresponding labeled amount. The results are shown in table 2.

The developed method was optimised and validated in spiked rat plasma. Liquid-liquid extraction method was selected for extraction of drugs from plasma. Good extraction efficiency was observed with methanol and volume of the solvent used was optimised by performing trials with 300µL and 500 µL. The absorbance of the extracted samples was measured and absorption spectrum was recorded for the spiked plasma solutions and better extraction was observed with 500 µL of solvent volume.

The method was validated in rat plasma by performing linearity and recovery studies. The method showed good linearity in the concentration range of100-1000ng/ml of TDF & LAM and 200-2000ng/ml of EFV in plasma. Overlay spectrum of linearity of TDF, LAM and EFV in rat plasma is shown in fig.6 and recovery studies data in table 3.

CONCLUSION

The developed method is effective for simultaneous estimation of tenofovirdisoproxilfumarate, lamivudine and efavirenz in fixed dose combination tablets and in rat plasma without any interference of other constitute in the formulation. The multicomponent method can be used for routine analysis of three drugs in fixed dose combination tablets and its application can be extended to preclinical and pharmacokinetic studies.

ACKNOWLEDGEMENT

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