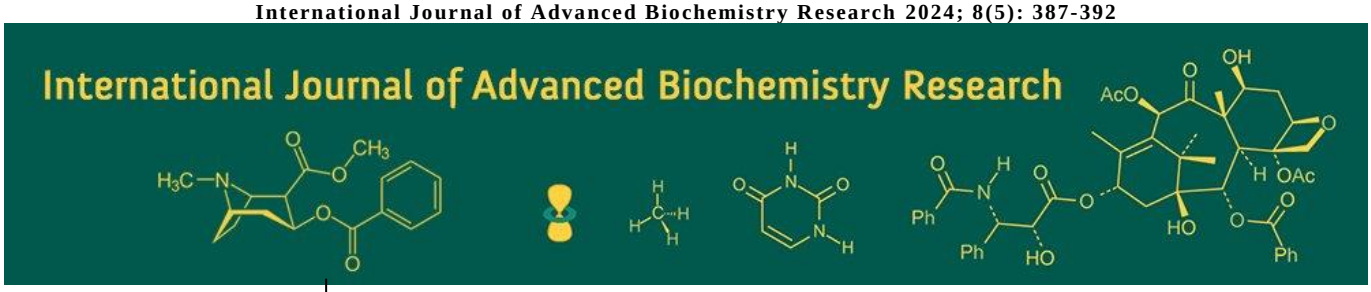

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Simultaneous UV-spectrophotometric determination of tertiary components in FDC of lamivudine, tenofovir disoproxil fumarate, and efavirenz in solid dose formulation

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Abstract
Developing a single spectroscopic analytical approach for estimating individual medications in formulations with multiple components is a difficult task. A simultaneous UV-spectrophotometric approach was used to analyze the antiretroviral (ARV) medications including Lamivudine (3TC), Tenofovir disoproxil fumarate (TDF), and Efavirenz (EFV) in a fixed drug combination (FDC). TDF, EFV, and 3TC had maximum absorptions at 230, 247, and 270 nm in 0.1 HCl, respectively. Method validation demonstrated repeatability and robustness, with linearity ranging from 2-10 ug mL^{-1} for 3TC, 1-5 ug mL^{-1} for TDF, and 10-50 ug mL^{-1} for EFV, respectively, with correlation coefficients (r) of 0.999, 0.998, and 0.998. The percentage drug contents for 3TC, TDF, and EFV were 98.44±1.26%, 97.52±0.72%, and 99.43±1.44%, respectively. The approach was free of excipient interference and could be utilized to detect multicomponent ARVs in FDCs.

Keywords: Lamivudine, tenofovir, efavirenz, antiretrovirals, fixed-dose

Introduction
Antiretrovirals (ARVs) are a class of medications that suppress various processes in human immunodeficiency virus (HIV) replication [1]. ARVs are an essential component of acquired immunodeficiency syndrome (AIDS) routine management [2]. ARV medications include viral entry or fusion inhibitors [3], uncoating inhibitors [4], nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs) [5], non-nucleoside reverse transcriptase inhibitors (NNRTIs) [6], integrase inhibitors [7], protease inhibitors (PIs) [8], virion release inhibitors [9], etc. HIV-related deaths have reduced in recent decades due to the use of ARVs [10]. Thus, persons living with HIV now have a higher quality of life and a longer lifespan [11].
The World Health Organization (WHO) recommends using Highly Active Anti-Retroviral Therapy (HAART) [12]. Reverse transcriptase inhibitors were the first medications approved for HIV/AIDS treatment [13]. NRTIs like Zidovudine, Lamivudine, Abacavir, Emtricitabine, Tenofovir, etc., impede the replication cycle by inhibiting viral reverse transcriptase enzyme required for the synthesis of viral DNA [14, 15]. NNRTIs, including Nevirapine, Efavirenz, and Etravirine, have structural similarities to NRTIs [6, 16, 17, 18]. However, they attach to an allosteric site on the reverse transcriptase enzyme [19]. Indinavir, Ritonavir, Lopinavir, Atazanavir, Darunavir, and other PIs inhibit the activity of viral protease enzymes necessary to proliferate new viral particles [20, 21].
HIV infects immune cells by inserting itself into their DNA via viral enzymes on their surface (CD4+ T-helper cells). The virus then uses the host cell's processes to multiply and infect other cells [22]. In the cytoplasm, viral reverse transcriptase transforms HIV RNA into viral DNA, which enters the cell nucleus and is integrated into the host DNA strand by the viral integrase enzyme. As normal cellular DNA transcription occurs, HIV DNA within the human strand is transcribed, creating HIV-derived mRNA [23, 24]. Viral protease then transforms the mRNA into the proteins needed to generate new HIV viral particles. If this process is not regulated, the HIV infection will quickly spread throughout all CD4+ immune cells, eventually depleting and eradicating them [25, 26].

Analytical Techniques are the procedures used for the qualitative and quantitative determination of a compound's concentration utilizing various techniques such as titrations [27, 28], spectroscopy [29], and chromatography [30, 31, 32], among others. A literature search turned up a few analytical methods for detecting lamivudine, tenofovir disoproxil fumarate (TDF), and efavirenz in pharmaceutical products in mixed formulations. The use of reverse-phase high-performance liquid chromatography (RP-HPLC) [33]. Emtricitabine, rilpivirine, and tenofovir in bulk dosage forms were separated and quantified using RP-HPLC, which was regarded as simple, rapid, precise, and dependable. The method was validated as a final verification of the development of the method concerning precision, linearity, accuracy, ruggedness, and robustness, and was successfully adapted to the commercially available pharmaceutical dosage form, yielding very good and reproducible results [17, 34, 35]. A first-order derivative spectrophotometric approach, a derivative spectroscopy method, and a simultaneous UV spectroscopic method were used to determine combination formulations in solid dosage form using RP-HPLC [36, 37].

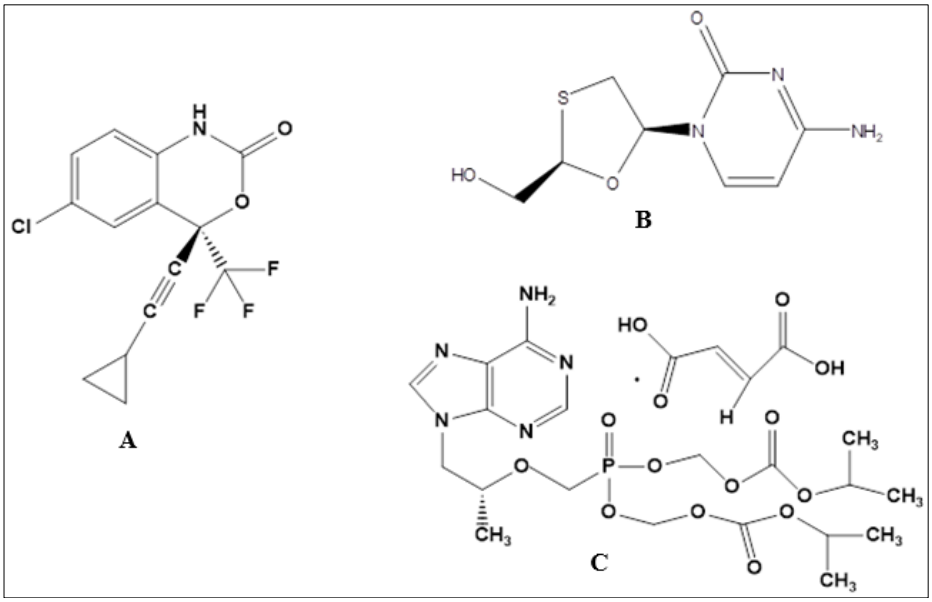

Fig 1: Structure of Efavirenz (A), Lamivudine (B), and Tenofovir disoproxil fumarate (C).

A simple, sensitive, quick, cost-effective, and accurate first-order derivative spectrophotometric approach has been devised to estimate TDF, 3TC, and EFV in bulk and tablet dose forms. The methods were validated according to ICH recommendations [38]. The use of HPTLC for determining tertiary component FDC has also been documented. Separation was accomplished using an HPTLC precoated plate with silica gel60F254 and a mobile phase with toluene: methanol (27:6%v/v). The regression line had the best fitting using a second-order polynomial equation for each of the APIs with r2 ≥ 0.98 at concentrations ranging from 375-900 ng/spot for 3TC, TDF, and 750-1800 ng/spot for EFV, respectively, with a satisfactory level of accuracy, mean recoveries in the range of 98 to 103%, and a high degree of selectivity for the APIs [39]. Several other methods for simultaneous qualitative measurement of pharmaceutical goods have been proposed [40, 41]. The study's purpose was to develop a simple, accurate, precise, fast, cost-effective, and reproducible UV spectrophotometric method for measuring LAM, TDF, and EFV, as well as bulk and pharmaceutical formulations, using commonly available materials, reagents, and equipment.

Materials and Methods

Reagents and equipment: All spectral measurements were performed on a JENWAY 6305 model ultraviolet-visible spectrophotometer equipped with 1.0 cm matched quartz cells, a centrifuge, a plastic cuvette, and a UV lamp at 254 nm. MeOH (Guangdong Guanghua Chemical China) and toluene (Mallinckrodoc Baker Inc. Phillipsburg, NJ 08865 USA; PH (908)852-215). All reagents used were analytical grade. Analytical weighing balance and micropipette (Microlux) were previously calibrated. The fixed-dose version of LAM, TDF, and EFV containing 300mgLAM/300mgTDF/600mgEFV was manufactured by Fabrique par: Mylan Laboratories, India.

Preparation of 0.1M HCl, 3TC, TDF, and EFA reference standard solutions

In a 100 ml volumetric flask, 0.85 ml of concentrated hydrochloric acid was mixed with an equal volume of pure water and diluted to the mark. To prepare stock standard solutions of 3TC, TDF, and EFV, 50 mg of each powder (≥ 99.4% purity) were weighed into separate 50 mL volumetric flasks. 5 mL of MeOH was added, shaken gently to homogenize, and marked with MeOH to obtain a standard stock concentration of 1000µg/mL, from which all working concentrations were prepared.

Determination of maximum absorption (λ_{max}), and Calibration curve for 3TC, TDF, and EFV

A working dilution of 5ug/mL of the standard stock solutions was produced in 0.1M HCl and scanned from 200 to 350nm to achieve the maximal absorption of 3TC, TDF, and EFV. Serial dilutions of 3TC, TDF, and EFV reference standards were generated at concentrations ranging from 2 to 10 ug/mL, 10 to 50 ug/mL, and 1 to 5 ug/mL, respectively, at each corresponding maximum absorption. The calibration curves were shown as absorbance versus concentration.

Thin layer chromatography (TLC) solvent system, Sample, and Reference standard

The solvent system for TLC was developed by mixing chloroform and MeOH toluene in a 9:1.2:0.3 ratio. A weight equivalent to 25mg of 3TC/TDF and 50 mg EFV in powdered FDC tablet was put into a 25ml volumetric flask containing 3mL methanol, which was gently agitated for 2 minutes to dissolve the drug components before being diluted to mark with the same solvent. TLC was performed using each of the prepared reference standards.

Identification and separation of 3TC, TDF, and EFV using TLC: The TLC plate was pre-washed with MeOH and activated in an oven at 50 ℃ for 5 minutes, and allowed to saturate for 20 minutes. The FDC drug solution and standard sample from the stock solution of 3TC, TDF, and EFV were spotted on the TLC plate with capillary tube and transferred to the TLC chamber having chloroform: MeOH: toluene [9:1.2:0.3 (v/v/v)] as mobile phase, for 10 minutes. The developed TLC was viewed under a UV lamp, and areas of compounds were marked. The Rf values of LAM, TDF, and EFV were computed respectively using the expression: Retention Factor (Rf) = Distance moved by eluent (Solute)/Distance moved by solvent.

Simultaneous Equation Method for quantification

This analytical method was based on drug absorption at their respective wavelength maxima (λ_{max}). To build simultaneous equations, three wavelengths (230, 247, and 270nm) representing the λ_{max} of three APIs were selected. The absorbances of EFV, TDF, and LAM were measured, and absorptivity values were determined for all three wavelengths.

Results and Discussion

Identification of EFV, 3TC, and TDF in drug samples by TLC: Figure 2 depicts the TLC for the FDC of EFV/TDF/3TC, as well as the individual reference standards. Three unique and well-separated spots were discovered in the FDC sample, and each drug component was identified using corresponding reference spots and retention factors (Rf) on the TLC plate. The presence of three distinct dots indicates the absence of contaminants or metabolites in the tablet FDC medication formulation. The Rf were as follows: Lamivudine 0.4 (2.4/6), Tenofovir disoproxil fumarate 0.75 (4.5/6), and Efavirenz 0.9 (5.4/6).

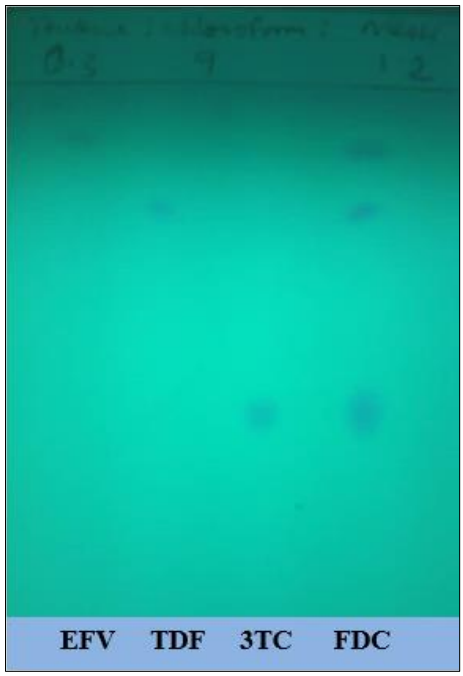

Fig 2: TLC plate for identification of reference standard and FDC of 3TC, TDF, EFV

Determination of maximum absorption (λ_{max})

Figure 3 depicts the spectra obtained for EFV, TDF, and 3TC reference standards, which had maxima absorptions at 230 nm, 247 nm, and 270 nm, respectively, and they overlapped. The overlain spectra made it difficult to quantify these ARVs in the FDC using the zero-order derivative.

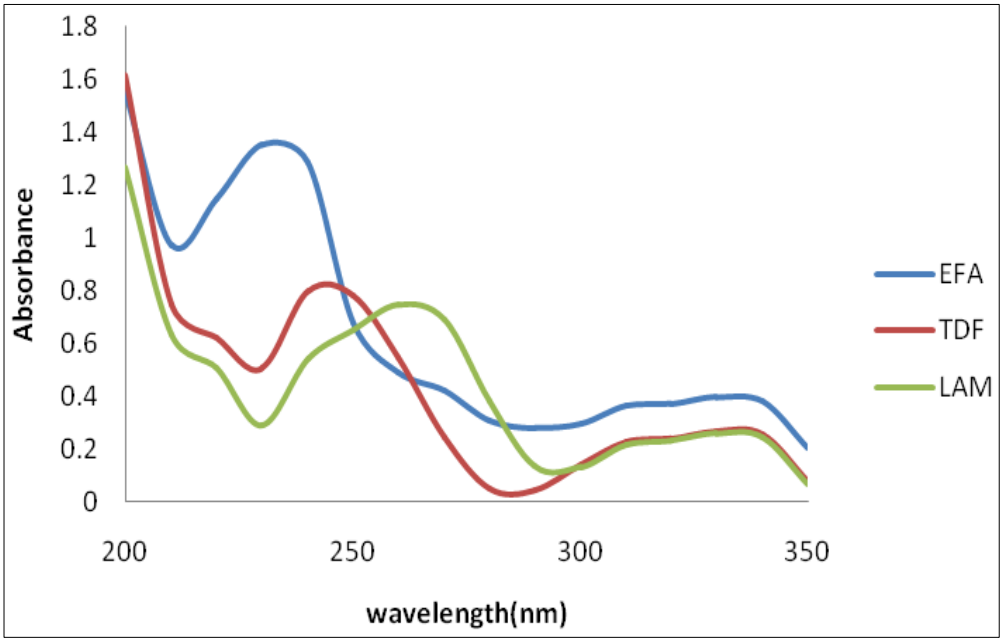

Fig 3: UV spectra of Efavirenz (EFV), Lamivudine (3TC) and Tenofovir disoproxil fumarate (TDF)

Analytical performance

The analytical performance of the proposed method was validated following the ICH guideline (ICH, 2005). The optimum experimental conditions for the quantification of EFV, 3TC, and TDF using simultaneous UV spectrophotometry are presented in Table 1. The calibration curve showed good linearity and obeyed Beer's Law with concentrations ranging from 2-10 ug/mL, 10-50 ug/mL, and 1-5 ug/mL for 3TC, TDF, and EFV respectively.

Table 1: Optical characteristics of lamivudine, tenofovir disoproxil fumarate, and efavirenz by simultaneous UV-spectrophotometric Method.

	Component		
Parameter	**3TC**	**TDF**	**EFV**
Max. absorption (λ_{max}) (nm)	270	247	230
Beer's law limit (ug/mL)	2-10	10-50	1-5
Correlation coefficient (r)	0.999	0.998	0.998
Regression equation			
Slope (m)	0.057	0.022	0.077
Intercept	0.436	0.497	0.352
LOD	0.89	1.85	0.33
LOQ	2.67	5.55	0.99

The regression equations were obtained with the aid of Microsoft Excel, using the least-square method. The intercept and slope ranged from 0.022-0.077 and 0.352 to 0.496 respectively for the three ARVs. The correlation coefficient (r) was 0.999, 0.998, and 0.998 for 3TC, TDF and EFV respectively. The LOD values were 0.89, 1.85, and 0.33 for 3TC, TDF, and EFV respectively, with corresponding LOQs being 2.67, 5.55, and 0.99. These LOD and LOQ values confirm the repeatability and reliability of the method.

Simultaneous equation method for quantification of 3TC, TDF, and EFA: The application of simultaneous UV-spectrophotometry for the determination of 3TC, TDF, and EFV in a 0.1M HCl solvent system is based on the inertness or non-reactiveness between any of these three drug components. The concentrations of 3TC, TDF, and EFV were calculated using the simultaneous equations given below:

Equations,

$A_1 = E_{x1}C_x + E_{y1}C_y + E_{z1}C_z$. at λ_1 (230 nm, λ_{max} of EFV) (1)

$A_2 = E_{x2}C_x + E_{y2}C_y + E_{z2}C_z$. at λ_2 (247 nm, λ_{max} of TDF) (2)

$A_3 = E_{x3}C_x + E_{y3}C_y + E_{z3}C_z$. at λ_3 (270 nm, λ_{max} of 3TC) (3)

Where,

A_1, A_2, A_3 are the absorbance of the mixture at 230 nm (λ_1), 247 nm (λ_2), 270 nm (λ_3) respectively and,

C_x, C_y, and C_z are the concentrations of EFV, TDF, and 3TC respectively

E_{x1}, E_{x2}, and E_{x3} are the absorptivity of EFV at 230, 247, and 270 nm respectively

E_{y1}, E_{y2}, and E_{y3} are the absorptivity of TDF at 230, 247, and 270 nm respectively

E_{z1}, E_{z2}, and E_{z3} are the absorptivity's of 3TC at 230, 247 and 270 nm respectively

Recovery studies

Table 2 illustrates the recovery studies that were used to assess the performance of the simultaneous UV-spectroscopic approach. The study involved spiking pure 3TC, TDF, and EFV at three concentration levels with FDC powdered tablets in 0.1 M HCl solution containing 2, 2, and 4 µg mL-1, respectively. The aforementioned were fortified with doses ranging from 1 to 6 µg mL-1 of the reference standards (3TC, TDF, and EFV). The average percentage recoveries for spiked analytes ranged from 93.82±0.94% to 105.92±2.96%. These figures reflected the method's robustness and reliability.

Table 2: Recovery Studies for EFV, 3TC, and TDF in FDC

Drug	Amount of drug in sample taken (ugmL^{-1})	Amount Pure drug spiked (ugmL^{-1})	Amount found (ugmL^{-1})	% Recovery	Mean Recovery (%) ±SD
3TC	2	2	2.12	106.00	
	2	3	3.29	109.51	
	2	4	4.09	102.25	105.92±2.96
TDF	2	1	0.93	92.70	
	2	2	1.9	95.00	
	2	4	3.75	93.75	93.82±0.94
EFV	4	2	2.24	112.00	
	4	4	3.98	99.51	
	4	6	6.19	103.21	104.89±5.25

Application of the method for the quantification of 3TC, TDF, and EFV **in dosage formulation**

The proposed method was effectively used in the analysis of FDC medication with 3TC, TDF, and EFV. The results are shown in Table 3. The amount of medicines discovered in FDC was consistent with the label claims for the three ARVs. The percentage contents of 3TC, TDF, and EFV were 98.44±1.26, 97.52±0.72, and 99.43±1.44%, respectively. The student t-test for drug quantities in FDC formulation was ≤ 1.224 for four replicates, which is less than the tabulated value of 3.182 at a 95% confidence level with three degrees of freedom (v). The student t-test results indicate that there is no significant difference between the label claim and the amount of ARVs discovered in FDC for the method used. The simultaneous technique offers the advantage of being free of interferences induced by excipients such as lactose, starch, and glucose. These excipients are insoluble in water, and the formulations do not contain any chromogenic substances that could interfere with UV absorbance measurements. The absence of interference demonstrates the method's great selectivity and usefulness for the study of solid dose formulations.

Table 3: Application of simultaneous UV-Spectrophotometric method to tablet formulation

Drug Components	Label claim (mg/tablet)	Quantity found ±SD (mg/tablet)	%RSD	SEM	Content (%)
3TC	300	295.33±3.77	1.26	2.18	98.44±1.26 t = 1.224
TDF	300	292.57±2.15	0.72	1.24	97.52±0.72 t = 1.095
EFV	600	596.59±8.63	1.44	4.98	99.43±1.44 t = 0.984

*Student *t*-test is concerning the label claim of ARVs in FDC. Theoretical *t*-distribution (at $v = 3$, n = 4) at 95% confidence level is 3.182.

Conclusion

The current approach relies on simultaneous UV spectroscopy in 0.1 M HCl. The suggested method was effectively applied to the investigation of EFV, TDF, and 3TC in fixed dosage combination form, demonstrating its ability to resolve overlapping or overlain spectra in multi-component formulations. Furthermore, it has demonstrated remarkable precision, accuracy, selectivity, and repeatability while remaining interference-free. However, applying the simultaneous equation for tertiary components in medication formulations needs a strong mathematical background, which may be a barrier for certain scientists.

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