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Comparative application of derivative spectrophotometric and HPLC techniques for the simultaneous determination of lamivudine and tenofovir disoproxil fumarate in fixed-dose combined drugs

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Abstract

Background Lamivudine (LAM) and tenofovir disoproxil fumarate (TDF) are part of a fixed-dose combination (FDC) therapy recommended by WHO. Both drugs exhibit similar solubility in many solvent systems and tend to have overlapping spectra with maxima at 260 and 270 nm, respectively, in the UV spectrum—thus making their spectrophotometric assay difficult in FDCs. A third-order derivative (D_3, $d^3A/d\lambda^3$) spectrophotometric technique was applied to simultaneously evaluate TDF and LAM in FDC drugs, with amplitudes at 240 and 262.5 nm, respectively. Pharmacopoeia-recommended chromatographic method was also applied for comparative purpose.

Results Method performance by the proposed D_3 technique showed linearity for LAM and TDF from 2–10 µg mL^{-1} to 8–24 µg mL^{-1}, respectively ($R^2 \geq 0.998$), while for HPLC method both drugs ranged from 0.25 to 5.0 µg mL^{-1} ($R^2 \geq 0.999$). The intercepts and slopes of the regression equations were $\leq 1.62 \times 10^{-4}$ and $\leq 3.58 \times 10^{-5}$, respectively, while calculated standard errors were $\leq 8.04 \times 10^{-5}$. Limits of detection and quantification for both methods were ≤ 0.46 µg mL^{-1} and ≤ 1.40 µg mL^{-1}, respectively, for LAM, while corresponding limits for TDF were ≤ 2.61 and ≤ 7.90 µg mL^{-1}. The percentage recovery for both drugs and methods ranged from 94.80 to 100.33%. The amount of LAM and TDF in brands I and II was $\geq 99.59 \pm 1.19\%$ and $\geq 99.39 \pm 0.63\%$, respectively, for the proposed D_3 spectroscopic method, while corresponding values for the HPLC method were $\geq 99.86 \pm 0.50$ and $\geq 99.87 \pm 0.32\%$. Statistically, both methods were adjudged to have no significant difference at 95% confidence level as the student's *t*-test values; experimental paired *t*- and *F*-test values were found satisfactory.

Conclusion The D_3 spectrophotometric technique was time saving, cheap, simple and more environmental friendly and shows reliability, precision and accuracy and could be used for routine analysis of FDCs where HPLC is not available.

Keywords Derivative spectrophotometry, Lamivudine, Tenofovir disoproxil fumarate, Third order, Comparative, Antiretrovirals

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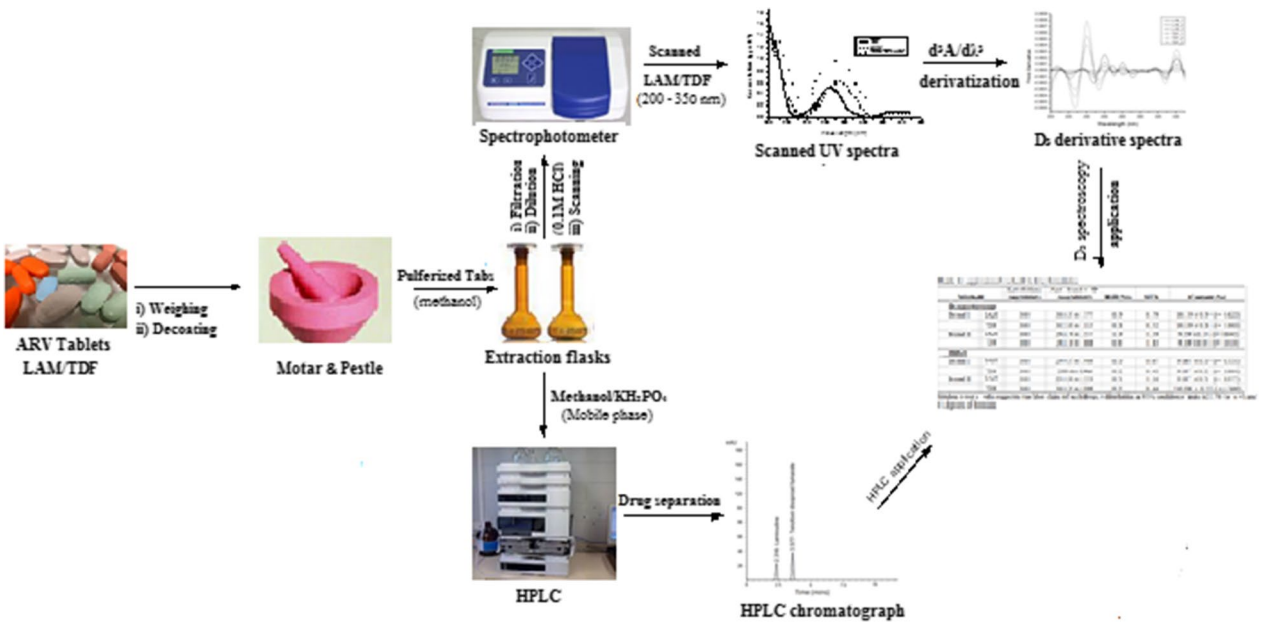
Graphical abstract



Background

Fixed-dose combination (FDC) therapy of lamivudine (LAM) and tenofovir disoproxil fumarate (TDF) is part of the three antiretroviral drugs recommended by WHO—and it is among the preferred first- and second-line regimens for adolescents, adults, children and infants [1]—the third being efavirenz, a generation non-nucleoside reverse transcriptase inhibitor (NNRTI). This therapy is also referred to as highly active antiretroviral therapy (HAART), and it is believed to be the most effective treatment in slowing HIV-1 infection progression and retarding the emergence of resistant mutants [2, 3]. Generally, its use has improved and alleviated the challenges faced in the management and treatment of people living with AIDS [4, 5].

Lamivudine, 2′,3′-dideoxy-3′-thiacytidine-4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one (Fig. 1a)—a nucleoside reverse transcriptase inhibitor (NRTI) prodrug analogue of dideoxycytidine—is known to be active against human immune deficiency virus (HIV) [6]. It requires three phosphorylation steps intracellularly, to elicit its pharmacological active anabolite, lamivudine triphosphate [7, 8]. The nucleoside analogue is infused into viral DNA by HIV reverse transcriptase and HBV polymerase leading to the DNA chain termination. Tenofovir disoproxil fumarate (TDF) (Fig. 1b) is an acyclic nucleoside

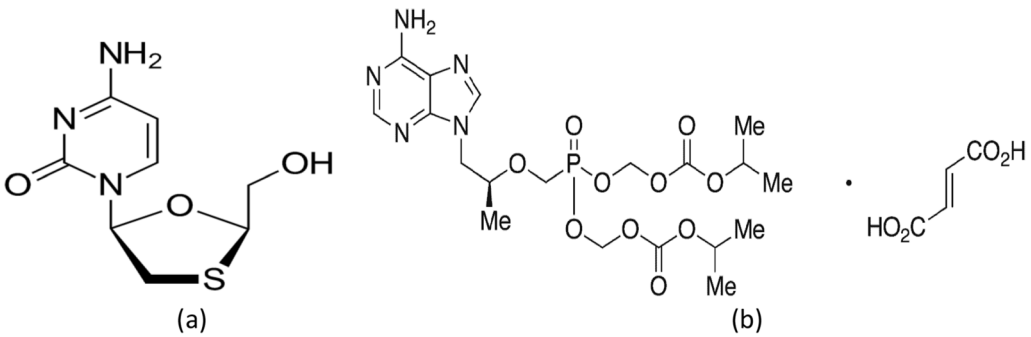


Fig. 1 Chemical structure of **a** lamivudine and **b** tenofovir disoproxil fumarate

phosphonate diester analogue of deoxyadenosine 5′-monophosphate, chemically named 9—[(R)-2-[[bis [[isoproxy carbonyl) oxy] methoxy] phospinyl] methoxy propyl], that belongs to the class of antiretrovirals—nucleotide reverse transcriptase inhibitors (NtRTIs). It acts by blocking the enzyme reverse transcriptase that is pivotal to viral production in persons infected by HIV [9, 10]. The active form of TDF is the diphosphate metabolite—tenofovir-diphosphate (TFV-DP) that arises from its inhibition of reverse transcriptase, by competing with the natural substrate deoxyadenosine 5′-triphosphate through intracellular phosphorylations [11], leading to the termination of the DNA chain by its incorporation [12].

LAM and TDF, like most anti-retrovirals, do exhibit similar solubility in many solvent systems (such as ethanol, methanol, dilute mineral acids) and have maximum absorptions (λ_{max}) between 250 and 280 in these solvents [5, 13, 14]. The spectra of these drugs are either overlaying or overlapping, hence the difficulty in separating multicomponent FDCs into their components [5]. The aforementioned difficulties associated in assaying multicomponent antiretrovirals have been resolved with the application of HPLC techniques as recommended by various pharmacopeias [15, 16]. However, in developing countries, access to functional HPLC is limited, because of its astronomical cost and the availability of associated accessories and consumables [5]. The UV–visible spectrophotometry is relatively cheaper and easier instrument for assaying pharmaceuticals. However, to overcome the occurrence of overlain spectra, the use of derivative UV-spectrophotometry has been applied for the evaluation of different drug compounds [17–19]. Other reported methods are simultaneous UV spectrophotometric method [13, 14, 20] and TLC-UV-spectrophotometric method [5]. Derivative spectrophotometry (DS) offers a range of applications, which are more reliable with respect to the normal spectrophotometry. Other spectra derivative techniques such as ratio derivative (RDS), difference derivative (DD) and compensation method (CM) have been found very usefulness in the assay of pharmaceuticals in binary mixtures [21]—these techniques are computer oriented. It has been used to resolve overlaying or overlapping spectra of multicomponent FDCs simultaneously [17, 22], determination of trace analytes in various matrices, amino acids and protein assay, environmental analysis, identification of organic and inorganic substances [23]. In addition, it has been widely applied for quantitative analysis, characterization and quality assurance in the agro and pharmaceutical industries and in biomedical-related disciplines [17, 22]. These outstanding features are mainly due to its enhanced sensitivity, selectivity, specificity and the elimination of spectral interference [24, 25]. It is also characterized by simplicity, rapidity and reproducibility. The aforementioned advantages are as a result of its spectral differential and resolution enhancement, quantitative and qualitative methods that distinguishes small variation between almost similar spectra [22]. The versatility of derivative spectroscopy (DS) is hinged on the associated data processing techniques—which comprise of zero-crossing, least-square deconvolution, Fourier transforms, etc.

This study is aimed at evaluating the application of derivative spectrophotometric method—by referencing it with the pharmacopeia recommended HPLC–UV technique for the estimation of LAM and TDF in FDC formulations.

Methods

Apparatus

Ultraviolet–Visible spectrophotometer—JENWAY 6305 model, with 1.0-cm quartz cells, was used for all spectral measurements. The analytical weighing balance (Sartorius MSU66S Model) and Eppendorf micropipettes used were previously calibrated. Other instruments/equipments used were Hp Probook 6550b laptop, Microsoft excel 2007 and OriginLab, 2019 software.

Agilent HPLC, model 1200 series was used for the quantification of LAM and TDF. The instrument was inter-phased to a UV-detector and quaternary pump, using a RP18, ODS, octadecyl column (5 µm, 150 × 4.6 mm, ZORBAX Eclipse XDB-C18), for chromatographic separation. Elutions were performed using mobile phase made up of methanol (70%, v/v) and 10 mM KH_2PO_4 (30%, v/v), with a flow rate of 1 mL min^{-1} at ambient temperature.

Materials and reagents

Chemicals used were of HPLC and spectroscopic grade. The potassium dihydrogetn phosphate and methanol were manufactured by SIGMA-ALDRICH GmbH, Germany, while the concentrated HCl was manufactured by Merck, Darmstadt, Germany. Lamivudine (LAM) and tenofovir disoprixil fumarate (TFD) standards were gifted from NAFDAC, Yaba, Lagos, and made by European Directorate of Quality Medicine (EDQM). The FDC drug samples—lamivudine/tenofovir disoprixil fumarate (300/300 mg), were gifted by the Federal Medical Center, Yenagoa, Nigeria, and manufactured by Mylan Laboratories Ltd., Hyderabad, Telangana, India, and Hetero Laboratories Ltd Telangana, India.

Preparation of reagents

(i) Hydrochloric acid (0.1 M) reagent: Transfer 8.5 mL concentrated HCl acid to 100 mL of distilled water

in 1-L volumetric flask, and make to mark with distilled water.

(ii) Lamivudine and tenofovir disoprixil fumarate standard solutions: Weigh 50 mg of LAM and TDF standards into separate 50-mL volumetric flasks, dissolve with 5 mL methanol, and make to volume with 0.1 M HCl to obtain a stock solutions of 1000 µg mL^{-1} each.

Analytical techniques

Zero-order derivative spectra and determination of maximum wavelength (λmax)

Procedure by Vaikosen et al. [5] was adopted. Separate solutions of LAM and TDF reference standards and combined standards (LAM/TDF) as formulated in binary FDC were prepared from stock solutions in 0.1 M HCl. These solutions were scanned in the UV region (200 to 350 nm) to obtain individual and combined drug spectra. The maximum absorptions (λ_{max}) of each drug in 0.1 M HCl were then obtained from the spectra.

Derivative of spectra

To resolve LAM and TDF spectra overlap, their derivatives (1st–4th) were calculated and corresponding spectra plots were done using OriginLab and Microsoft Excel 2007 softwares. The most appropriate of the four derivatives was chosen.

HPLC–UV method

Aliquots of clear drug solutions were diluted with the mobile phase mixture to obtain appropriate concentrations, and 10 µL was injected into instrument. Three injections per FDC brand were made, while peak areas of each drug were computed. The amounts of the anti-retrovirial drugs—LAM and TDF—were determined from their calibration curves.

Method validation

The analytical performances of methods were assessed in accordance with ICH guidelines [26] and in addition, by applying the proposed methods to formulated FDC drugs. Under the ICH guidelines, the following parameters, specificity, interference, precision, accuracy, linearity, sensitivity, ruggedness, and robustness, were studied. The linearity and sensitivity of the methods were established by carrying out a five point calibration curve for standards. The least squares method was used to obtain the regression equations and other parameters. The limits of detection (LOD) and quantification (LOQ)—which depicted sensitivity of methods—were evaluated using the expressions, $LOD = 3.3\ S_d/x$; $LOQ = 10\ S_d/x$ (where S_d is the standard deviation of the intercept of regression line and "x" is the slope of the regression line) [27, 28]. The ruggedness methods were assessed by applying both to assay brands of FDC antiretroviral drugs—this measured the reliability of methods for routine laboratory quality assessment. The recovery studies were done by spiking drug samples containing 1.0, 1.5, 2.0 and 2.5 mg of LAM, with drug standard at concentrations 2.0, 1.5, 1.0 and 2.5 mg, respectively, while for TDF, sample containing 10.0, 12.5, 15.0 and 10.0 mg was spiked with 2.0, 2.5, 5.0 and 10.0 mg pure drug standard. The intra-day and inter-day precision was determined by replicate analysis at four concentration levels—4, 6, 8, 10 µg mL^{-1} for LAM and 8, 10, 15 and 20 µg mL^{-1} for TDF, while for HPLC–UV, levels were at 0.5, 1.0, 2.5 and 4.0 µg mL^{-1}; these were spiked with 1, 2.5, 5, 2.5 and 1.0 µg mL^{-1}, respectively, for recovery studies. The concentrations for intra-day and inter-day studies were 0.5, 1.0, 2.5, and 5 µg mL^{-1}. The drugs were replicated thrice on the same day, while the inter-day assay was done on 3 days—every other day, within a week using the same concentrations and two brands of the FDC.

Calibration graphs of D_3 spectra for drug standards

A five-point calibration curve for the third–order derivative spectra was prepared by carrying out serial dilutions from stock solutions of reference standards. The spectra were measured at two wavelengths with respect to the order of the derivative, where zero crossing was observed for TDF and maximum for LAM (λ_{max} for LAM) and conversely zero crossing for LAM and a maximum for TDF (λ_{max} for TDF). The values for D_3 amplitudes were obtained for concentration ranges of 8–24 µg mL^{-1} and 2–10 µg mL^{-1} for TDF and LAM, respectively. The absorbance values were plotted against the concentrations of the solutions to obtain a straight line calibration curve, while amount of drugs in FDC was deduced for test samples.

The standard solutions for the calibration of LAM and TDF for the HPLC method were prepared from stock solutions to obtain co-mixed standards in the mobile phase. The working concentrations for a five-point calibration curve and drug quantification in brands ranged from 0.25 to 5.0 µg mL^{-1} for both drugs.

Procedure for simultaneous extraction and application of methods to drugs

Derivative spectroscopic method

An equivalent of 50 mg each of LAM and TDF in pulverized FDC tablets was weighed and transferred into a 25-ml calibrated volumetric flask, shaken gently with 10 ml of methanol for about 2–3 min and then made to volume with methanol. This solution was filtered into a clean volumetric flask, with the first 5 mL of the filtrate

discarded. Appropriate dilutions were made to obtain concentrations within the working range for each analyte using 0.1 M HCl. The absorbance of the solution was measured at two wavelengths from the derivative spectra, where zero crossings were observed for TDF (λ_{max} for LAM) and LAM (λ_{max} for TDF).

HPLC–UV method

For the high-performance liquid chromatographic, suitable aliquots of clear drug extract were diluted with the mobile phase and 10 µl was injected into instrument. Three runs were made for brand and the peak areas of the drugs were computed, while the quantities of each in FDC tablets were determined from the regression equations obtained.

Statistical analysis

The statistical analyses were carried out using OriginLab80 (Origin, China, 2019 version) and Microsoft Office Excel 2010.

Result

Zero- and higher-order derivative spectra of TDF and LAM

Figure 2 shows the zero-order derivative spectrum of TDF, LAM and overlay of co–mixed standards (TDF/LAM as found in FDC tablets). The maximum absorptions for LAM and TDF were observed at 270 and 260 nm, respectively, and there was significant spectra overlap between LAM and TDF reference standards, while the co–mixed showed a single maximum absorption at 260 nm. Figure 3 represents four different orders of derivative UV spectra for LAM and TDF standards. The third-order (D_3) and fourth-order derivative spectra (D_4) were found satisfactory; however, the D_3 spectrum was found to be the more appropriate, with the overlapping of both drugs properly resolved (Fig. 3c). The amplitudes at 240 nm and 262.5 nm showed maxima for TDF and LAM, respectively—these also corresponded to the zero-crossing points for LAM and TDF. The observed maximum amplitude for LAM in this study is close to the amplitude of 265.6 nm reported by Uslu and Özkan [17] in the first derivative spectra of lamivudine. Figure 4 shows the linear response of both drugs at three levels of concentration for the third-order derivative spectra with alternate maxima and zero-crossing points for LAM and TDF to enable their simultaneous quantification.

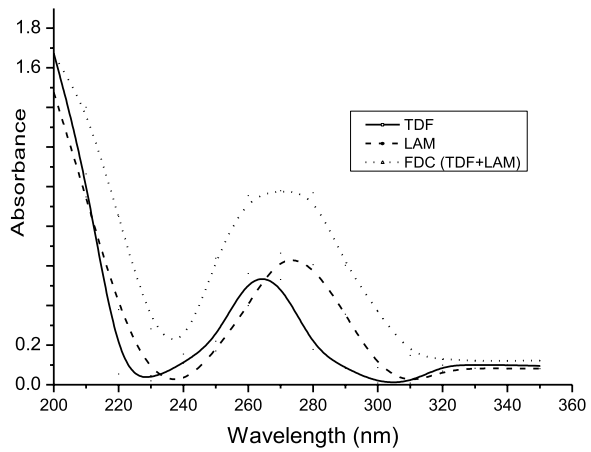


Fig. 2 Zero-order derivative spectra for TDF, LAM, and mixture of TDF + LAM standards

HPLC assay of FDC TDF and LAM

Figure 5 shows the HPLC chromatogram for LAM and TDF standards, with retention times at 2.316 and 3.577 min, respectively. Both drugs were detected at an optimum wavelength of 254 nm, and their peaks were well resolved.

Analytical performance of methods: derivative spectroscopic and HPLC methods

The results obtained for the measurement of performance of methods are as enumerated in the sections below.

Linearity range and sensitivity

The calibration graphs for the D_3 spectrophotometry using Beer's law plot (n = 5) for LAM and TDF showed good linearity at concentration ranges of 2–10 µg mL^{-1} and 8–24 µg mL^{-1}, respectively (Table 1). The regression equations were obtained using the least squares method, with very small intercepts ($\leq 1.62 \times 10^{-4}$) and slopes ($\leq 3.58 \times 10^{-5}$). Calculated standard errors were $\leq 8.04 \times 10^{-5}$, with correlation coefficient (R^2) of 0.998 for both drugs. These values were considered satisfactory and indicated good sensitivity of the proposed derivative method. Similarly, the HPLC curves were linear, with correlation coefficients for LAM and TDF being 0.999. Also, the intercepts and slopes were 175.05 and 1214.50, respectively, for LAM, with corresponding values of 124.74 and 2040.60 for TDF. These values depicted good sensitivity and accuracy of both methods. The standard errors of the intercept and slope were ≤ 2.32 and ≤ 20.35, respectively.

The LOD for the proposed D_3 spectrophotometric method was 0.46 and 2.61 µg mL^{-1} for LAM and TDF, respectively, with corresponding LOQ values as 1.40 and 7.90 µg mL^{-1}. These values confirmed the reliability and repeatability of the D_3 method.

Precision and accuracy

The results for the intra-day and inter-day studies are presented in Tables 2 and 3 for D_3 and HPLC methods,

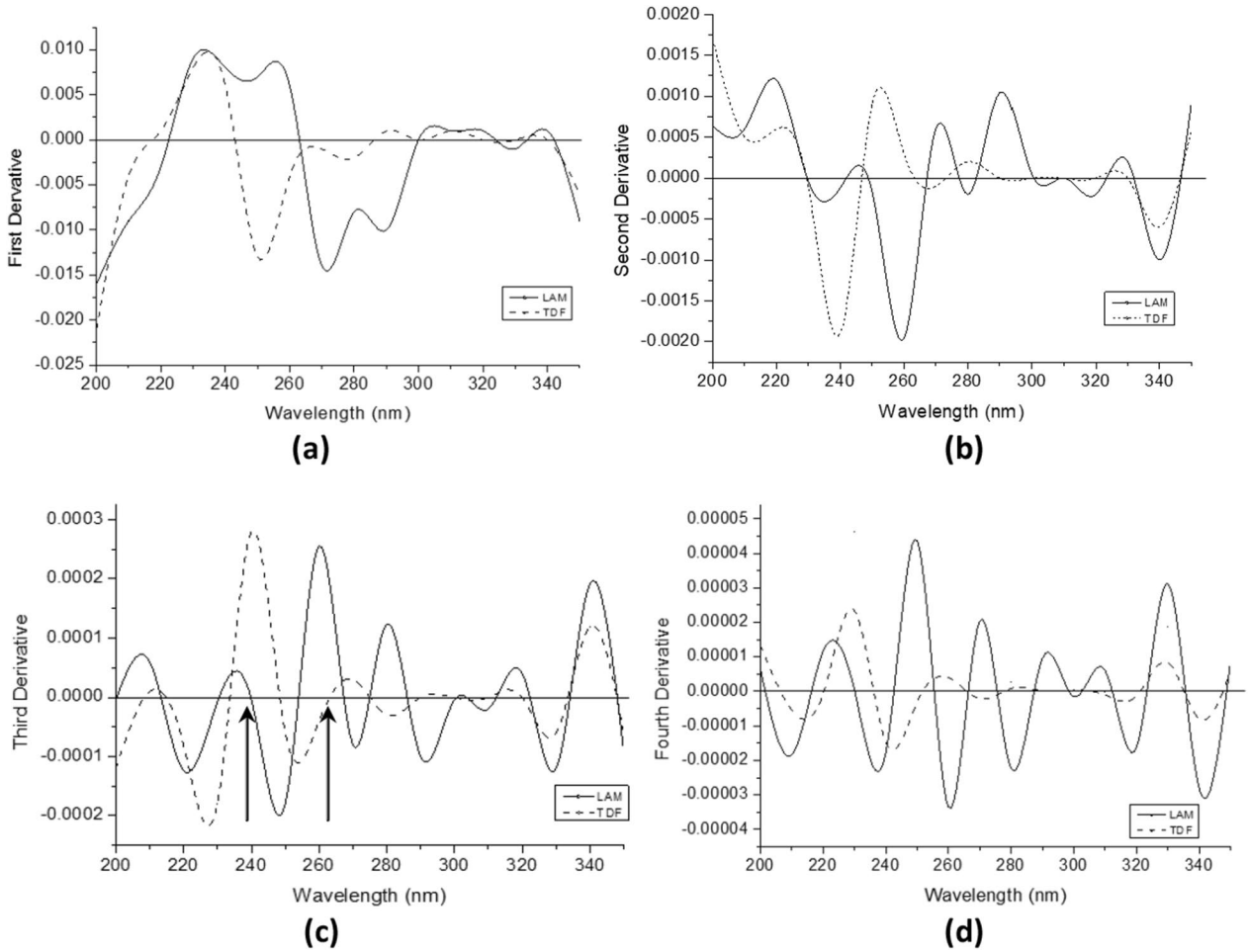


Fig. 3 Overlay spectra of **a** first ($dA/d\lambda$)-, **b** second ($d^2A/d\lambda^2$)-, **c** third ($d^3A/d\lambda^3$)- and **d** fourth ($d^4A/d\lambda^4$)-order derivatives of TDF and LAM

respectively. The computed relative standard deviation (RSD) for intra-day and inter-day assays for D_3 method ranged from 0.16 to 1.90% for both drugs, while for HPLC it was from 0.16 to 1.99%. The RSD was found to be less than 2%—this shows that the methods are precise and accurate [29]. The standard errors (SEs) were ≤ 0.08 and ≤ 0.02 for all runs in the D_3 and HPLC methods, respectively. These values depicted high reproducibility, good precision and accuracy of both methods [30].

Ruggedness, robustness and recovery studies

The ruggedness of the third derivative and HPLC methods was assessed by applying methods to assay two FDC brands of LAM/TDF—thus evaluating the reliability of both methods for routine laboratory quantification. Results obtained in varying some experimental conditions/parameters—such as brands, standard addition, varying of drug concentrations and comparative studies with established pharmacopeia HPLC methods [15, 16]—were useful indices for the evaluation of the reliability of the D_3 method (Table 4). The percentage recovery for the proposed method ranged from 94.80–100.13% to 96.63–99.93% for LAM and TDF, respectively, while values obtained for HPLC method were from 96.00–100.33% and 96.00–100.13%. Observed variations in results were insignificant; hence, the D_3 method is considered reliable, rugged and robust.

Application of analytical methods to dosage form

Table 5 shows the results obtained for the successful application of the proposed D_3 spectroscopic and the HPLC methods for the assay of two FDC brands containing LAM/TDF. The amounts of drugs found in the formulations were within the BP and USP specifications [15, 16] and also agree with the label claim. The content of LAM and TDF in brand I was $100.19 \pm 0.59\%$ and $100.89 \pm 0.38\%$, respectively, with corresponding values for brand II being $99.59 \pm 1.19\%$ and $99.39 \pm 0.63\%$ for the proposed D_3 spectroscopic method. For the HPLC method, the amounts of LAM in brands I and II were

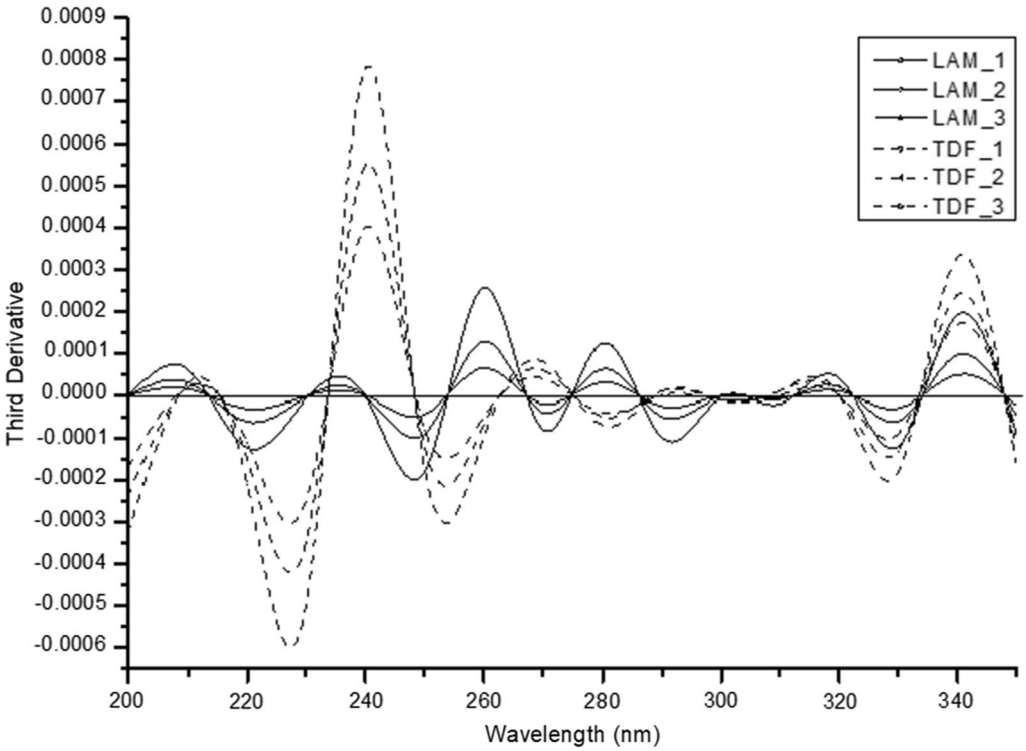


Fig. 4 Third-order derivative spectra of lamivudine (1) 2 µg mL^{-1}, (2) 4 µg mL^{-1}, (3) 6 µg mL^{-1} and tenofovir disproxal fumarate (1) 16 µg mL^{-1}, (2) 20 µg mL^{-1}, (3) 24 µg $mL.^{-1}$

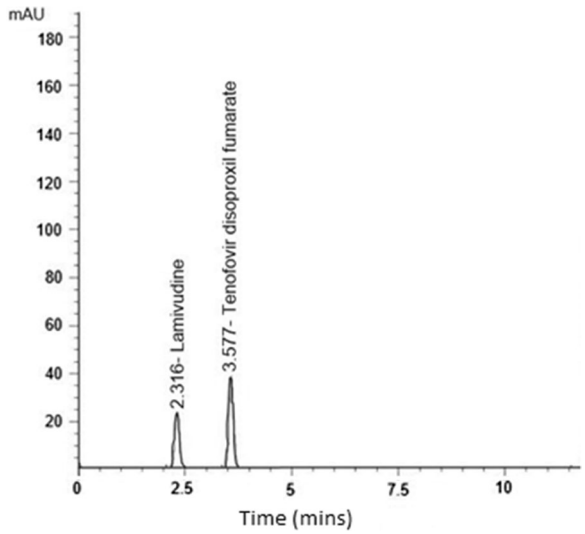


Fig. 5 Chromatogram of mixed standards of lamivudine and tenofovir disoproxil fumarate

99.86 ± 0.50 and 99.87 ± 0.71, respectively, while those for TDF percentage content were 99.87 ± 0.32% and 100.06 ± 0.35%. The Student-t test for accuracy with respect to the amount of drugs in formulations for both methods was between 0.075 and 1.016 for 5 replicates—this implies that no significant difference between the claims on brands and values obtained in evaluating the both methods at 95% confidence level [31].

Discussion

Maximum and zero-crossing amplitudes

The proposed D_3 method has effectively shown the propensity to resolve overlaying/underlaying and overlapping problem observed with zero derivative spectra for LAM/TDF FDC and has been used for the quantification of both drugs simultaneously. The amplitudes at 240 nm and 262.5 nm showed maxima for TDF and LAM, respectively, with corresponding zero-crossing points for LAM and TDF, thus making the simultaneous determination feasible.

Specificity and interference

Interference of extraneous materials on the proposed method was negligible, while specificity was enhanced. Drug samples were in solid dosage form, and the quantification of analytes was evaluated in the UV-region; hence, there is no chromophore-bearing compound, as co-extractive that is likely to interfere in the methanol used for the extraction of LAM and TDF. In addition,

Table 1 Optimum conditions for drug assay by proposed methods

Parameter	D_3 spectroscopic method		HPLC method	
	Lamivudine	Tenofovir disoproxil fumarate	Lamivudine	Tenofovir disoproxil fumarate
Wavelength (nm) D_0	270	260	254	254
Wavelength (nm) D_3	262.5	240	–	–
Molar absorptivity (L mol^{-1} cm^{-1}) (D_0)	0.945×10^4	1.009×10^4	–	–
Beer's conc. range (µg mL^{-1})	2–10	8–24	0.25–5.0	0.25–5.0
Limit of detection (LOD) (µg mL^{-1})	0.46	2.61	0.014	0.009
Limit of quantification (LOQ) (µg mL^{-1})	1.40	7.90	0.043	0.027
Regression equation				
Slope	3.2×10^{-5}	3.58×10^{-5}	1214.50	2040.60
Standard error of slope	8.16×10^{-7}	8.04×10^{-5}	18.99	20.35
Intercept	2.0×10^{-6}	1.62×10^{-4}	175.05	124.74
Standard error of intercept	5.42×10^{-6}	1.36×10^{-5}	2.32	2.22
Correlation coefficient (R^2)	0.998	0.998	0.999	0.999

D_0, zero-order derivative; D_3, third-order derivative

Table 2 Summary of precision and accuracy studies for third derivative spectroscopy method

Brand I	Amount of drug (µg mL^{-1})		RSD (%)	Standard error	Amount found (%)
	Taken	Found ± SD			
Intra-day assay (n = 3)					
Lamivudine	4	3.90 ± 0.03	0.87	0.02	97.58 ± 0.85
	6	5.91 ± 0.08	1.38	0.05	98.50 ± 1.36
	8	8.01 ± 0.01	0.16	0.01	100.08 ± 0.16
	10	9.94 ± 0.06	0.62	0.04	99.37 ± 0.61
(Mean content, %)					99.58 ± 0. 74
Tenofovir disoproxil fumarate (TDF)	8	7.89 ± 0.09	1.15	0.05	98.63 ± 1.14
	10	10.11 ± 0.09	0.85	0.05	101.07 ± 0.86
	15	14.94 ± 0.10	0.66	0.06	99.58 ± 0.66
	20	20.04 ± 0.06	0.29	0.03	100.20 ± 0.29
(Mean content, %)					99.87 ± 0.73
Inter-day assay (n = 3)					
Lamivudine	4	3.88 ± 0.07	1.90	0.04	96.92 ± 1.84
	6	5.87 ± 0.10	1.79	0.06	97.89 ± 1.75
	8	7.99 ± 0.06	0.75	0.03	99.83 ± 0.75
	10	9.88 ± 0.09	0.96	0.05	98.77 ± 0.95
(Mean content, %)					98.35 ± 1.32
Tenofovir disoproxil fumarate (TDF)	8	8.02 ± 0.08	1.00	0.05	100.25 ± 1.01
	10	9.95 ± 0.15	1.47	0.08	99.53 ± 1.46
	15	15.00 ± 0.09	0.60	0.05	99.98 ± 0.60
	20	20.02 ± 0.07	0.36	0.04	100.08 ± 0.36
(Mean content, %)					99.96 ± 0. 86

solid dosage formulations are made up of pharmaceutical inorganic excipients or aids such as—magnesium stearate, sodium lauryl sulfate, starch sodium glycolate, carboxymethylcellulose (CMC), talc, lactose spray dried, titanium dioxide, microcrystalline cellulose, pre-gelanitizated starch and hydroxypropylcellulose [30, 32]. These substances are chromophore-free and insoluble in methanol and ethanol. Also, the presence of dyes or colored

Table 3 Summary of precision and accuracy studies for HPLC method

Brand I	Amount of drug (μgmL^{-1})		RSD (%)	Standard error	Amount found (%)
	Taken	Found ± SD			
Intra-day assay (n = 3)					
Lamivudine	0.5	0.50 ± 0.01	1.63	0.00	100.00 ± 1.63
	1	1.00 ± 0.02	1.96	0.01	100.00 ± 1.96
	2.5	2.50 ± 0.01	0.57	0.01	100.46 ± 0.57
	5	4.97 ± 0.04	0.87	0.02	99.40 ± 0.86
(Mean content, %)					99.42 ± 1.31
Tenofovir disoproxil fumarate (TDF)	0.5	0.49 ± 0.00	0.96	0.00	98.67 ± 0.94
	1	1.00 ± 0.02	1.99	0.01	100.00 ± 2.16
	2.5	2.51 ± 0.01	0.50	0.01	100.27 ± 0.50
	5	5.01 ± 0.01	0.16	0.00	100.20 ± 0.16
(Mean content, %)					99.66 ± 0.94
Inter-day assay (n = 3)					
Lamivudine	0.5	0.50 ± 0.01	1.87	0.01	100.67 ± 1.89
	1	1.00 ± 0.01	1.41	0.01	100.00 ± 1.41
	2.5	2.50 ± 0.01	0.50	0.01	99.87 ± 0.50
	5	5.02 ± 0.02	0.41	0.01	100.33 ± 0.41
(Mean content, %)					98.98 ± 1.05
Tenofovir disoproxil fumarate (TDF)	0.5	0.50 ± 0.00	0.95	0.00	99.33 ± 0.94
	1	1.01 ± 0.01	0.50	0.00	100.50 ± 0.50
	2.5	2.51 ± 0.01	0.50	0.01	100.27 ± 0.50
	5	5.01 ± 0.01	0.28	0.01	100.20 ± 0.28
(Mean content, %)					100.00 ± 0.56

Table 4 Recovery studies for lamivudine and tenofovir disoproxil fumarate in FDC

FDC sample brand	Analyte drug in FDC tablet	Third derivative spectroscopic method				HPLC method			
		Amount of drug in weighed tablet (mg)	Amount of pure drug spiked (mg)	Total quantity of drug found (mg)	Percent recovery of drug spiked (%)	Amount of drug in weighed tablet (mg)	Amount of pure drug spiked (mg)	Total quantity of drug found (mg)	Percent recovery of drug spiked (%)
I	Lamivudine	1.0	2.0	2.90 ± 0.09	96.56	0.5	1.5	1.93 ± 0.04	96.50
		1.5	1.5	2.99 ± 0.01	99.67	1.0	2.5	3.45 ± 0.04	98.48
		2.0	1.0	2.95 ± 0.05	98.44	2.5	2.5	5.02 ± 0.02	100.33
		2.5	2.5	5.01 ± 0.06	100.13	4.0	1.0	5.01 ± 0.04	100.20
	Tenofovir	10	2.0	11.98 ± 0.12	99.86	0.5	1.5	1.93 ± 0.06	96.50
	Disoproxil	12.5	2.5	14.88 ± 0.08	99.22	1.0	2.5	3.44 ± 0.05	98.38
	Fumarate	15	5.0	19.82 ± 0.23	99.10	2.5	2.5	5.01 ± 0.01	100.13
		10	10	19.33 ± 0.39	96.63	4.0	1.0	5.00 ± 0.04	100.07
II	Lamivudine	1.0	2.0	2.99 ± 0.08	99.56	0.5	1.5	1.95 ± 0.05	97.67
		1.5	1.5	2.88 ± 0.02	96.11	1.0	2.5	3.45 ± 0.04	98.48
		2.0	1.0	2.91 ± 0.20	97.00	2.5	2.5	5.00 ± 0.06	100.00
		2.5	2.5	4.74 ± 0.18	94.80	4.0	1.0	4.96 ± 0.05	99.27
	Tenofovir	10	2.0	11.84 ± 0.14	98.69	0.5	1.5	1.92 ± 0.03	96.00
	Disproxil	12.5	2.5	14.69 ± 0.17	97.93	1.0	2.5	3.45 ± 0.04	98.67
	Fumarate	15	5.0	19.99 ± 0.02	99.93	2.5	2.5	5.00 ± 0.02	100.07
		10	10	19.96 ± 0.05	99.82	4.0	1.0	5.00 ± 0.09	100.03

Table 5 Application of methods to drug formulation

Method		Label claim (mg/tablet)	Amt. found ± SD (mg/tablet)	RSD (%)	SEM	Content (%)
D3 spectroscopy						
Brand I	LAM	300	300.37 ± 1.77	0.59	0.79	100.19 ± 0.59 (t = 0.624)
	TDF	300	302.05 ± 1.15	0.38	0.52	100.89 ± 0.38 (t = 1.004)
Brand II	LAM	300	298.76 ± 3.57	1.19	1.59	99.59 ± 1.19 (t = 0.645)
	TDF	300	298.16 ± 1.88	0.63	1.83	99.39 ± 0.63 (t = 1.016)
HPLC						
Brand I	LAM	300	299.57 ± 1.49	0.50	0.67	99.86 ± 0.50 (t = 0.533)
	TDF	300	299.6 ± 0.96	0.32	0.43	99.87 ± 0.32 (t = 1.004)
Brand II	LAM	300	299.90 ± 2.59	0.71	1.16	99.87 ± 0.71 (t = 0.075)
	TDF	300	300.35 ± 0.99	0.35	0.44	100.06 ± 0.35 (t = 0.360)

Student t-test is with respect to the label claim of each drugs; t-distribution at 95% confidence limits is 2.776 for $n = 5$ and 4 degrees of freedom

Table 6 Paired t-test/F-test for D_3 spectroscopy and HPLC methods

Method/test	Fixed drug combination (FDC) (mg/tablet)			
	Band I		Brand II	
	LAM	TDF	LAM	TDF
Label claim	300	300	300	300
D_3 spectroscopy	300.37 ± 1.77	302.05 ± 1.15	298.76 ± 3.57	298.16 ± 1.88
HPLC	299.57 ± 1.49	299.6 ± 0.96	299.6 ± 0.76	300.35 ± 0.99
F-test	1.41	1.44	2.81	3.61
Paired t-test	0.21	0.96	0.61	1.32

Theoretical values for t-distribution and F-distribution (at 4 degree of freedom) are 2.776 and 6.39, respectively

substances that are alcohol soluble would absorb in the visible region. The absence of interference implied that the proposed D_3 spectra method is highly selective for FDC tablet formulations of LAM/TDF and could be used for routine laboratory quality control analysis of pure and solid dosage forms.

Comparison between D_3 spectroscopy and HPLC methods

Both analytical techniques were compared using statistical analysis. The Student's t-test values for both methods and analytes were ≤ 1.016 (Table 5), while the tabulated value for 5 replicates at 95% confidence is 2.776. This implies that there was no significant difference in the quantities as claimed by the manufacturers and the results obtained in applying both methods [33]. Table 6 shows the calculated paired *t-test* and variance ratio *F-test* values between both methods and the amount of actives found in the brands. The experimental t- and F-values ranged from 0.21 to 1.32 and 1.41 to 3.61, respectively—none of these values exceeded the stipulated critical values (t = 2.776, F = 6.39) for four degrees of freedom. The aforementioned statistical values suggest also that there was no significant difference between the proposed D_3 spectroscopy and HPLC methods at 95% confidence level [34].

The LOD and LOQ values for both methods suggested that the HPLC method was more sensitive than D_3 method. However, the D_3 technique is simpler, more economic, more time saving, more robust and sufficient samples can be run within a day compared to the HPLC method (without automation devices). In addition, the D_3 spectroscopy method has shown the inclination of being free from interferences associated with excipients such as starch, glucose, talc, lactose and/or from frequent degradation products compared to the HPLC method—where residual analytes and impurities build-up in columns and are likely to interfere with the assay [30, 31, 33]. For method validation with respect to precision and accuracy, the D_3 method seemed better than the HPLC method. Tables 2 and 3 reaffirm D_3 statistical preference—where the %RSD for two brands of LAM/TDF antiretrovirals (Heteros and Mylan brands) ranged from 0.08–0 1.86% to 0.16–1.99% for D_3 spectrophotometric and HPLC methods, respectively. From the aforementioned, both assay techniques do not exhibit any significant difference and do resolve the overlaying challenges often encountered in FDC analyses using zero-order derivative spectrophotometric method.

Conclusion

The study presents a comparative use of third-order derivative spectroscopic method with the pharmacopoeia recommended HPLC method for the assay of antiretroviral FDC containing LAM and TDF. Statistically, both methods were adjudged to have no

significant difference and do have the ability and capacity to resolve overlaying problems often associated with zero derivative spectrophotometry in the assay of FDCs. Although the D_3 spectrophotometric technique is mathematical at deducing the wavelengths of interest, it was considered more time saving, cheaper, simpler, more environmental friendlier and more economical in terms of consumables and the generation of waste, than the HPLC method. The reliability, precision and accuracy of the proposed method are reflected in the validation parameters assessed and could be used for routine analysis of FDCs where HPLC is not available.

Abbreviations

AIDs	Acquired immune developed syndrome
CM	Compensation method
CMC	Carboxymethylcellulose
D_3	Third order
D_4	Fourth order
DD	Difference derivative
DNA	Deoxyribonucleic acid
DS	Derivative spectrophotometry
EDQM	European Directorate of Quality Medicine
FDC	Fixed-dose combination
HAART	Highly active antiretroviral therapy
HIV	Human immune deficiency virus
HPLC	High-performance liquid chromatographic
LAM	Lamivudine
LOD	Limits of detection
LOQ	Limits of quantification
NAFDAC	National Agency for Food, Drugs, Administration and Control
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside reverse transcriptase inhibitor
NtRTIs	Nucleotide reverse transcriptase inhibitors
RDS	Ratio derivative spectrophotometry
RSD	Relative standard deviation
SEs	Standard errors
TDF	Tenofovir disoproxil
TFV-DP	Tenofovir-diphosphate
TLC–UV	Thin-layer chromatography–ultraviolet
UV	Ultraviolet
WHO	World Health Organization

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Author contributions
ENV took part in conceptualization, supervision, methodology, software; data curation; formal analysis; investigation; validation; visualization; writing—review and editing. SJB involved in supervision, software; data curation; formal analysis; validation; visualization; writing review and editing. JNO took part in formal analysis; investigation; visualization; formal analysis; writing—review and editing. FD involved in formal analysis; investigation; software; data curation; validation; visualization; writing—original draft. All authors read and approved the final manuscript.

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Declarations

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The authors declare that they have no competing interests.

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References

1. World Health Organization (WHO) (2016) Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection: recommendations for a public health approach, 2nd edn. WHO
2. Iyidogan P, Anderson KS (2014) Recent findings on the mechanisms involved in tenofovir resistance. Antivir Chem Chemother 23(6):217–222. https://doi.org/10.3851/IMP2628
3. Arts EJ (2012) Hazuda DJ (2012) HIV-1 antiretroviral drug therapy. Cold Spring Harb Perspect Med 2:a007161
4. Wan X, Ma P, Zhang X (2014) A promising choice in hypertension treatment: Fixed-dose combinations. Asian J Pharm Sci 9:1–7
5. Vaikosen NE, Kashimawo JA, Soyinka OJ, Orubu S, Elei S, Ebeshi UB (2020) Simple thin layer chromatography-ultraviolet spectrophotometric method for quality assessment of binary fixed-dose-combinations of lamivudine/tenofovir disoproxil fumarate and lamivudine/zidovudine in tablet formulations. J Sep Sci 43(11):2228–2239. https://doi.org/10.1002/jssc.201901117
6. Quercia R, Perno CF, Koteff J, Moore K, McCoig C, Clair SM, Kuritzkes D (2018) Twenty-five years of lamivudine: current and future use for the treatment of HIV-1 infection. J Acquir Immune Defic Syndr 78(2):125–135. https://doi.org/10.1097/QAI.0000000000001660
7. Anderson PL, Rower JE (2010) Zidovudine and lamivudine for HIV infection. Clin Med Rev Ther 2:a2004–a2023
8. Else LJ, Jackson A, Pils R, Hill A, Fahey P, Lin E, Amara A, Siccardi M, Watson V, Tjia J, Emery S, Khoo S, Back DJ, Boffito M (2012) Pharmacokinetics of lamivudine and lamivudine triphosphate after administration of 300 milligrams and 150 milligrams once daily to healthy volunteers: results of the ENCORE 2 study. Antimicrob Agents Chemother 56(3):1427–1433
9. Patel PH, Zulfiqar H (2022) Reverse transcriptase inhibitors. [Updated 2022 May 19]. In: StatPearls [Internet]. StatPearls Publishing, Treasure Island. Available from: https://www.ncbi.nlm.nih.gov/books/NBK551504/. Accessed 18 Sept 2022
10. Singh AB, Das K (2022) Insight into HIV-1 reverse transcriptase (RT) inhibition and drug resistance from thirty years of structural studies. Viruses 14:1027. https://doi.org/10.3390/v14051027
11. Ng HH, Stock H, Rausch L, Bunin D, Wang A, Brill S, Gow J, Mirsalis JC (2015) Tenofovir disoproxil fumarate: toxicity, toxicokinetics, and toxicogenomics analysis after 13 weeks of oral administration in mice. Int J Toxicol 34(1):4–10
12. Cihlar T, Ray AS (2010) Nucleoside and nucleotide HIV reverse transcriptase inhibitors: 25 years after zidovudine. Antiviral Res 85:39–58
13. Sangshetti NJ, Bhojane S, Rashid SB, Gonjari I (2014) Spectrophotometric method for simultaneous estimation of lopinavir and ritonavir in bulk and tablet dosage form. Int J Chem Tech Res 6:823–827

14. Venkatesan S, Kannappan N (2014) Simultaneous spectrophotometric method for determination of emtricitabine and tenofovir disoproxil fumarate in three-component tablet formulation containing rilpivirine hydrochloride. Int Sch Res Not 2014, Article ID 541727. https://doi.org/10.1155/2014/541727
15. The British Pharmacopoeia Commission (2009) Monographs on medicinal and pharmaceutical substances market towers 1 Nine Elms Lane London, I & II
16. United States Pharmacopoeia (2018) 41 edn. The United States Pharmacopeial Convention, Rockville
17. Uslu B, Ozkan SA (2002) Determination of lamivudine and zidovudine in binary mixtures using first derivative spectrophotometric, first derivative of the ratio-spectra and high-performance liquid chromatography-UV methods. Anal Chimica Acta 466:175–185
18. Sharma R, Mehta K (2010) Simultaneous spectrophotometric estimation of tenofovir disoproxil fumarate and lamivudine in three component tablet formulation containing efavirenz. Indian J Pharm Sci 72:527–530
19. Mohite PB, Pandhare RB, Khanage SG (2011) Derivative spectrophotometric method for estimation of antiretroviral drugs in fixed dose combinations. Adv Pharm Bull 2:115–118. https://doi.org/10.5681/apb.2012.016
20. Sudha T, Ravikumar RV, Hemalatha VP (2010) Validated HPTLC method for simultaneous determination of lamivudine and abacavir sulphate in tablet dosage form. Int J Pharm Sci Res 1:107–111
21. Turak F, Dinç M, Dulger O, Ozgur UM (2014) Four derivative spectrophotometric methods for the simultaneous determination of carmoisine and ponceau 4R in drinks and comparison with high performance liquid chromatography. Int J Anal Chem 2014:650465. https://doi.org/10.1155/2014/650465
22. Redasani KV, Patel RP, Marathe YD, Chaudhari RS, Shirkhedkar AA, Surana JS (2018) A review on derivative uv-spectrophotometry analysis of drugs in pharmaceutical formulations and biological samples review. J Chil Chem Soc 63:34129
23. Kus S, Marczenko Z, Obarski N (1996). Chem Anal 41:899–927
24. Karpinska J (2012) Basic principles and analytical application of derivative spectrophotometry. Jamal Uddin
25. Owen A (1995) Uses of derivative spectroscopy, UV–visible spectroscopy, application note, Agilent Technologies, 4. D. Cameron, D. Moffatt. Apl Spec 41:539–544, 1987
26. ICH. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human use, ICH Harmonized Tripartite Guideline, ICH Q2A. Text on validation of analytical procedures, November 2005. Accessed 4 Sept 2022
27. Bohm DA, Stachel CS, Gowik P (2010) Confirmatory method for the determination of streptomycin in apples by LC-MS/MS. Anal Chim Acta 672:103–106. https://doi.org/10.1016/j.aca.2010.03.056
28. Shrivastava A, Gupta VB (2011) Methods for the determination of limit of detection and limit of quantitation of the analytical methods. Chron Young Sci 2:21–25
29. Annapurna MM, Malineni S, Vellanki SVS (2017) Simultaneous determination of brimodine tartrate and timolol maleate by first derivative and ratio derivative spectroscopy. J Anal Pharm Res 4(6):2–7
30. Vaikosen NE, Bioghele J, Worlu CR, Ebeshi UB (2020) Spectroscopic determination of two beta-blockers—atenolol and propanolol by oxidative derivatization using potassium permanganate in alkaline medium. Rev Anal Chem 3:56–64. https://doi.org/10.1515/revac-2020-0103
31. Karthik VV (2016) Excipient used in the formulation of tablets. Res Rev J Chem 5(2):143–154
32. Omara AH, Amin SA (2010) Spectrophotometric microdetermination of anti-parkinsonian and antiviral drug amantadine HCl in pure and in dosage forms. Arab J Chem 4:287–292. https://doi.org/10.1016/j.arabj.2010.06.048
33. Venkatesh P, Daggumati M (1999) Development and validation of a normal-phase HPTLC method for the simultaneous analysis of lamivudine and zidovudine in fixed-dose combination tablets. J Pharm Anal 2:152–155. https://doi.org/10.1016/j.jpha.2011.11.002
34. Miller JC, Miller JN (2005) Statistics and chemometrics for analytical chemistry, 5th edn. Pearson Education Limited, Harlow

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