

Liquid Chromatographic Determination of Roxithromycin: Application to Stability Studies

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A simple, stability-indicating, reversed-phase liquid chromatographic method has been developed for the determination of roxithromycin in the presence of its forced alkaline, oxidative and ultraviolet degradation products. Reversed-phase chromatography was conducted using an ODS C18 (150 × 4.6 mm i.d.) column at ambient temperature with ultraviolet detection at 215 nm. A mobile phase consisting of 0.03 M potassium dihydrogen phosphate buffer–methanol (40:60, v/v) adjusted to pH 4.5 was used for the separation of the studied drug and its degradation products at a flow rate of 1 mL/min. The method showed good linearity over the concentration range of 10.0–150.0 µg/mL with a detection limit of 2.5 µg/mL and quantification limit of 8.4 µg/mL. The proposed method was successfully applied for the analysis of roxithromycin in its commercial tablets; the obtained results were favorable compared with those obtained by the official method. Furthermore, content uniformity testing of the studied tablets was also conducted. The method was also utilized to investigate the kinetics of the different degradation products of the drug. The first-order rate constant, half-life time and activation energy of the degradation reactions were calculated.

Introduction

Roxithromycin (ROX) is a semi-synthetic 14-membered-ring macrolide antibiotic (Figure 1), in which the erithronolide A lactone ring has been modified by the replacement of the 9 keto group with an etheroxime side chain to prevent deactivation in the gastric milieu (1). The *in vitro* activity of ROX is well known and is as effective as, or more effective than, other macrolide antibiotics against a wide range of infections. *In vivo*, ROX has mostly been used to treat respiratory, urinary and soft tissue infections (2). Gastrointestinal disturbances are the most frequent adverse effects, but are less frequent than with erythromycin (3). ROX exhibits increased chemical stability and higher concentrations of antibiotic in the serum after oral administration than erythromycin (4). ROX has been analyzed using thin layer chromatography and high-performance liquid chromatography (HPLC) procedures (5–9). Different methods for the determination of ROX and its analysis in human plasma, urine and serum by HPLC using electrochemical and spectrophotometric detection have also been described (10–13). Neither of the previously reported methods conducted a thorough stability study with detailed kinetic investigation for ROX as suggested by this work, which gives this manuscript a remarkable advantage.

The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) (14) was organized to harmonize stability testing

requirements for new drug applications within the European Union, the United States and Japan. The ICH guideline Q1A on stability testing of new drug substances and products emphasizes that the testing of those features that are susceptible to change during storage and are unlikely to influence quality, safety and/or efficacy must be done by validated stability-indicating testing methods. It is also mentioned that forced decomposition studies (stress testing) should be performed on the drug substance to establish the inherent stability characteristics and degradation pathways to support the suitability of the proposed analytical procedures (14).

The aim of the present work is to focus on the development of an efficient liquid chromatographic method for the determination of ROX in the presence of its different degradation products in a short chromatographic run, and to study the results kinetically to prove the stability-indicating property of the method.

Experimental

Apparatus

Separation was performed with a Shimadzu C-R6A Chromatopac equipped with a Rheodyne injector valve with a 20-µL loop and an ultraviolet-visible (UV-VIS) detector.

A Shimadzu UV 1601 PC spectrophotometer equipped with a pair of 1-cm matched cells was used. Recording range: 0–2; wavelength: 200–400 nm; factor: 1; number of cells: 1; cycle time: 0.1 min.

Materials

ROX of purity 98.94% was provided by El-Obour Modern Pharmaceutical Industries Company (Cairo, Egypt). Methanol (Sigma–Aldrich, St. Louis, MO) was HPLC grade. The buffer, 0.03 M potassium dihydrogen phosphate (BDH, Poole, UK), was prepared in distilled water. The pH was adjusted to 4.5 using 0.03 M phosphoric acid and 0.03 M sodium hydroxide. Sodium hydroxide (2 M solution), hydrochloric acid (2 M solution) and hydrogen peroxide (6% v/v solution) were provided by BDH. Roxicin tablets, labeled to contain 150 and 300 mg ROX per tablet (El-Obour Modern Pharmaceutical Industries Company) were obtained from commercial sources in the local market.

Chromatographic conditions

Separation was achieved on an EC nucleosil C18-SN: 4115568 column (150 × 4.6 mm i.d. (5 µm) combined with a guard column (Merck, Darmstadt, Germany). The columns were operated at ambient temperature. The analytical system was washed daily with 60 mL of a 1:1 mixture of water and

Results and Discussion

The proposed liquid chromatographic method allows the separation of ROX from its degradation products with satisfactory resolution. Figures 2A, 2B and 2C show good resolution of ROX from each of its alkaline, oxidative and UV degradation products, respectively. It was also possible to conduct a stability study for the degradation kinetics of the drug. The drug was highly stable to acidic degradation, which could be attributed to the incorporation of the *N*-oxime side chain attached to the lactone ring of ROX, increasing its stability to acid catalyzed degradation. It was also found that only 10% of the drug was decomposed after exposure to direct sunlight for 20 h.

Selection and optimization of the chromatographic conditions

Well-defined symmetrical peaks were obtained upon measuring the response of the eluent under the optimized conditions after thorough experimental trials. Three columns were used for performance investigations, including EC nucleosil C18-SN: 4115568 column, Hibar prepacked column RT-250-4-L-100-RP8 and Zorbax SB-Phenyl column (250 × 4.6 mm i.d. (5 μm) column. Experimental studies revealed that the first column was appropriate, giving symmetrical, well defined peaks and allowing good separation of ROX from its degradation products.

Several modifications in the mobile phase composition were performed to study the possibilities of changing the selectivity of the chromatographic system. These modifications included the change of the type and ratio of the organic modifier, the

pH, the strength of phosphate buffer and the flow rate. The results are summarized in Table I. Methanol and acetonitrile were investigated for selection of the proper organic modifier for the assay, but the latter resulted in excessively tailed peaks. Methanol was the organic modifier of choice, giving symmetrical, narrow and well-resolved peaks.

The effect of changing the ratio of organic modifier on the selectivity and retention times of the test solutes was investigated using mobile phases containing methanol–phosphate buffer in a ratio of (45:55–70:30, v/v). Ratios higher than 65% of methanol resulted in a decrease in the number of theoretical plates (*N*), accompanied by a loss of peak symmetry. Meanwhile, mobile phases containing ratios less than 60% of methanol caused unacceptably long retention times of the drug and relatively broad peaks. Finally, a mobile phase consisting of methanol–0.03 M potassium dihydrogen phosphate mixture in the ratio of 60:40, v/v (pH 4.5), was considered to be optimal because it provided a good compromise between retention times, resolution factor, number of theoretical plates and peak shape.

To investigate the effect of the pH of the mobile phase on the selectivity and retention times of the test solutes, mobile phases of pH values ranging from 2.5 to 6.0 were attempted. With mobile phases of pH values higher than pH 5, the peak of oxidative degradation product overlapped with that of the drug, while at pH lower than 4, distorted peaks and a remarkable decrease in *N* resulted. Therefore, pH 4.5 was the most appropriate, permitting good separation of the drug from all of its degradation products.

The effect of changing the ionic strength of the phosphate buffer on the selectivity and retention times of the test solutes was investigated using mobile phases containing concentrations of 0.0075–0.05 M of phosphate buffer. Mobile phase containing phosphate buffer of ionic strengths less than 0.02 M resulted in a gradual decrease in *N* and alteration of the peak

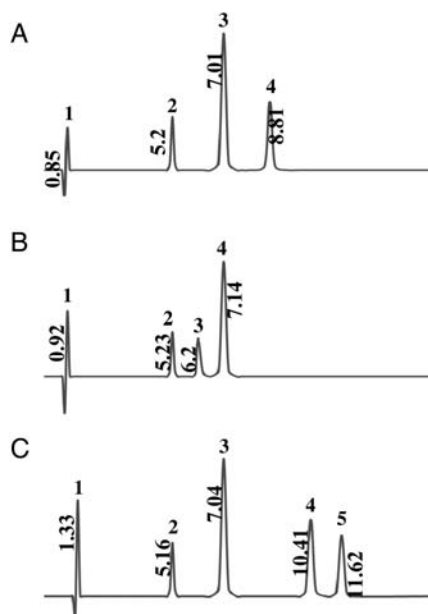


Figure 2. Typical chromatograms showing 1: solvent front, 2: erythromycin internal standard (30.0 μg/mL), 3: remaining ROX, 4: alkaline degradation product of ROX (50.0 μg/mL) with 2 M NaOH at 100°C for 40 min (A); 1: solvent front, 2: erythromycin internal standard (30.0 μg/mL), 3: oxidative degradation product of ROX (50.0 μg/mL) with 6% H₂O₂ at 80°C for 20 min, remaining ROX (B); 1: solvent front, 2: erythromycin internal standard (30.0 μg/mL), 3: remaining ROX, 4: first UV degradation product, 5: second UV degradation product of ROX after exposure to UV lamp at 254 nm for 8 h (C).

Table I

Optimization of the Chromatographic Conditions for Separation of ROX from its Degradation Products by the Proposed Method

Parameter	Number of theoretical plates (<i>N</i>)				Resolution (<i>R_s</i>)				
	A*	B	C	D	A	B	C	D	
Ratio of organic modifier	70:30	5,864	7,005	3,850	10,731	3.5	7.2	1.8	9.8
	65:35	6,840	7,739	4,958	10,826	4.3	7.9	2.3	10.6
	60:40	6,833	7,744	4,963	10,816	4.5	8.2	2.2	10.5
	55:45	6,441	6,113	3,210	9,334	4.1	7.5	1.6	9.4
	50:50	6,231	5,660	2,015	8,234	3.5	6.1	0.92	8.3
pH	45:55	6,100	5,170	1,156	7,881	2.6	5.4	0.82	7.2
	2.5	5,633	6,821	3,512	8,754	2.4	7	1.5	8.8
	3.0	5,931	7,035	3,954	9,834	3.2	7.5	1.8	9.1
	3.5	6,214	7,620	4,025	10,264	4	8	2.1	9.5
	4.0	6,811	7,739	4,973	10,824	4.4	8.3	2.3	10.4
	4.5	6,833	7,744	4,963	10,816	4.5	8.2	2.2	10.5
	5.0	6,811	7,740	4,885	10,789	4.2	8.1	0.92	10
Ionic strength of phosphate buffer	5.5	6,746	7,689	4,874	10,654	3.8	7.8	0.81	9.5
	6.0	6,513	7,621	4,877	10,503	3.1	7.6	0.62	9
	0.0075	6,238	7,103	4,764	10,689	2.8	6.5	1.3	9.5
	0.01	6,654	7,325	4,877	10,845	3.8	7.3	1.5	9.8
	0.02	6,829	7,752	4,970	10,820	4.4	8.2	2.3	10.4
	0.03	6,833	7,744	4,963	10,816	4.5	8.2	2.2	10.5
	0.04	6,835	7,731	4,959	10,835	4.5	8.4	2.1	10.3
0.05	6,841	7,728	4,971	10,840	4.3	8.3	2.3	10.4	

*Note: A is ROX, B is alkaline degradation product, C is oxidative degradation product, and D is UV degradation product.

symmetry of the drug and its degradation products. Table I shows that 0.03 M phosphate buffer (pH 4.5) was found to be the most suitable, providing good resolution and the highest number of theoretical plates. The effect of flow rate on the separation of peaks of the studied drug and its degradation products was investigated and a flow rate of 1 mL/min was found to be optimal for good separation within a reasonable time. Flow rates greater than 1 mL/min caused broad peaks, while flow rates lower than 1 mL/min caused long retention times.

Method validation

Linearity and range

Under the previously described experimental conditions, a linear relationship was established by plotting peak area ratio for ROX to the internal standard against ROX concentrations. The concentration range was found to be 10.0–150.0 µg/mL. The high value of the correlation coefficient (r -value > 0.999) and small value of intercept indicate the good linearity of the calibration graph over the working concentration range. Statistical analysis of the data gave small values of the standard deviation (SD) of the residuals ($S_{y/x}$), slope (S_b) and intercept (S_a) (Table II) (16), thus indicating low scattering of the points around the calibration curve.

Limit of quantitation and limit of detection

Detection limit (LOD) is the lowest concentration of the drug that can be detected, but not necessarily quantitated, under the stated experimental conditions. The LOD is generally quoted as the concentration yielding a signal-to-noise ratio of 3:1 (15) and is confirmed by analyzing a number of samples near this value using the following equation:

Signal-to-noise ratio, $s = H/h$; where H = height of the peak corresponding to the drug; and h = absolute value of the largest noise fluctuation from the baseline of the peak of a blank solution.

The limit of quantification (LOQ) is the lowest concentration of the analyte that can be determined with acceptable precision and accuracy. It is quoted as the concentration yielding a signal-to-noise ratio of 10:1 and is confirmed by analyzing a number of samples near this value (15). The calculated values are listed in Table II.

Accuracy

The accuracy of an analytical method is defined as the similarity of the results obtained by this method to the true values. To

Table II
Performance Data of the Proposed Method

Parameter	Proposed method
Concentration range (µg/mL)	10.0–150.0
LOD (µg/mL)	2.5
LOQ (µg/mL)	8.4
Correlation coefficient (r)	0.9998
Slope	0.049
Intercept	-0.019
SD of residuals ($S_{y/x}$)	0.0156
SD of intercept (S_a)	1.03×10^{-3}
SD of slope (S_b)	1.2×10^{-4}

test the validity of the method, it was applied to the determination of pure samples of ROX over the concentration range of 10.0–150.0 µg/mL. The high percentage recoveries, with an average value of 100.14, and small value of SD (± 0.55) indicate the accuracy of the proposed method. The accuracy of the proposed HPLC method was also evaluated by studying the accuracy as percent relative error (%Error) and precision as percent relative standard deviation (%RSD), and the results are shown in Table III.

Precision

The intra-day precision was evaluated through replicate analysis of three concentrations of ROX in pure form on three successive times. The inter-day precision was also evaluated through replicate analysis of three concentrations for a period of three successive days. The results of intra-day and inter-day precision are summarized in Table IV. The small values of RSD and % Error indicate the high accuracy and precision of the proposed method, respectively.

Specificity

The specificity of the proposed HPLC method was proven by its ability to determine ROX in its commercial tablets, confirming that there was no interference by common excipients and additives such as anhydrous calcium phosphate, microcrystalline cellulose, pre-gelatinized starch, talk and colloidal silicon dioxide. The placebo was prepared, consisting of all these

Table III
Determination of ROX in its Pure Form Using the Proposed Method

Parameter	Taken (µg/mL)	Found (µg/mL)	% Found	Official method (15), % Found
ROX pure form	10.0	9.99	99.92	100.25
	40.0	39.66	99.15	100.61
	60.0	59.89	99.82	99.45
	80.0	80.36	100.45	
	100.0	100.67	100.67	
	120.0	120.31	100.25	
	150.0	151.08	100.72	
$X^- \pm SD$			100.14 ± 0.55	100.11 ± 0.59
t test			0.18 (1.94)*	
F test			1.15 (5.14)	
%RSD			0.55	
%Error			0.21	

*Figures within parentheses are tabulated t and F values at $P = 0.05$ (16).

Table IV
Precision Data for the Determination of ROX in Pure Form by the Proposed Method

ROX pure form	10.0 µg/mL	70.0 µg/mL	150.0 µg/mL
Intra-day precision, % Found	100.63	100.35	100.98
	100.12	100.84	100.13
	99.63	99.56	99.45
$X^- \pm SD$	100.13 ± 0.51	100.25 ± 0.65	100.19 ± 0.77
%RSD	0.51	0.65	0.77
%Error	0.29	0.38	0.44
Inter-day precision, %Found	99.12	99.32	100.45
	100.54	100.52	99.12
	100.38	100.12	99.81
$X^- \pm SD$	100.01 ± 0.78	99.99 ± 0.61	99.79 ± 0.67
%RSD	0.78	0.61	0.67
%Error	0.45	0.35	0.39

excipients with the active ingredient omitted and 20 μ L was injected under the described chromatographic conditions for the assay. As shown in Figure 3A, these matrix components did not show any interfering peaks at the retention times of either the drug or any of its degradation products. Specificity of the method was also confirmed by its ability to unequivocally measure the drug in the presence of all degradation products, as revealed by the results of the stability study (Figures 2A–2C).

Robustness

The robustness of the adopted method was demonstrated by the consistency of the values of the peak area ratios with deliberately minor changes in the experimental parameters, such as buffer pH (4 ± 0.5), molar strength (0.02 M–0.05 M) and phosphate buffer ratio–methanol ratio (35:65–40:60, v/v), which did not greatly affect the peak area ratios.

Solution stability and mobile phase stability

The stability of the stock solution was determined by quantitation of ROX and comparison to a freshly prepared standard solution. No significant change was observed in standard solution response relative to the freshly prepared standard. Similarly, the stability of the mobile phase was checked. The results obtained in both cases proved that the sample solution and mobile phase used during the assay were stable up to seven and three days, respectively.

Applications

Dosage form analysis

The proposed method was successfully applied to the assay of ROX in commercial tablets (Roxicin tablets, 150 and 300 mg).

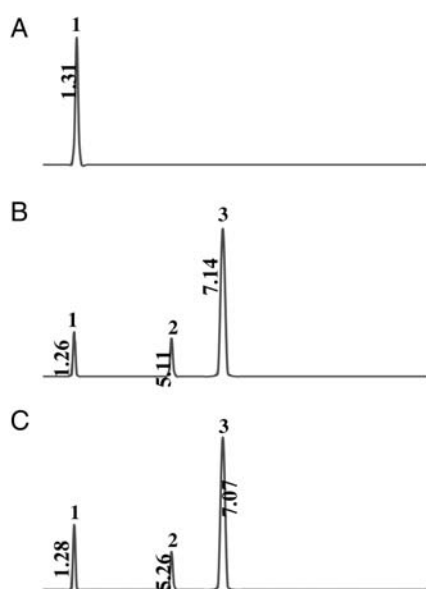


Figure 3. Typical chromatograms showing placebo (A); ROX in Roxicin tablet extract (150 mg ROX/tablet), 1: solvent front, 2: erythromycin internal standard (30.0 μ g/mL), 3: ROX (50.0 μ g/mL) (B); ROX in Roxicin tablet extract (300 mg ROX/tablet), 1: solvent front, 2: erythromycin internal standard (30.0 μ g/mL), 3: ROX (50.0 μ g/mL) (C).

The average percent recoveries of different concentrations were based on the average of three replicate determinations (Table V). Figures 3B and 3C show representative chromatograms for determination of ROX in its tablets.

Content uniformity testing

Due to the high precision of the method and its ability to rapidly estimate the concentration of the drug in a single tablet extract with sufficient accuracy, the method is suited for content uniformity testing, which is a time-consuming process when using a conventional assay technique. The steps of the test were adopted according to the United States Pharmacopoeia (15) procedure. The acceptance value (AV) was calculated for each of the commercially available tablets and was found to be smaller than the maximum allowed acceptance value (L1). The results demonstrated reasonable drug uniformity, as shown in Table VI.

Degradation behavior of ROX and study of its degradation kinetics

To establish whether the analytical method was stability-indicating, ROX was stressed under various conditions to conduct forced degradation studies (14). Methanol was used as a co-solvent in all forced degradation studies.

For the kinetic study, 2 M sodium hydroxide or 6% hydrogen peroxide were used for alkaline or oxidative degradation of the drug. The degradation was found to be temperature-dependent, as presented in Figures 4 and 5. The first-order degradation rate constant and the half-life time at each temperature were calculated (Table VII) according to the following equations:

In $a/a-x = Kt$; where a is the initial concentration of the drug; x is the concentration of the resulting degraded solution after time t ; and K is the reaction rate constant.

The half life time could be calculated as follows: $t_{1/2} = 0.693/K$.

Table V
Determination of ROX in its Dosage Forms Using the Proposed Method

Parameter	Taken (μ g/mL)	Found (μ g/mL)	%Found	Official method (15), %Found
Roxicin tablets (150 mg ROX/tablet)	10.0	9.93	99.25	100.45
	40.0	39.77	99.43	100.61
	60.0	60.28	100.46	99.46
	80.0	80.66	100.83	
	100.0	100.84	100.92	
	120.0	119.93	99.94	
	150.0	149.01	99.34	
$X \pm SD$			100.02 ± 0.72	100.17 ± 0.63
t test			0.81 (1.94)*	
F test			1.31 (5.14)	
Roxicin tablets (300 mg ROX/tablet)	10.0	9.91	99.14	99.61
	40.0	39.94	99.86	99.72
	60.0	59.49	99.16	100.43
	80.0	80.11	100.14	
	100.0	100.19	100.26	
	120.0	121.02	100.85	
	150.0	149.16	99.44	
$X \pm SD$			99.84 ± 0.63	99.92 ± 0.45
t test			0.91 (1.94)	
F test			1.96 (5.14)	

*Figures within parentheses are tabulated t and F values at $P = 0.05$ (16).

Table VI
Content Uniformity Testing of ROX in its Dosage Forms Using the Proposed Method

Parameter	Percentage of the label claim	
	Roxicin tablets (150 mg ROX/tablet)	Roxicin tablets (300 mg ROX/tablet)
	100.25	100.45
	99.45	100.75
	99.62	100.34
	100.14	99.77
	100.34	99.46
	100.75	100.48
	99.68	100.37
	99.45	99.33
	100.47	100.78
	99.34	99.64
Mean X	99.95	100.14
SD	0.45	0.54
%Error	0.14	0.17
AV	1.08	1.3
L1	15	

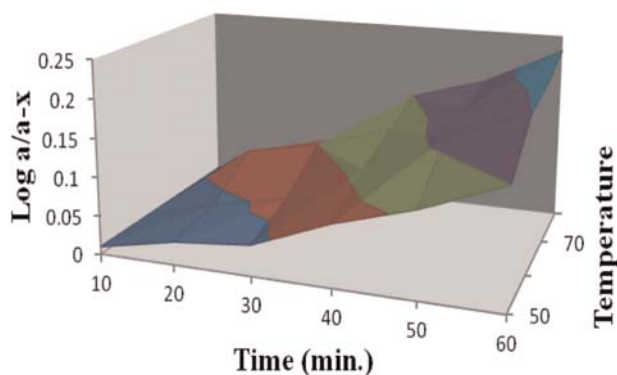


Figure 4. Three-dimensional plot showing the effect of heating time of ROX (50.0 µg/mL) with 2 M NaOH at different temperatures.

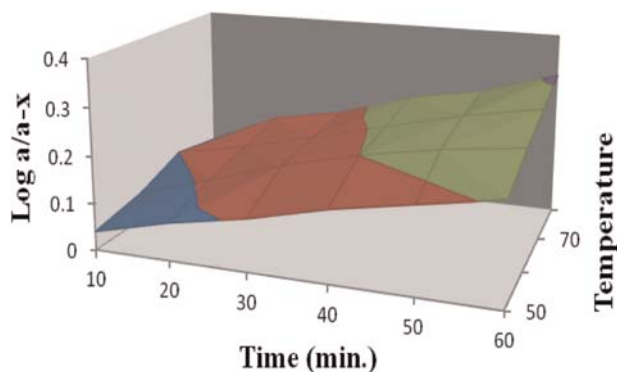


Figure 5. Three-dimensional plot showing the effect of heating time of ROX (50.0 µg/mL) with 6% H₂O₂ at different temperatures.

Plotting log K_{obs} values versus $1/T$, the Arrhenius plots were obtained (Figure 6). The activation energy for each type of degradation was also calculated by the equation.

In $K = -E_a/RT + \ln A$; where E_a is the activation energy; K is first order reaction rate constant; R is the gas constant; T is the temperature in Kelvin; and A is the frequency factor.

Table VII
Kinetic Parameters for ROX Degradation under Different Temperature Settings

Temperature (°C)	Alkaline degradation		Oxidative degradation	
	K (min ⁻¹)	t _{1/2} (min)	K (min ⁻¹)	t _{1/2} (min)
50	4×10^{-3}	173	7.4×10^{-3}	204
60	1.4×10^{-2}	49	2.6×10^{-2}	182
70	2.7×10^{-2}	26	5×10^{-2}	173
80	5.3×10^{-2}	13	9.8×10^{-2}	157
E _a = (K/Cal/mol ⁻¹)	19.7		18.3	

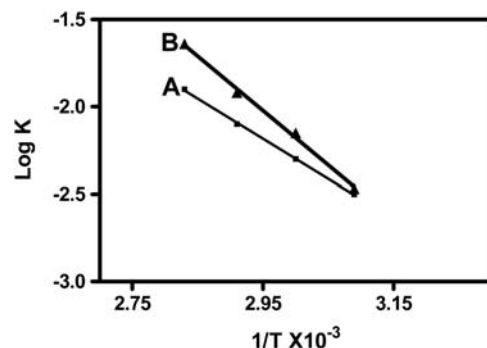


Figure 6. Arrhenius plot for the degradation of ROX (50.0 µg/mL) in: 2 M NaOH (A); 6% H₂O₂ (B).

pH-rate profile study

The effect of different pH values on the degradation of ROX was studied using the Britton-Robinson buffer of pH values ranging from 2–11 at 100°C for different time intervals. The apparent first-order degradation rate constants were calculated at each pH and plotted versus the pH values, resulting in a pH-rate profile curve (Figure 7). The lowest degradation rate constant is at pH 5, at which the drug solution is most stable.

Pathway of degradation

By analogy to previous reports (17–18) concerned with the degradation of some macrolide antibiotics, different degradation pathways of ROX could be explained. The alkaline degradation is proposed to proceed as presented in Figure 8, in which the lactone ring is hydrolyzed to its parent compound, and the straight chained bi-functional compound takes place.

Oxidative degradation is suggested to proceed as in Figure 9, in which formation of the *N*-oxide derivative of the amino-sugar moiety occurs.

UV degradation presented in Figure 10 explains the degradation pathway to occur through breakage of the glycosidic linkages with loss of either desosamine sugar or both desosamine and cladinose sugars.

These postulations depend on previous reports to suggest different degradation pathways of ROX, owing to the high similarity of its chemical structure with both erythromycin and azithromycin, which were chosen as examples of macrolide antibiotics that were subjected to detailed stability studies (17–18). Consequently, by simplifying the analytical procedure and avoiding the use of sophisticated and relatively expensive

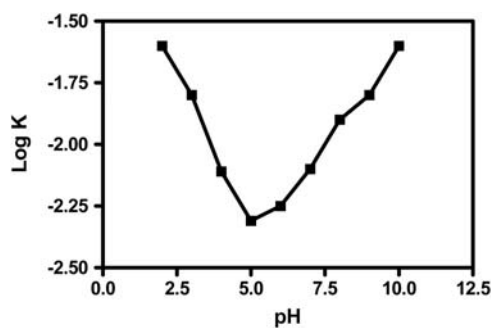


Figure 7. pH rate profile curve of ROX (50.0 µg/mL) using Britton Robinson buffer at 100°C.

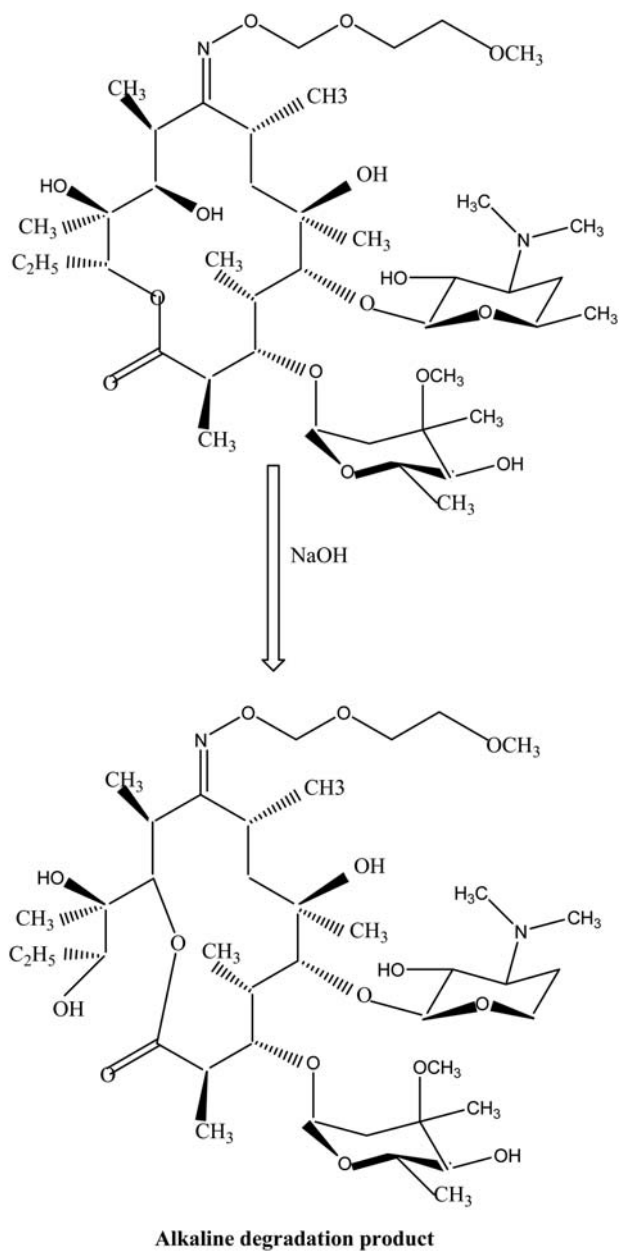


Figure 8. Pathway of alkaline degradation.

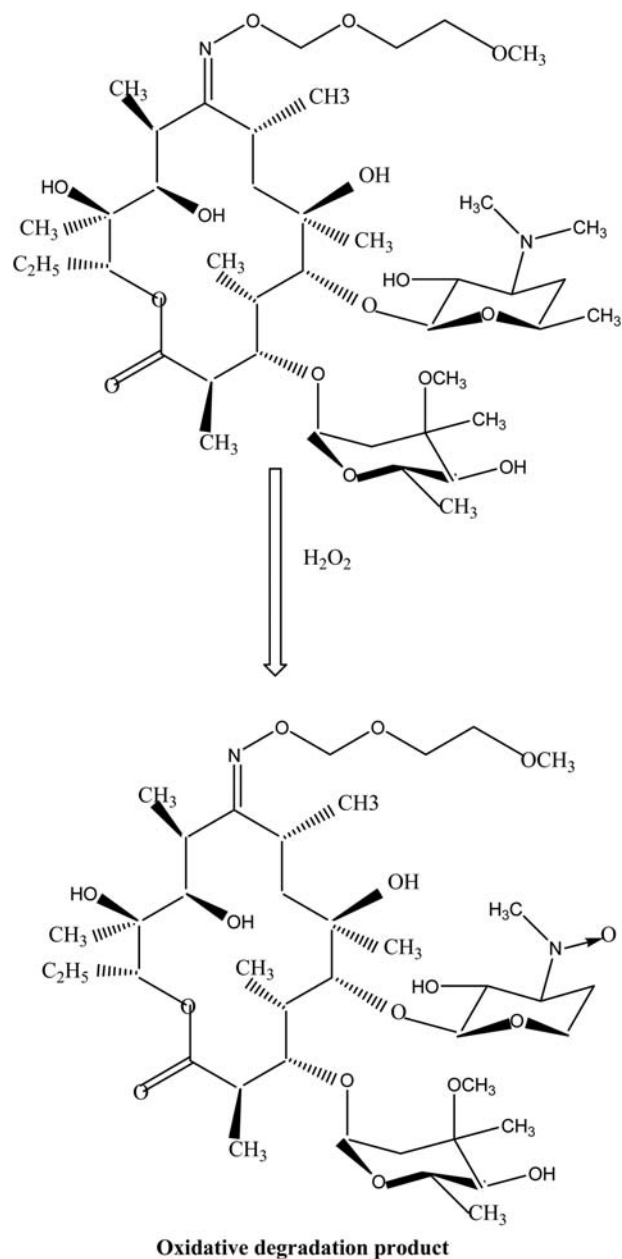


Figure 9. Pathway of oxidative degradation.

instrumentations such as liquid chromatography–mass spectrometry for separating the resultant peaks, it will be possible to apply the proposed method for routine work in quality control laboratories.

Conclusion

A simple, rapid, stability-indicating, reversed-phase liquid chromatographic method was developed to determine ROX in the presence of its different degradation products. The method succeeded in analyzing the drug in its dosage forms, and content uniformity testing of the studied pharmaceutical formulations was also performed. The kinetics of the different

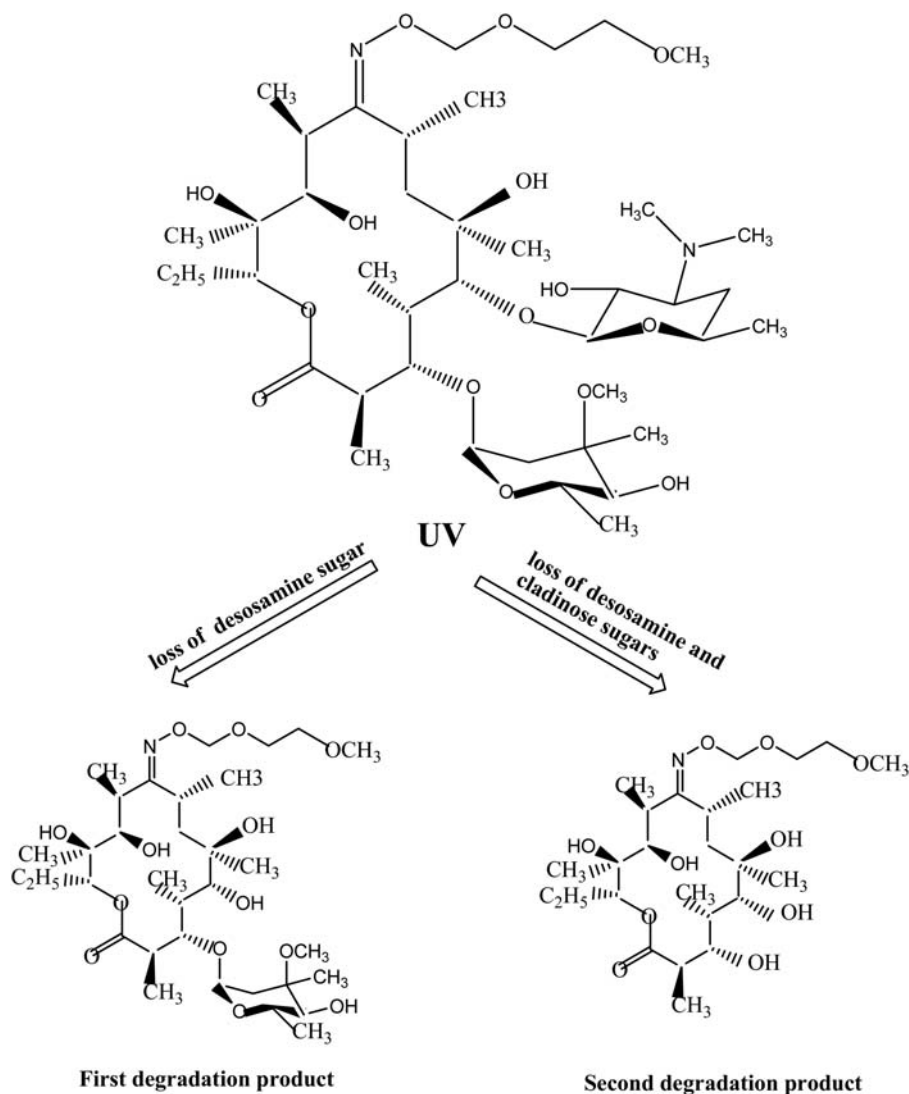


Figure 10. Pathway of UV degradation.

degradation products of the drug were investigated. The first-order rate constant, half-life time and activation energy of the degradation reactions were calculated.

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