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Kinetic Study of the Alkaline Degradation of Oseltamivir Phosphate and Valacyclovir Hydrochloride using Validated Stability Indicating HPLC

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ABSTRACT: Aqueous alkaline degradation was performed for oseltamivir phosphate (OP) and valacyclovir hydrochloride (VA). Isocratic stability indicating the use of high-performance liquid chromatography (HPLC) was presented for each drug in the presence of its degradation product. The separations were performed using the Nucleosil ODS column and a mobile phase consisting of phosphate buffer (pH = 7), acetonitrile, and methanol 50:25:25 (v/v/v) for OP. For VA separation, a Nucleosil CN column using phosphate buffer (pH = 7) and methanol 85:15 (v/v) was used as a mobile phase. Ultraviolet detection at 210 nm and 254 nm was used for OP and VA, respectively. The method showed high sensitivity concerning linearity, accuracy, and precision over the range 1–250 μg mL^{-1} for both drugs. The proposed method was used to determine the drug in its pharmaceutical formulation and to investigate the degradation kinetics of each drug's alkaline-stressed samples. The reactions were found to follow a first-order reaction. The activation energy could also be estimated. International Conference on Harmonisation guidelines were adopted for method validation.

KEYWORDS: oseltamivir phosphate, valacyclovir hydrochloride, degradation kinetic, stability indicating high-performance liquid chromatography

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Introduction

Oseltamivir phosphate (OP) (Fig. 1A) is an ester-type prodrug of a neuraminidase inhibitor, which was developed for the treatment of the A and B strains of the influenza virus. OP is a white crystalline solid that has the chemical name (3R,4R,5S)-4-acetylamino-5-amino-3(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid, ethyl ester, phosphate (1:1). Its chemical formula is $C_{16}H_{28}N_2O_4.PO_4$. The molecular weight is 410.4 m for OP salt. It attracted much attention after the 2009 flu pandemic occurred, as it was a global outbreak of a new strain of the H1N1 influenza virus, often referred to colloquially as the "swine flu." The detected virus contains a combination of genes from swine, avian (bird), and human influenza viruses.[1] People in at-risk groups have to be treated with antivirals (oseltamivir or zanamivir) as soon as possible after the first experienced flu symptoms.[2,3] Therapy with a neuraminidase inhibitor is considered especially important for patients with underlying risk factors, including pregnancy[4] and those with severe or progressive clinical illness.[5] Chemically, valacyclovir (VA) is a l-valine-2-[(2-amino-1, 6-dihydro-6-oxo-9-hipurin-9yl) methoxy] ethyl ester (Fig. 1B). It is a (l-valyl ester) prodrug of acyclovir. It is an antiherpes simplex virus type 1, 2, and varicella zoster virus.[6] VA acts specifically by inhibiting the deoxyribonucleic acid polymerase of the virus.[7,8] VA is converted rapidly into acyclovir after its oral administration via first-pass metabolism.[9] The oral bioavailability of VA is relatively higher than that of acyclovir.[10,11]

A literature survey regarding oseltamivir analysis revealed that there are several methods, which are based on different techniques. Such as bioassay and capillary electrophoresis,[12] cyclic voltammetry,[13] spectrophotometry,[14,15] fluorimetry,[16] Fourier transform infrared spectrometry.[17] To date, few liquid

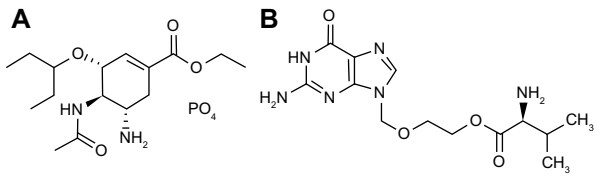

Figure 1. Chemical structure of oseltamivir phosphate (**A**) and valacyclovir (**B**).

chromatographic methods with ultraviolet (UV) detection,[18–21] photodiode array,[22] fluorescence detection,[22] or with mass spectrometry (MS) detection have been reported.[23–27]

A literature review of VA revealed that few analytical methods based on spectrophotometry,[28–30] high-performance liquid chromatography (HPLC) with UV detection,[21,31–34] HPLC with MS detection,[35,36] and micellar electrokinetic chromatography[37] were reported. The objective of the proposed methods is to develop simple and accurate methods for the estimation of OP and VA hydrochloride in bulk and in pharmaceutical dosage forms by reversed phase (RP)-HPLC. The method aimed to include kinetic studies, which are important for the quality control of pharmaceutical products. To our knowledge, the suggested method represents the first kinetics study of OP degradation and VA hydrochloride using HPLC. In addition, the method used for the separation of OP is more efficient and accurate when compared with its official HPLC method,[21] as indicated by the presented statistical data.

Experimental

Instruments. A liquid chromatograph consisting of an Agilent HPLC instrument quaternary pump (Model G 1311 A pump, Agilent 1200 series; Agilent Technologies, Santa Clara, CA, USA) connected with a UV detector (Model G1314B, Agilent 1200series; Agilent Technologies). The injector was a manual Rheodyne® injector (Model 7725I; IDEX Health & Science LLC, Rohnert Park, CA, USA) equipped with a 20 µL injector loop (Agilent Technologies). The chromatographic conditions for OP and VA included two stationary phases, which were Nucleosil® 300–5 C18 (Sigma-Aldrich, St Louis, MO, USA) and Macherey-Nagel EC 250/4 Nucleosil® 120–7 CN (Macherey-Nagel GmbH & Co. KG, Düren, Germany), respectively. The mobile phases used for OP and VA consisted of phosphate buffer (pH = 7), acetonitrile, methanol 50:25:25(v/v) (A), and phosphate buffer (pH = 7) and methanol 85:15(v/v) (B), respectively. Each mobile phase was filtered through a 0.45 µm Millipore membrane filter (EMD Millipore, Billerica, MA, USA) and was degassed for 30 minutes in an ultrasonic bath prior to use. UV detection was done at 210 nm and 254 nm for OP and VA, respectively. The system operated at ambient temperature. The flow rate was isocratic at 1.0 mL/minute^{-1} and 0.8 mL/minute^{-1} for OP and VA, respectively. The samples were filtered through a 0.45 µm membrane filter and were injected by the aid of a 25 µL Hamilton® analytical syringe (Sigma-Aldrich).

Materials and reagents. Reference OP, with a molecular weight of 410.4 m (CAS number 204255–11–8) and VA (CAS number 124832–27–5), as well as powder were kindly supplied by Nile Company for Pharmaceuticals and Chemical Industries SAE (Cairo, Egypt) and GlaxoSmithKline (Brentford, UK), respectively. The purity of OP and VA was checked and found to be 99.8% w/w and 98.4% w/w, respectively. Pharmaceutical dosage forms (Taminil® tablets) manufactured by the Nile Company for Pharmaceuticals and Chemical Industries SAE, batch number 19419, claim to contain 75 mg of OP, while Valtrex® tablets manufactured by GlaxoSmithKline (batch number 5286A) claim claimed to contain 500 mg of VA; these were purchased from the local market.

All chemicals used were of analytical grade, and deionized water was of HPLC grade. Hydrochloric acid, methanol, acetonitrile for HPLC, monobasic potassium phosphate, anhydrous dibasic sodium phosphate, and sodium hydroxide were obtained from El-Nasr Pharmaceutical Chemicals Co. (ADWIC; Cairo, Egypt).

Standard solutions. OP stock solution (5 mg/mL^{-1}) was prepared in methanol (OP stock solution). VA hydrochloride stock solution (5 mg/mL^{-1}) was prepared in the phosphate buffer (pH = 7) (VA stock solution). A working standard solution of OP (A) (1 mg/mL^{-1}) was prepared from an OP stock solution in methanol. A working standard solution of OP (B) (0.1 mg/mL^{-1}) was prepared from the OP stock solution in methanol. A working standard solution of VA (A) (1 mg/mL^{-1}) was prepared from a VA stock solution in the phosphate buffer (pH = 7). A working standard solution of VA (B) (0.1 mg/mL^{-1}) was prepared from a stock solution in phosphate buffer (pH = 7).

General procedure. *Accelerated alkaline degradation of OP.* From the OP working standard solution (A), the aliquot equivalent to OP (10 mg) was transferred into a measuring flask (100 mL), then 10 mL of 0.1 M NaOH was added; the flask was then completed to volume with methanol. Complete hydrolysis was achieved at room temperature after 3 hours (180 min.), as investigated by liquid chromatography using the chromatographic conditions described under Instruments section.

Accelerated alkaline degradation of VA. From the VA working standard solution (A), 50 mL was transferred into a measuring flask (100 mL), and the flask was then completed to volume with 0.1 M NaOH. Complete hydrolysis was achieved at room temperature after 2.5 hours (150 min.), as investigated by liquid chromatography using the chromatographic conditions described under Instruments section.

Linearity. Volumes equivalent to (1–250 µg) from the standard solutions and the working standard solution (A) and (B) of OP and VA, respectively, were transferred separately into two series of measuring flasks (10 mL); they were then completed to volume with methanol and phosphate buffer (pH = 7) for OP and VA, respectively. Twenty µL of each of the previously described solutions were injected into the

liquid chromatograph using the chromatographic conditions described under Instruments section. The corresponding peak areas were used to construct the calibration curves using area under peaks (AUPs) versus the corresponding concentrations in $\mu g/mL^{-1}$; two regression equations were calculated for OP and VA, respectively.

Assay of OP in pharmaceutical formulation. The powder content of 20 capsules was evacuated, and this content was weighed. A quantity equivalent to OP (25 mg) was accurately weighed and transferred into a volumetric flask (25 mL) and methanol (10 mL) was added. The flask was then sonicated for 5 minutes, and its volume was completed with that of methanol. The above solution was filtered, and aliquots from the filtrate were taken and diluted with methanol to obtain a concentration range of 1–250 $\mu g/mL^{-1}$. The procedure was then followed as described under the Linearity section.

Assay of VA in pharmaceutical formulations. Twenty tablets were weighed and finely powdered. A quantity of the powdered tablets equivalent to VA (25 mg) was accurately weighed and transferred into a volumetric flask (25 mL), and phosphate buffer (pH = 7) (10 mL) was added. The flask was then sonicated for 5 minutes and the volume was completed with phosphate buffer (pH = 7). The above solution was filtered, and aliquots from the filtrate were taken and diluted with phosphate buffer (pH = 7) to obtain a concentration range of 1–250 $\mu g/mL^{-1}$. The procedure was then followed as described under the Linearity section.

Method validation. Method validation was carried out in accordance with the International Conference on Harmonisation guidelines.[38]

Kinetic studies. *Studying the kinetic order of the reaction of OP.* From the OP stock solution, the aliquot equivalent to OP (100 mg) was transferred into a measuring flask (50 mL), and then 0.1 M of NaOH (10 mL) was added and the volume was completed with methanol. A total of 5 mL sample solutions were taken at 30-minute intervals, placed into measuring flasks (10 mL), neutralized with 0.1 M HCl (1 mL), and the volume was completed with methanol. An aliquot equivalent to initial concentration (C_0 = 40 $\mu g/mL^{-1}$) was transferred into a measuring flask (5 mL) and completed to volume with methanol. The solution was injected into the liquid chromatograph using the chromatographic conditions described under Instruments section. The concentration of OP was calculated, and the log percentage of the remaining concentration was plotted against time.

Studying the kinetic order of the reaction of VA. From the VA working standard solution (A), the aliquot equivalent to VA (50 mg) was transferred into a measuring flask (100 mL) and completed to volume with 0.1 M of NaOH. Then, 4 mL sample solutions were taken every 15 minutes, placed into measuring flasks (10 mL), neutralized with 2 mL of 0.1 M HCl, and the volume was then completed with the phosphate buffer (pH = 7). Aliquot equivalent to the initial concentration (C_0 = 100 $\mu g/mL^{-1}$) were transferred into a measuring flask (5 mL) and completed to volume with the phosphate buffer (pH = 7). The solution was injected into the liquid chromatograph using the chromatographic conditions described under Instruments section. The concentration of VA was calculated, and the log percentage of the remaining concentration was plotted against time.

Studying the effect of NaOH concentration and temperature on the reaction rate of OP and VA. Into two separate series of measuring flasks (5 mL), aliquots equivalent to OP or VA (1 mg) were transferred, and 1 mL volume of NaOH (0.1 M, 0.2 M, and 0.3 M) were added and completed to the mark with methanol and phosphate buffer (pH = 7) for OP and VA, respectively. These solutions were transferred into clean, dry, conical flasks and refluxed in a thermostatically controlled water bath at 40 °C, 50 °C, and 60 °C for OP and at 30 °C, 40 °C, and 50 °C for VA. From each flask, 1 mL of the sample solutions were taken into a measuring flask (5 mL) every 10 minutes for OP, or after every 5 minutes for VA; the samples were then neutralized with 0.2 mL, 0.4 mL, and 0.6 mL of 0.1 M HCl, respectively. The volume was then completed with the respective solvent. Further dilution was done to obtain solutions with concentrations of 40 $\mu g/mL^{-1}$ and 100 $\mu g/mL^{-1}$ of OP and VA, respectively. Each solution was injected into the liquid chromatograph, as described under Instruments section. The log percentage of the remaining concentration against time was constructed for the different molarities of NaOH, and the rates constants and $t_{1/2}$ were calculated.

Studying the effect of acidic, oxidizing, and photolytic conditions on the stability of OP and VA. Aliquots of OP or VA equivalent to 10 mg were transferred separately into two separate series of volumetric flasks (100 mL). To the first flask of each series, 0.1 M of HCl (10 mL) were added; the solutions were left for 4 hours then neutralized. To the second flask of each series, 5 mL of 50% H_2O_2 were added; the solution was left for 4 hours. The last flask of each series was completed to volume, as directed, and it was subjected to a UV lamp for 24 hours. The flasks were completed to volume with methanol or phosphate buffer (pH = 7) for OP and VA, respectively, in all cases. After the mentioned time, 20 µL of the solutions were injected into the liquid chromatograph using the chromatographic conditions described under Instruments section.

Results and Discussion

A simple isocratic HPLC method was developed for the determination of OP and VA in the presence their degradation products without prior separation. A trial for accelerated degradation was done for both drugs. Aqueous alkaline degradation was found to affect the drugs' stability, and it led to 100% degradation at 3 hours and 2.5 hours for OP and VA, respectively (Fig. 2).

High-performance liquid chromatographic developments. To optimize the HPLC assay parameters, the type of columns used, its dimensions, its mobile phase conditions, and its choice of detection wavelength were carefully investigated.

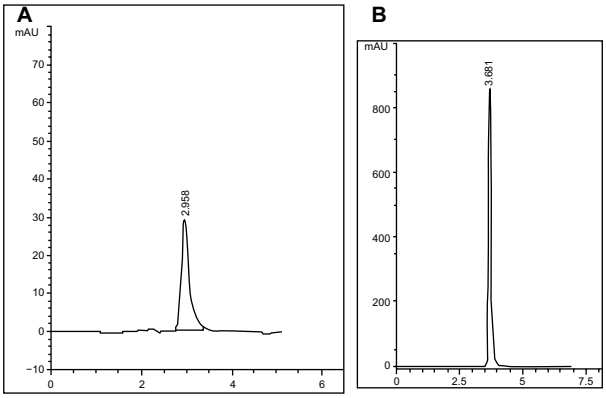

Figure 2. The chromatograms of the degradation products of OP (**A**) and VA (**B**).

Different types of stationary phases, columns with different dimensions, and particle sizes were tried. It was found that the Nucleosil® 300–5 C18 column (250 mm × 4.6 mm) with a particle size of 5 µm and Macherey-Nagel EC 250/4 Nucleosil® 120–7 CN with a particle size of 7 µm gave the most suitable resolution for OP and VA, respectively. The mobile phase was chosen after several trials to reach the optimum stationary/mobile phase matching. Several mobile phase compositions with different buffer pH values were employed. The optimum separation for OP or VA, as well as the corresponding degradation product were obtained with a mobile phase consisting of phosphate buffer (pH = 7), methanol, and acetonitrile 50:25:25 (v/v), or phosphate buffer (pH = 7) and methanol 85:15 (v/v), respectively. The retention times were 3.3 ± 0.15 minutes for OP and 2.8 ± 0.15 minutes for the OP degradation product, or 6 ± 0.15 minutes for VA and 3.6 ± 0.15 minutes for the VA degradation product (Fig. 3). System suitability tests were used to ensure adequate performance of the chromatographic system. Items were evaluated for three replicate injections of the drugs at concentrations of 80 µg/mL^{-1} and 100 µg/mL^{-1} for OP and VA, respectively. Results were of the acceptable limits (Table 1).

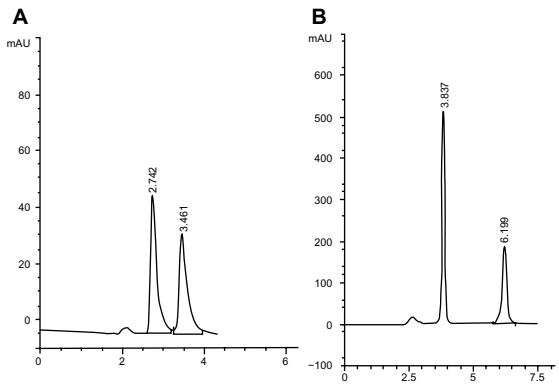

Figure 3. Representative chromatograms of OP and its degradation product (**A**) and VA and its degradation product (**B**).

The chromatographic separation in this work allowed for the complete baseline separation of OP from its degradation product in less than 5 min., and of VA from its degradation product in less than 8 minutes.

HPLC validation. *Linearity and range.* Linear relationships were obtained between the AUPs at the selected wavelengths (210 nm and 254 nm for OP and VA, respectively). The linearity of the calibration curves and adherence of the system to Beer's law were verified by the high correlation coefficient. The analytical data for the calibration curves are summarized in Table 2.

Specificity. The specificity of separation can be demonstrated by the resolution of the two components (2.61, 10.54) for OP and VA separation from their degradation products under the experimental condition respectively. Good peaks, a clear baseline, and the absence of interference are demonstrated in Figure 3. The specificity of the proposed method was shown by the analysis of laboratory-prepared mixtures containing known amounts of the standard drugs at 25%–100%, which were fortified to the degradation samples. The percentage recovery rate at each level was in good agreement, irrespective of the percent of the added degradation (Table 3). Moreover; the specificity of the method was ascertained by successful application to Taminil® tablets for OP and Valtrex® tablets for VA, without any peak distortions or variations that resulted from the excipients. The small relative standard deviation percentage (0.526% for OP, 0.215% for VA) indicates that the methods were specific (Table 2).

Accuracy. The standard addition technique was used to ascertain the accuracy of the results (Table 2). The accuracy of the drug substances was ascertained by comparing the results of the proposed method with an official method[21] for OP and the reported HPLC method[34] for VA. Results indicate a nonsignificant difference, proving the method's accuracy (Table 4).

Precision. Precision was determined by applying the procedures to three concentrations for three replicates. The precision of the suggested methods was also expressed in terms of relative standard deviation of the interday and intraday analysis (Table 2).

Robustness. The robustness of the HPLC method was investigated via an analysis of samples under a variety of experimental conditions, such as small changes in pH

Table 1. System suitability results for the proposed HPLC method.

PARAMETER	OP	OP DEGRADATE	VA	VA DEGRADATE
[a]N	1986	2102	6784	10989
[b]R	2.61		10.54	
[c]K	1.48	0.968	1.63	0.63
[d]α	1.529		2.587	
[e]T	1.69	1.65	0.94	1.17

Notes: [a]Number of theoretical plates. [b]Resolution. [c]Capacity factor. [d]Selectivity factor. [e]Tailing factor.

Table 2. Assay parameters and methods validation for the proposed HPLC method.

PARAMETER	OP			VA		
Retention time (min.)	3.3 ± 0.15			6 ± 0.15		
Wavelength of detection, nm	210			254		
Calibration range, μg ml^{-1}	1–250			1–250		
Regression equation	y = 31.37x–31.7400			y = 49.71 x + 8.4311		
Correlation coefficient (r)	0.9998			0.9999		
[a]S_b	0.244			0.200		
[b]S_a	0.369			0.267		
[c]LOD	0.055			0.29		
[d]LOQ	0.165			0.879		
Confidence limit of the slope	31.37 ± 0.002			49.7 ± 0.010		
Confidence limit of the intercept	31.74 ± 0.007			8.43 ± 0.001		
Standard error of the estimation	51.9457615			43.73		
Intra day: Mean of concentrations (μg/ml) n=3	79.68	99.87	150.97	102.40	116.98	142.55
[e]RSD,%	0.680	1.580	0.808	0.217	0.165	0.221
Inter day: Mean of concentrations (μg/ml) n=3	79.44	98.74	151.52	101.96	118.75	142.27
RSD,%	1.11	0.95	0.37	1.07	1.30	1.18
Results: Drug in dosage form,%	96.31121 ± 0.506964			100.1937 ± 0.215629		
Drug added,%	100.2444 ± 1.63194			100.2633 ± 0.362817		

Notes: [a]Standard deviation of the slope. [b]Standard deviation of the intercept. [c]Limit of detection. [d]Limit of quantification. [e]Relative standard deviation. LOD and LOQ calculated by styx's.

(6.0–7.5), or changing the column using a 250 mm × 4.6 mm id, C18 Lichrosorb 10 m analytical column for OP, and using the column 250 × 4 id Waters Spherisorb 5 μ CN for VA. The effect on retention time and peak parameters was studied. It was found that the method was robust when the column and the mobile phase pH were varied. During these investigations, the retention times were modified; however, the areas and peak symmetries were conserved. Changes in instruments or personnel did not alter the results, which indicated the ruggedness of the proposed method.

Limits of detections and quantifications. The detection and quantification limits were calculated based on the standard deviation of the response and the slope of the calibration curves, as follows:

$$LOD = 3.3 \times \sigma/S, \quad (1)$$

$$LOQ = 10 \times \sigma/S, \quad (2)$$

where σ is the standard deviation of the response and S is the slope of the regression line of the calibration curve, indicating the sensitivity of the method.

Table 3. Accuracy determination of OP and VA and their degradation by the proposed HPLC method.

DRUG% / DEGRADATION%		CONC.		FOUND CONC.		RECOVERY%	
OP	VA	OP	VA	OP	VA	OP	VA
100/37.5	100/100	40	50	39.8	49.88	99.5	99.76
100/33.3	100/40	75	50	75.6	50.88	100.8	101.76
100/26.6	100/25	60	80	59.4	80.61	99	100.76
100/18.7	100/50	80	60	79.04	59.96	98.8	99.93
100/38.8	100/66.6	90	15	90.9	15.08	101	100.53
100/11.1	100/33.3	90	15	90	15.06	100	100.40
Mean						99.85	100.52
SD						±0.916	±0.711

Table 4. Statistical analysis of the results obtained by applying the proposed HPLC methods.

STATISTICAL TERMS	OP		VA	
	REFERENCE METHOD [21]	HPLC METHOD	REFERENCE METHOD [34]	HPLC METHOD
Mean	100.375	100.345	99.452	99.997
S.D.±	1.368	0.727	1.134	0.583
S.E.±	0.559	0.297	0.463	0.238
R.S.D,%	1.363	0.725	1.141	0.583
N	6	6	6	6
V	1.872	0.529	1.287	0.340
t(1.81)	0.008		1.047	
F(5.05)	3.541		3.780	

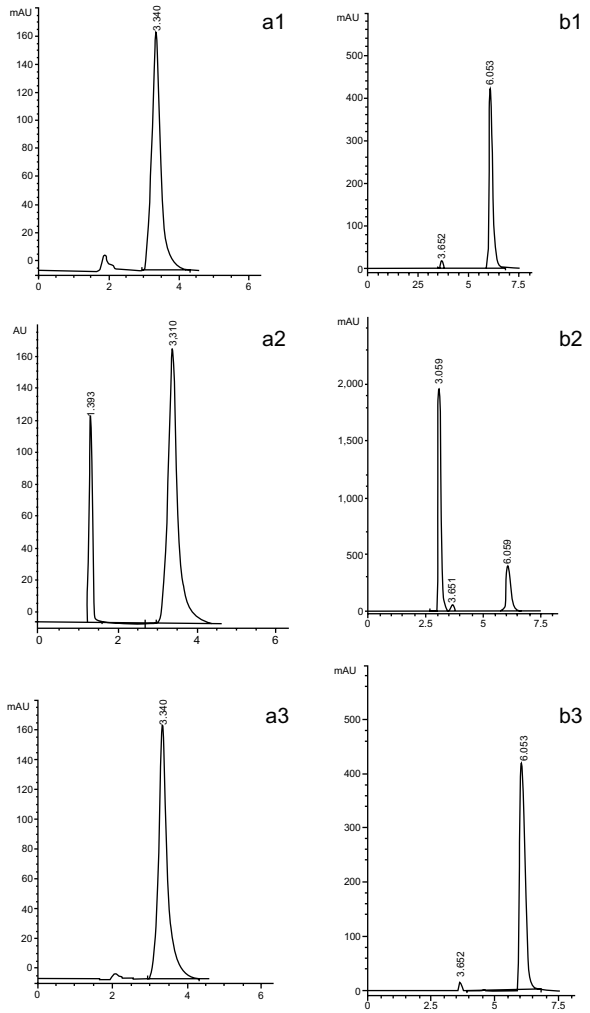

Figure 4. Chromatogram representing results of acid induced degradation of OP (a1) and VA (b1), oxidizing agent-induced degradation of OP (a2) and VA (b2), UV- induced degradation of OP (a3) and VA (b3).

Stability study. Treatment of both drugs with acid, an oxidizing agent—namely, hydrogen peroxide—and, finally, exposure to light for a fixed period of time (as mentioned under the Studying the effect of acidic, oxidizing, and photolytic conditions on the stability of OP and VA section) resulted in nearly no change of the drugs' chromatogram, as shown in Figure 4.

Kinetics study of the degradation. Treatment of OP and VA with alkali resulted in the gradual decomposition of both drugs. The linear relationship (Fig. 5) between the percentage log of the remaining concentration against time indicated first-order degradation. Since the hydrolysis was performed in a large excess of NaOH (0.1 mol/L^{-1}), it followed a pseudo first-order reaction rate.[39] The term is used when two reactants are involved in the reaction, but one of them is in such a large excess (NaOH) that any change in the concentration is negligible compared with the change in the concentration of the other reactant (drug).

Different parameters that affect the rate of the reaction were studied. The temperature dependence and effect of base strength on OP and VA degradation was studied by conducting the reaction at different temperatures using different base strengths (Fig. 6). At each temperature, the rate constant and $t_{1/2}$ were calculated. It was concluded that as the temperature increased, the rate of hydrolysis also increased, with a decrease noted in the $t_{1/2}$ (Table 5). Also, the energy of activation was determined by calculating the rate constant from the following equation:[40]

$$\text{Log } K_2/K_1 = Ea/2.303R^*T_2\text{-}T_1/T_2T_1 \quad (3)$$

Where "Ea" is the activation energy, "T_1" and "T_2" are the two temperatures in Kelvin, "R" is the gas constant, and "k_1" and "k_2" are the rate constants at the two temperatures used. The calculated "Ea" was found to be 30.013 k joule mol^{-1} and 8.79 k joule mol^{-1} for OP and VA, respectively. Another factor that affects the rate of the reaction is the base strength of NaOH. Different base strengths were used to study its effect upon the hydrolysis, and it was found that increasing the base strength results in a uniform increase in the rate constant and a decrease in the $t_{1/2}$ for OP. It is worth mentioning that attempts to use higher base strengths for VA result in

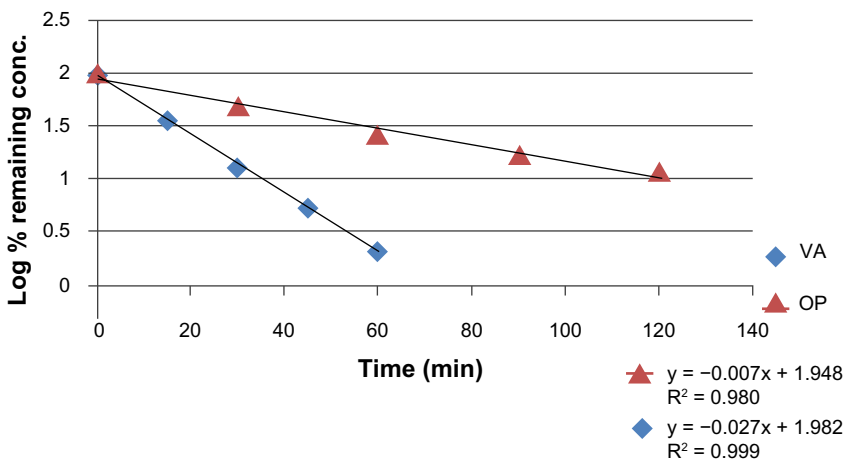

Figure 5. First order plot of hydrolysis of OP and VA (4 mg%) with o.1 M NaOH.

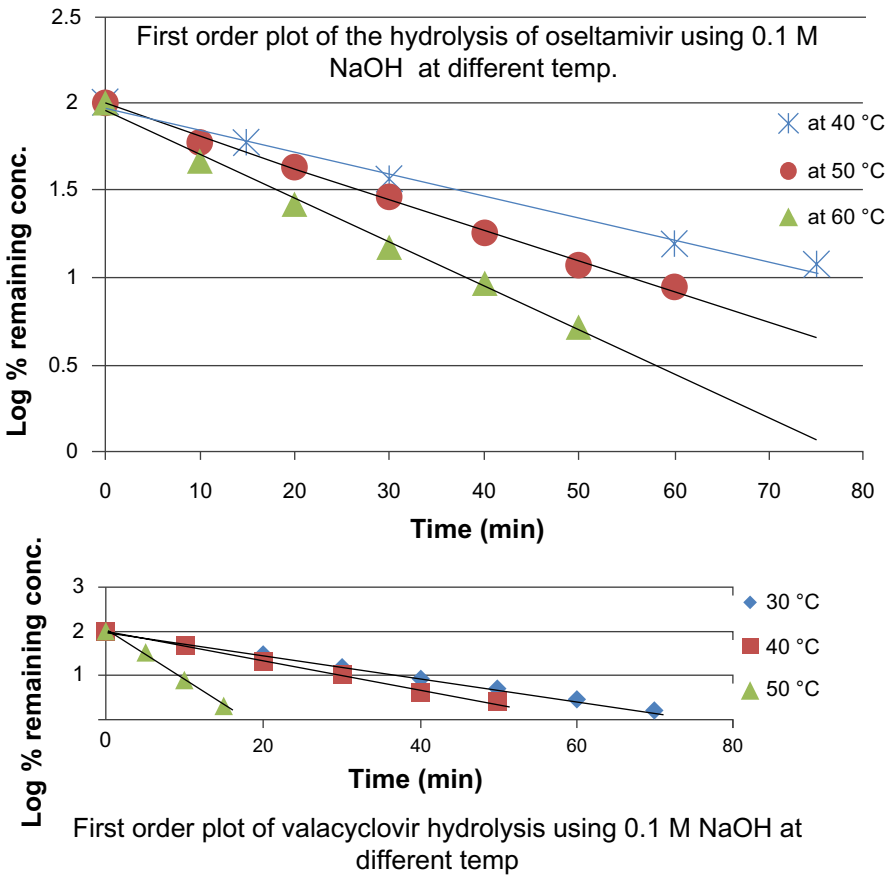

Figure 6. First order plot of the hydrolysis of OP and VA (10 mg%) with o.1 M NaOH at different temperature.

a spontaneous degradation, revealing high instability of the drug towards higher base strengths that was also confirmed by its law value of the activation energy.

Conclusion

The proposed HPLC methods provide simple, sensitive, and specific methods suitable for the quantitative analysis of both drugs in the presence of their degradation products for the routine quality control analysis of OP and VA, either in their pure form or in available pharmaceutical dosage forms, with no interference from the excipients or the degradation product. The reaction kinetic of the degradation was found to be a pseudo first-order reaction under the experiment's basic stress conditions. Other stress conditions did not affect the drugs investigated. The methods proved that the selectivity, accuracy, and simple mobile phases used provide simple and economic applications, and they reflect suitability for quality control laboratories.

Table 5. Kinetic data of the stress alkaline hydrolysis OP and VA using the proposed HPLC method.

STRENGTH OF NAOH	TEMPERATURE (°C)		K(H^{-1})		$T_{1/2}$ (H)	
	OP	VA	OP	VA	OP	VA
0.1M	Room temp.	Room temp.	0.016	0.062	42.987	11.145
	40	30	0.029	0.058	24.073	11.036
	50	40	0.041	0.074	17.001	9.403
	60	50	0.0578	0.253	11.988	2.736
0.2 M	40	---	0.062	---	11.145	---
	50	---	0.124	---	5.572	---
	60	---	0.168	---	4.122	---
0.3 M	40	---	0.081	---	8.597	---
	50	---	0.120	---	5.787	---
	60	---	0.210	---	3.307	---

Author Contributions

Conceived and designed the experiments: RIB, AAZ, FAM. Analyzed the data: RIB, AAZ, FAM, MMF. Wrote the first draft of the manuscript: AAZ, MMF. Contributed to the writing of the manuscript: FAM, MMF. Agree with manuscript results and conclusions: RIB, AAZ, FAM. Jointly developed the structure and arguments for the paper: RIB, AAZ, MMF. Made critical revisions and approved final version: RIB, AAZ, FAM. All authors reviewed and approved of the final manuscript.

DISCLOSURES AND ETHICS

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance

with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

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