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Green stability indicating UV- spectrophotometric techniques for estimation of tenofovir alafenamide in bulk form and dosage forms

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Abstract: Four simple, precise and sensitive UV- spectrophotometric procedures were achieved for estimation of tenofovir alafenamide in the presence of its alkaline degradate. Dual wavelength was the first one which based on determination of the drug at 235.5 nm and 261.5 nm. First – derivative spectrophotometric method was the second one which the amplitude values were measured at 274 nm using Δλ of 8 nm and a scaling factor of 20. Third one was ratio difference which peak's amplitudes ratio spectra difference of tenofovir alafenamide was measured between 261.5 nm and 252 nm using devisor of 10 µg mL^{-1} of its alkaline degradate. While the last method was a first derivative of ratio spectra using Δλ = 8 nm and a scaling factor = 10 to measure the amplitude at 275.6 nm. The linearity range was 5-35 µg mL^{-1} in all procedures. The suggested procedures were effectively utilized for the estimation of the cited drug in bulk form as well as its dosage forms. According to ICH guidelines, all procedures were validated. Moreover, Analytical eco-scale and Green Analytical Procedure Index (GAPI) were used to estimate the greenness of the suggested methods compared with a reported one as two assessment tools.

Keywords: Green; Stability indicating; degradate; Tenofovir Alafenamide; UV- spectrophotometry.

1. INTRODUCTION

Tenofovir alafenamide (TAF); chemically known as isopropyl (2S)-2-[[[(1R)-2-(6-aminopurin-9-yl) -1- methyl-ethhoxy] methyl-phenoxy phoshoryl] amino] propionate is a nucleoside reverse transcriptase inhibitors (NRTIs) and a novel ester prodrug of the antiretroviral tenofovir. Tenofovir alafenamide is related to a HIV drugs groups which called nucleoside reverse transcriptase inhibitors (NRTIs) which acts by blocking reverse transcriptase, to prevent HIV from multiplying [1]. The literature revealed that various predetermination methods of tenofovir alafenamide either alone or with other drugs by spectroscopic methods [2- 5], HPLC [6-15], LC [16], LC- MS/ MS [17,18]. However, a few stability indicating methods have been carried for determination of the drug using spectrophotometric method [19,20], HPLC [21- 25] and RP-UPLC-PDA in multidrug combination [26]. Green chemistry's main idea is seen as a tool for enhancing reasonable development in the method of analysis. In the field of analytical chemistry, many studies have been conducted to reduce the cost and to avoid the threats of analytical procedures. In order to make chemical analysis green, green chemistry is used as it confirms the use of safe, less toxic and gentler solvents or removal of solvents and the use of small quantities of reagents [27]. Therefore, this work focused on the development of first eco-friendly, simple, fast, economic and delicate UV - Spectroscopic procedures for estimation of tenofovir alafenamide in presence of its alkaline degradate as well as in its dosage forms. The greenness of the four established methods as well as the reported one were evaluated by using analytical Eco-Scale and Green Analytical Procedure Index (GAPI) methods to confirm the effect of each approach on the environment.

2. METHODS

2.1. Instruments

Shimadzu double beam UV-visible spectrophotometer 1601 PC Ultra with matched pair supplied with 10 mm matched quartz cells (Tokyo, Japan), with UV-Probe personal spectroscopy with software of version 2.10.

2.2. Materials and reagents

Tenofovir alafenamide, B.N. TV0020516 was obtained from PHARMED Healthcare; Cairo, Egypt. The purity was reported to be 97.60 % as stated by the supplier. Methanol (Sigma-Aldrich, Darmstadt, Germany). NaOH, HCL and H_2O_2, (Qualikems fine chemical Pvt. Ltd, India). Freshly distilled water was used through all steps of the work.

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2.3. Dosage form

HEPBEST® tablets; B. N. 8069912, one tablet badged to contain 25 mg tenofovir alafenamide which produced from Mylan Company for Pharmaceutical Industry, USA.

2.4. Standard solutions

In methanol, tenofovir alafenamide (1 mg mL^{-1}) was prepared to obtain the normal stock solution. In order to achieve a final concentration of 100 µg mL^{-1}, the operating standard solutions were prepared with further dilution of the stock solution with the same solvent.

2.5. Degraded samples

A weight of 100 mg of pure tenofovir alafenamide was accurately weighed and refluxed with 25 mL of 3 N NaOH for 3 h or with 25 mL 5 N HCl for 4 h at 100 ° C for alkaline and acidic degradation, respectively. Both solutions were cooled and neutralized at pH 7, then evaporated until dryness under vacuum. The obtained residues were extracted three times each with 25 mL methanol then filtered into two separate 100- mL volumetric flasks and diluted to the volume with methanol to obtain a stock solution claimed to contain alkaline or acid degradate derived from 1 mg mL^{-1} intact drug.

2.6. Procedures

2.6.1. Spectral characteristics

Tenofovir alafenamide's zero-order spectra and its alkaline and acidic degradate were scanned over the 200-400 nm range, as shown in Figure 1.

2.6.2. Constitution of calibration curves

From the drug working standard solutions (100 µg mL^{-1}), different volumes equivalent to (5-35) µg tenofovir alafenamide were transferred to a series of 10 mL volumetric flasks and the volume was completed with the same solvent. The absorption spectrum of prepared solutions has been scanned from 200 to 400 nm and stored in the computer to be manipulated by the following procedures.

DW method: Absorbance of the above solutions were measured between 235.5 and 261.5 nm where the difference in absorbance of the alkaline degradate is zero (Figure 1).

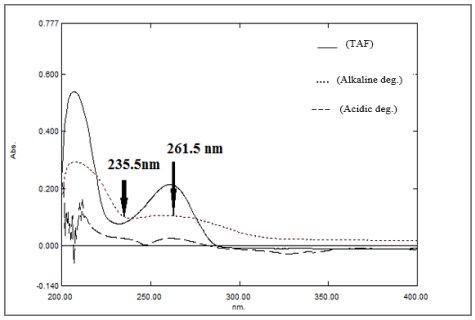
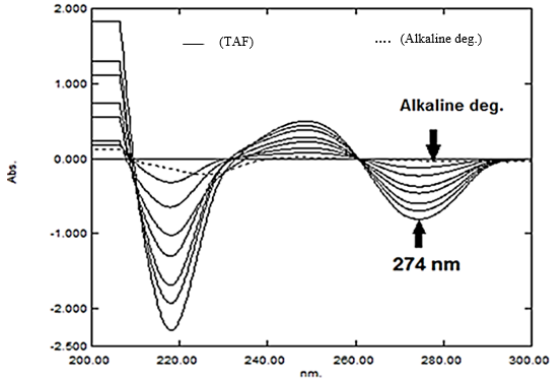


Figure (1): Zero absorption spectrum of 10 µg mL^{-1} tenofovir alafenamide overlaid with 10 µg mL^{-1} of its alkaline degradate and its acidic degradate.

^{1}D method:

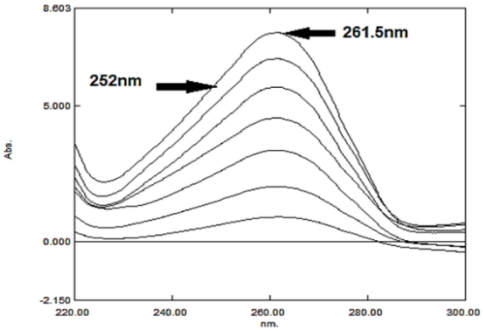
For the above solutions, the first absorption spectrum derivative stored in the computer was reported using Δ λ = 8 nm and a scaling factor = 20; At 274 nm, the amplitude values were calculated (Figure 2).



Figure(2): First derivative spectra of (5-35 µg mL^{-1}) tenofovir alafenamid in presence of its alkaline degradate in methanol.

RD method:

The stored zero order spectra of tenofovir alafenamide solutions were divided by the spectrum of 10 µg mL^{-1} of its alkaline degradate. The recorded difference values in peak amplitudes between 261.5 and 252 nm were calculated (Figure 3).



Figure(3): Smoothed ratio spectra of tenofovir alafenamide (5-35µg mL^{-1}) using (10 µg mL^{-1}) of its alkaline degradate as a divisor.

[1]DR method: [1]D with λ = 8 nm and scaling factor =10 of the previously reported ratio spectra and the amplitudes of the resulting spectra were calculated at 275.6 nm (Figure 4). In each of the above procedures, construction of calibration curve between response and drug concentration was done and regression equation was computed.

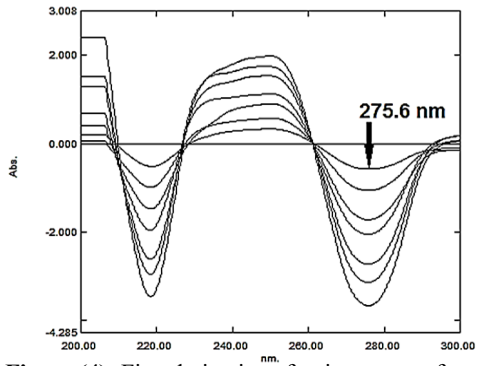


Figure (4): First derivative of ratio spectra of tenofovir alafenamide (5-35 µg mL^{-1}) using (10 µg mL^{-1}) of its alkaline degradate as a divisor.

2.6.3. Application to laboratory prepared mixtures

Into a series of 10 mL volumetric flasks containing (0.28 - 0.07 mg) of the degraded drug, aliquots containing (0.07- 0.28 mg) of intact tenofovir alafenamide solution were transferred to the label and then diluted with the solvent. The spectrum was calculated and stored on the computer for these mixtures. The protocol for each process was then applied and the concentrations of the drug in the prepared mixtures were calculated from the corresponding regression equation.

2.6.4. Application to dosage forms

Ten HEPBEST ® tablets containing 25 mg of tenofovir alafenamide were well weighed, powdered and blended. A quantity equal to 100 mg of the drug was transferred to a volumetric flask of 100 mL and diluted with the same solvent in the label. The flask was sonicated for 15 min then filtered. The clear filtrate was claimed to contain 1 mg mL^{-1} of tenofovir alafenamide. Ten mL of the filtrate was 10 folds diluted with the solvent to obtain a solution containing 100 µg mL^{-1} of the drug and analyzed by the developed procedures using the previously mentioned conditions. The concentration of the studied drug was calculated from the corresponding regression equation.

3. RESULTS

The goal of this work is to develop a new validated green stability indicating UV- spectroscopic methods capable of determining tenofovir alafenamide with appropriate sensitivity and selectivity in the presence of its alkaline degradation, either in bulk form or in its dosage forms.

3.1. Methods validation

Validation of the developed methods in compliance with ICH Guidelines [28].

3.1.1. Linearity and range – The calibration curves for the suggested methods were developed under the mentioned experimental conditions by plotting the response versus tenofovir alafenamide concentrations in µg mL^{-1}. For the cited compound, the regression plots were found to be linear over the 5-35 µg mL^{-1} range. For each of the suggested methods, the spectrum and equations of linear regression were computed and shown in Table 1.

3.1.2. Detection and Quantitation Limits – Based on LOD (3.3 SD/S) and LOQ (10 SD/S), the sensitivity of the proposed methods was assessed. The results shown in Table 1.

3.1.3. Accuracy – Three separate concentrations of pure tenofovir alafenamide samples covering the linearity spectrum, each in triplicate, were estimated. From their corresponding regression equations, concentrations were calculated. Good %R (99.69%, 99.40%, 99.77 % and 99.80%) for the proposed methods; respectively, as listed in Table 1, confirms excellent accuracy.

3.1.4. Precision – Three concentrations of tenofovir alafenamide (10, 20, 30 µg mL^{-1}) were tested, each in triplicate, within one day and on three successive days using the proposed UV- methods. RSD% of repeatability amounted to be 0.60%, 0.62%, 0.79% and 0.58%, While intermediate precision computed to be 1.38%, 0.95%, 1.21% and 0.84% for each established methods, respectively as shown in Table 1.

3.1.5. Selectivity – It was verified by applying the established processes along with its alkaline degradation to laboratory prepared mixtures of the intact drug. Good recoveries of intact tenofovir alafenamide were obtained by the proposed methods in the presence of up to 80 % of its degradate, as shown in Table 2.

3.2. Application to dosage forms

The methods developed have been successfully used in the estimation of tenofovir alafenamide to HEPBEST® tablets. The findings were appropriate and gave high reliability and good agreement with labelled quantity. The proposed methods were then tested using the standard addition technique which there was no interference from excipients and additives was observed, as shown in Table 3. The findings obtained were statistically compared to those obtained by applying the t-test and F-test to the reported RPHPLC method at 95% confidence level [29], showing no significant difference between

the established and reported methods, as shown in Table 4. The proposed procedures are therefore sufficiently detailed and effective. The developed UV-spectrophotometric techniques, however, were more sensitive and more selective.

Table 1: Assay parameters and validation results obtained by the proposed UV- spectrophotometric methods for determination of tenofovir alafenamide.

Method parameters	DW	^{1}D	RD	^{1}DR
Wavelength (nm)	**261.5 - 235.5**	**274**	**261.5 - 252**	**275.6**
Linearity range (µg mL^{-1})	**5-35**	**5-35**	**5-35**	**5-35**
Regression equation				
Slope± SD(S_Y)	**0.0274±0.005**	**0.023±0.003**	**0.0360±0.0004**	**0.1052±0.0012**
Intercept± SD(S_X)	**0.00457±0.0012**	**0.007±0.0024**	**0.0061±0.010**	**0.0077±0.0072**
SD of residual (S_{YX})	**0.527**	**0.371**	**0.910**	**0.517**
Correlation coefficient (r)	**0.9992**	**0.9996**	**0.9995**	**0.9996**
Accuracy (mean ± SD)	**99.69±0.38**	**99.40±0.21**	**99.77±0.38**	**99.80±0.78**
Precision (±%RSD)				
***Repeatability**	**0.60**	**0.62**	**0.79**	**0.58**
***Intermediate precision**	**1.38**	**0.95**	**1.21**	**0.84**
LOD	**0.144**	**0.344**	**0.916**	**0.225**
LOQ	**0.437**	**1.04**	**2.77**	**0.684**

*n= nine determinations.

Table 2: Determination of tenofovir alafenamide in laboratory prepared mixtures with its alkaline degradate by the proposed UV- spectrophotometric methods.

Intact	Degradate	DW	^{1}D	RD	^{1}DR
(µg mL^{-1})	(µg mL^{-1})	% of intact	% of intact	% of intact	% of intact
7	**28**	**100.20**	**102.00**	**97.40**	**100.60**
10	**25**	**99.81**	**97.70**	**101.20**	**98.60**
15	**20**	**98.6**	**98.40**	**97.93**	**97.93**
20	**15**	**101.05**	**99.05**	**99.15**	**100.90**
25	**10**	**101.24**	**99.44**	**97.56**	**100.24**
28	**7**	**99.20**	**97.96**	**99.56**	**100.33**
Mean % ± SD		**100.02±1.03**	**99.09±1.57**	**98.80±1.46**	**99.77±1.20**

Table 3: Assay of tenofovir alafenamide in dosage forms and application of standard addition technique for determination of the drug by the proposed UV- spectrophotometric methods.

		Tenofovir alafenamide			
		DW	**^{1}D**	**RD**	**^{1}DR**
HEPBEST® tablets	**Mean%±SD**	**99.31±0.79**	**99.15±1.28**	**99.86±1.51**	**99.21±0.89**
		Standard addition technique			
Dosage form taken (µg mL^{-1})	**Pure added (µg mL^{-1})**	**% recovery [a]**			
		DW	**^{1} D**	**RD**	**DR^{1}**
	5	**97.80**	**100.20**	**97.80**	**102.60**
10	**10**	**97.91**	**98.30**	**97.81**	**100.21**
	15	**100.15**	**99.40**	**100.53**	**99.53**
	25	**99.30**	**98.60**	**99.20**	**100.65**
Mean%±SD		**98.79±1.13**	**99.13±0.86**	**98.84±1.31**	**100.75±1.32**

[a] mean of 3 determinations

Table 4: Statistical comparison for the results obtained by the proposed methods and the reported method [21] for determination of tenofovir alafenamide.

Parameters	**Proposed methods**				
	DW	**^{1}D**	**RD**	**^{1}DR**	**Reported Method [21]**
Linearity range (µg mL^{-1})	**5-35**	**5-35**	**5-35**	**5-35**	**10-150**
N	**5**	**5**	**5**	**5**	**5**
Mean%	**99.31**	**99.15**	**99.86**	**99.21**	**100.29**
SD	**0.790**	**1.28**	**1.51**	**0.89**	**1.53**
Variance	**0.62**	**1.63**	**2.28**	**0.79**	**2.34**
Student's t-test (2.306)	**1.34**	**1.38**	**0.35**	**1.75**	**---**
F – value (6.388)	**3.74**	**1.42**	**1.02**	**2.91**	**---**

-The values between parenthesis are the theoretical values of t- and F-test at P = 0.05

-Ref [21] stability indicating assay method for the determination of tenofovir alafenamide tablets by RP-HPLC with UV detection was 260 nm.

4. DISCUSSION

4.1. Forced degradation

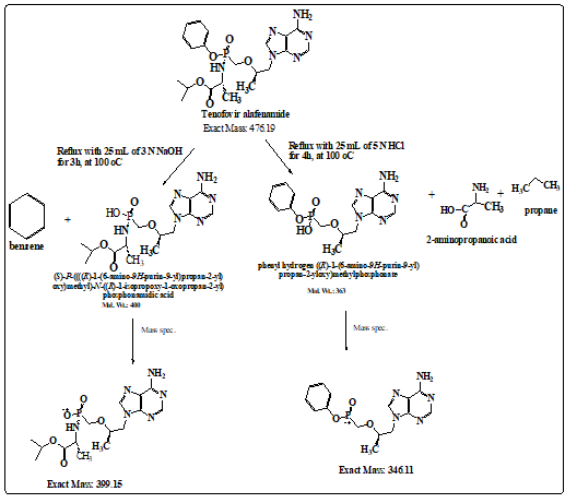
Study of stress degradation of tenofovir alafenamide was done by refluxing the drug with NaOH, HCl and aqueous H_2O_2 for different time intervals or under thermal conditions. The drug not affected by aqueous H_2O_2 or thermal check. Full degradation was reached only by refluxing the drug with 3 N NaOH for 3 hr or with 5 N HCl for 4 hr. This was certified by absence of band in the area of the degradate corresponding to the band of the intact drug.

Degradation products were supported by IR using KBr disc as follows; a wide band was observed for two (NH) groups in the IR spectrum of the pure drug at 3326 cm^{-1}. Bands for both the aromatic (CH) at 2982 cm^{-1} and ester carbonyl group at 1744 cm^{-1} in were also determined. The alkaline degradate, showed a wide band of phosphoric (OH) group at 3445 cm^{-1} and lacked the CH aromatic band. While in acidic degradate, IR spectrum revealed absence of the C=O band of the ester moiety at 1744 cm^{-1} and presence of phosphoric (OH) group broad band at 3423 cm^{-1} as shown in Figure 5 (a-c). Degradation was also confirmed by MS as follows; the intact drug displayed spectroscopic molecular ion peak electronic ionization mass at m/z = 476. The MS of the developed alkaline degradate represented a molecular ion peak at m/z = 399 with 44.89 %. Degradation with alkaline conditions, suggesting a lower molecular ion peak of 77 units. This implies phenyl group loss. In acid degradation, the molecular ion peak was shown by electronic ionization mass at m/z = 346, with high intensity (56.99%), this indicate decreasing in molecular ion peak equal 130 unit referring to loss of isopropyl d-alaninate moiety as shown in Figure 6 (a-c). In conclusion, the postulated alkaline degradate compound produced by the elimination of the phenyl moiety to carry the free phosphoryl group and the established acid degradate compound formed by isopropyl alaninate moiety removal to provide the free phosphate group from the previous conditions of IR and Electronic Ionization mass analyses. The system of degradation has been shown in scheme1.

4.2. Method optimization

Severe overlapping in spectrum consider the major problem presented during the assay of tenofovir alafenamide in presence of its alkaline degradate; as shown in Figure 1.

However; this overlapping can be resolved upon applying DW, ^{1}D, RD and ^{1}DR UV-spectrophotometric methods for determination of tenofovir alafenamide in presence of its alkaline degtadate. However; the presence of its acidic degradate, hindered the assigning of the drug by these methods. Thus, they were only used for determination of the dug in relation to its alkaline degradate.



Scheme 1. Suggested degradation pathway of tenofovir alafenamide.

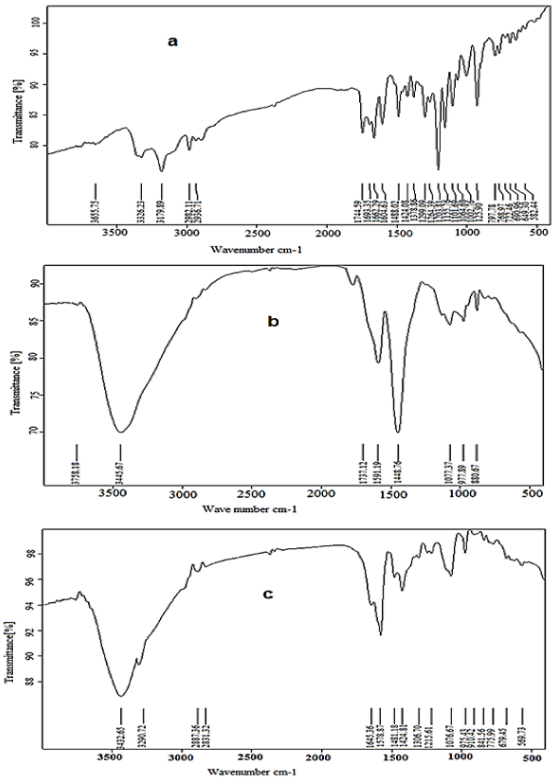


Figure (5): IR Spectra of (a) intact tenofovir alafenamide, (b) Its alkaline degradate and (c) its acidic degradate(c)) on KBr disc.

4.2.1. Dual wavelength (DW)

Absorbance between 235.5 and 261.5 nm was used for estimation of tenofovir alafenamide in presence of alkaline degradate which showed zero absorbance. Method of dual wavelength is simple, accurate and easy method as it doesn't need any particular

software programs and doesn't require additional processing.

4.2.2. First derivative (^1D)

^1D- spectrophotometric method eliminated the interference in spectra between intact drug and its alkaline degradate at 274 nm, using Δλ = 8 nm and a SF = 20.

4.2.3. Ratio Difference (RD)

In this method, good choice of the divisor concentration was of high importance. Thus, various concentrations of alkaline degradate were tried as a divisor (10, 15, 20 and 25 µg mL^{-1}). The preferable one was 10 µg mL^{-1}, as it gave better results with minimum noise in accordance with good selectivity. So, absorbance was measured between 261.5 and 252 nm for determination of pure drug in presence of its alkaline degradation product by using 10 µg mL^{-1} of the later as a divisor to obtain the ratio spectra.

4.2.4. Method of first Derivative of Ratio Spectra (^1DR)

Tenofovir alafenamide was estimated in presence of its alkaline degradate to obtain the ratio spectra by using (10 µg mL^{-1}) of degradate as a divisor, to get the ratio spectra. Then, the first derivative of ratio spectra with SF: 10 was obtained and finally smoothed at Δλ 8 nm. The amplitude of DR^1 spectra of (TAF/ alkaline degradate) is measured at 275.6 nm.

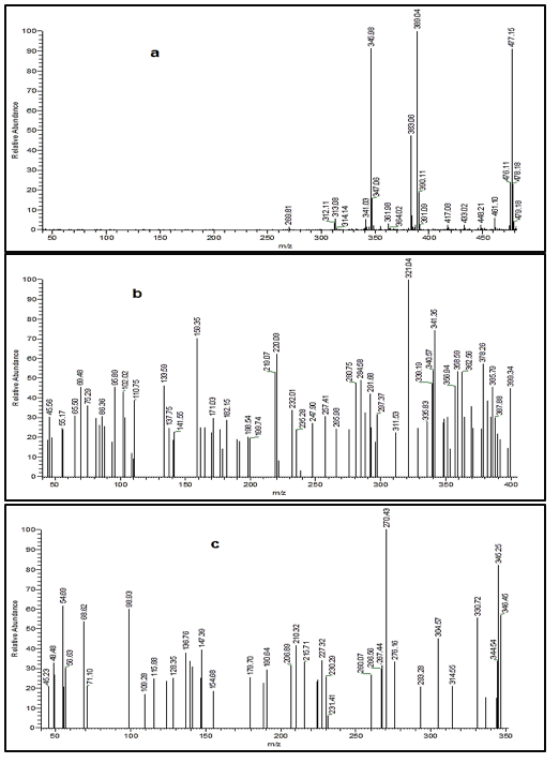


Figure (6): Mass spectra of (a) intact tenofovir alafenamide, (b) Its alkaline degradate and (c) Its acidic degradate.

5. EVALUATION OF GREENNESS OF THE SUGGESTED METHODS

Greenness of the suggested UV-spectrophotometric methods were evaluated following to the analytical Eco-Scale which was adjusted by estimating penalty points of each step during the whole procedure. The analytical Eco-Scale has characteristics comparing with other scales because of simplicity of calculating the score and pointing out to different aspects of the environmental effect of analytical procedure in the assessment procedure [27]. In GAPI, a specific symbol with five pentagrams could be used to evaluate and quantify-from green through yellow to red-the low, medium and high environmental impact involved for each step of the methodology[30]. The analytical Eco-Scale value of the proposed methods was calculated and its score was 83 which was considered as an excellent green analysis as shown in Table 5. Translation of the GABI pentagrams for the proposed spectrophotometric methods and its comparison with the reported one was illustrated in Figure 7. It is evident that our developed methods are greener than the reported one with good validation parameters. So, without harmful effects on the environment, the developed procedures can be used for routine analysis of the studied drug.

6. CONCLUSION

The suggested study described four different UV- spectroscophotometric methods used for estimation of tenofovir alafenamide in presence of its alkaline degradate in which the degradation conditions were documented by IR and mass spectra. All of these developed methods lead to spectra resolution and confirm their strength to be used for stability indicating determination of the drug. They provide to be green, simple, accurate and economic and do not require initial pretreatment steps. Using analytical Eco-Scale and Green Analytical Procedure Index techniques, greenness evaluation of the method was carried out, demonstrating the highest greenness of the developed methods. Consequently, these methods were efficiently performed for assessment, stability studies and regular quality control analysis of tenofovir alafenamide.

Figure (7): The green assessment profile for the proposed methods in comparison with the reported method, using the GAPI tool.

Table 5: The penalty points of the proposed method according to the analytical Eco-Scale.

Reagents/ Instruments	Penalty points
Methanol	12
spectrophotometer	0
Occupational hazards	0
Waste	5
Total pps	Σ17
Eco-Scale	83
	Excellent green analysis

List of Abbreviations: TAF: Tenofovir alafenamide; UV: Ultraviolet; HIV: Human Immunodeficiency; NRTIs: Nucleoside Reverse Transcriptase Inhibitors; SF: Scaling Factor; DW: Dual Wavelength; ^{1}D: First Derivative; RD: Ratio Difference; ^{1}DR: First Ratio Spectra Derivative; ICH: International Harmonization Meeting; LOD: Limit of Detection; LOQ: Limit of Quantification; MS: Mass spectroscopy; RSD: Relative Standard Deviation; SD: Standard Deviation; ICH: International Conference on Harmonization; GAPI: Green Analytical Procedure Index.

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Conflicts of interests: Authors also stated that there is no conflict of interest.

Ethical statement: NA

Authors' contributions: All authors had full access to all the information and took responsibility for data integrity and data analysis accuracy. Author SAR designed the study and wrote the protocol. Author NSS performed the experimental work and scanned the literature. Authors NSS and ZAN wrote the manuscript's first draught. Authors SAR and ZAN supervised the work. The final manuscript was read and accepted by all the contributors.

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