Isoxazoles. 9. Degradation Kinetics of 4-(Isoxazolylamino)-1,2-naphthoquinone in Acidic Aqueous Solution

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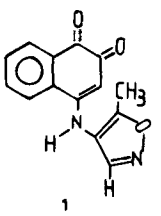
Abstract □ The degradation kinetics of *N*-(5-methyl-4-isoxazolyl)-4-amino-1,2-naphthoquinone (**1**) was studied in aqueous solution over a pH range of 0.65–7.50, at 35 °C and at a constant ionic strength of 0.5. The degradation rates were determined by high-pressure liquid chromatography and were observed to follow pseudo-first-order kinetics with respect to the concentration of **1**. The pH–rate profile was linear with slope −1 under acidic pH, becoming pH independent from 3.50 to 7.50. Good agreement between the theoretical pH–rate profile and the experimental data supports the proposed degradation process. The catalytic rate constants for hydrogen ion and water were $k_H = 0.901\ M^{-1}\ h^{-1}$ and $k_o = 1.34 \times 10^{-3}\ h^{-1}$, respectively. These data are appropriate to develop a stable dosage form of **1**. The accuracy, peak sharpness, and asymmetry factor for the analytical method were determined.

Introduction

Preliminary studies[1–3] of the chemical behavior of new isoxazolylnaphthoquinones have demonstrated that compounds with the amino group in the 4-position of the isoxazole ring show structural stability differences compared with the 3- and 5-aminomethylisoxazoles. These results and the importance of knowing the chemical stability of new active compounds prompted us to extend our studies[4–7] on the conditions and mechanism by which these compounds are degraded.

N-(5-Methyl-4-isoxazolyl)-4-amino-1,2-naphthoquinone (**1**) represents a relatively unexplored class of naphthoquinones with important potential biological properties. This paper describes the effect of pH and temperature on the stability of this compound.



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The quantitative analysis was performed by HPLC since **1** and its degradation product in acidic aqueous solution, 2-hydroxy-1,4-naphthoquinone (**2**) can be monitored simultaneously. N^1-2-pyrimidylsulfanilamide (**3**) was used as an internal standard.

Experimental Section

Materials—All chemicals and reagents were of analytical grade. Methanol was used for the mobile phase in HPLC and was treated with (2,4-dinitrophenyl)hydrazine according to the literature procedure.[8] The water used for HPLC and for buffer solutions was purified with a Milli-Rho Milli-Q system. The mobile phase (methanol:water, 50:50 v/v) was filtered (0.45 μm Nylon-66 membrane) and degassed by vacuum and sonication before use.

***N*-(5-Methyl-4-isoxazolyl)-4-amino-1,2-naphthoquinone (1)** was prepared and purified following a previously reported[3] procedure.

2-Hydroxy-1,4-naphthoquinone (2) was purchased from Sigma Chemical Co. and used as received.

N^1-2-Pyrimidylsulfanilamide (3) was of analytical grade.

4-Amino-5-methylisoxazole (4) was prepared using the method described in the literature.[9,10]

Buffer Solutions—Buffer solutions[11] consisted of suitable mixtures of analytical grade citric acid, sodium hydrogen phosphate, and potassium dihydrogen phosphate (Table 1). For very acidic solutions, aqueous hydrochloric acid solutions were used to obtain the desired pH. A constant ionic strength (μ) of 0.5 was maintained by adding an appropriate amount of sodium chloride.[12]

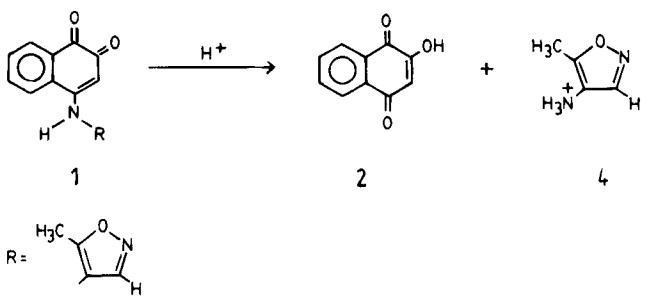
Internal Standard Solution—A sample of N^1-2-pyrimidylsulfanilamide (**3**) was accurately weighed (2.5000 mg), transferred to a volumetric flask (250 mL), and brought to volume with methanol. The solution was stored in the dark at 5 °C.

Apparatus—A Cahn Electrobalance (Model G-sensitivity 2×10^{-4} mg) was used to weigh the samples. The pH values were measured with an Orion Model SA 520 pH meter and SC glass electrode. For kinetic measurements, the constant temperature bath was regulated by a Haake F_3 with ±0.1 °C precision.

The HPLC system consisted of a Konik 500 G liquid chromatograph equipped with a loop injector (Rheodyne Model 7125), a Lichrosorb C_{18} 10-μm column (250 × 4 mm, Hibar-Merck), and a fixed-wavelength UV absorption detector (UVIS-200). The separation of **1** from its degradation product, **2**, was achieved at room temperature using methanol:water (50:50) as the mobile phase (Scheme 1). The column pressure was 120–130 atm; the flow rate was 1.4 mL/min; the detection was made at 264 nm and the detector sensitivity was 0.032 AUFS.

Table 1—Buffer Systems, Observed Rate Constants, t_{90}, and $t_{1/2}$ for the Degradation of 1 at 35 °C

pH	Buffer System	$10 \times k_{obs}$	(CV), h^{-1}	t_{90}, h	$t_{1/2}$, h
0.65	HCl	3.38	(0.37)	0.311	2.05
1.25	HCl:KCl	0.408	(0.67)	2.57	17.0
2.06	HCl:KCl	0.0639	(2.1)	16.4	108
3.51	Ac Cit:Na_2HPO_4	0.0216	(7.6)	48.6	320
4.47	Ac Cit:Na_2HPO_4	0.0191	(7.1)	55.0	363
5.40	Ac Cit:Na_2HPO_4	0.0150	(3.7)	70.0	462
7.32	NaOH:KH_2PO_4	0.0139	(5.2)	75.5	499



1 **2** **4**

R =

Scheme 1

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Table 2—Average Recovery, Peak Sharpness (Q), and Asymmetry Factor (A_s) of Compound 1

compd	Average Recovery		Q	A_s
	Nominal Value %	Recovery % ± CV		
1	100.00	98.20 ± 0.84	6.3	0.8

Peak areas of **1**, internal standard, and degradation product, **2**, were calculated by a programmable integrator. The linearity of the calibration curve of peak-area ratio versus **1** concentration showed excellent correlation coefficients (r) of 0.999 (pH 4) and 0.998 (pH 7). The concentration of **1** was determined by a method of peak-area ratio (drug/internal standard) of sample versus known drug concentrations from the calibration curve.

Kinetic Procedure—A stock solution of **1** was prepared in the appropriate buffer to produce a concentration of 8×10^{-5} M. Aliquots (10 mL) were transferred to a tight-closing snap flask and then placed in a constant-temperature bath. Samples were withdrawn at suitable time intervals, diluted to a known volume with internal standard solution and immediately stored in a freezer. Then, at the time of analysis, samples were removed from the freezer, thawed at room temperature, and mixed well before HPLC analysis. Triplicate samples were run for each storage condition.

Results and Discussion

Validation Method—The kinetics for the degradation of **1** were followed by HPLC. The stability-indicating nature of the method has been previously demonstrated.[3] The dependence of the chromatographic efficiency with flow rate was determined and the repeatability, precision, sensitivity, minimum detectable concentration (MDC), and minimum detectable quantity (MDQ) were also evaluated. In this paper the validity of the HPLC method was extended by determining the average recovery of **1**, the peak sharpness,[13,14] and the asymmetry factor.[15]

The average recovery was evaluated as the percent of observed concentration divided by the known concentration. Determinations were performed in triplicate and by direct comparison with a standard sample of sulfadiazine. The results (Table 2) show a percent recovery of 98.20 with a relative standard deviation of 0.84%, which indicates the effectiveness of the analytical method for our purposes.

The peak shape was evaluated by measuring peak sharpness and an asymmetry factor. The peak sharpness, Q, and the asymmetry factor, A_s, were calculated as follows:

$$Q = \frac{t_R}{t_W} \qquad A_s = \frac{\text{hwr}}{\text{hwf}} \tag{1}$$

where t_R is the retention time, t_W is peak width measured on a time scale, and hwr and hwf are partial peak widths measured from the peak maximum to peak rear and from the peak maximum to the peak front, respectively.

Table 2 summarizes the average recovery, relative sharpness and asymmetry factor of compound **1**. These results demonstrate that the chromatographic column is working efficiently.

Representative chromatograms are shown in Figure 1 for 0, 3, 11, and 31 h of degradation of **1**. The peak designated as **1** is the parent compound and those designated as **2** and **3** are the hydrolysis degradation product and the reference standard, respectively. The identification of **2** is based on a comparison of the HPLC retention time with that of an authentic sample (Table 3). Similar chromatograms were obtained using other solutions in acidic media under stressed conditions.

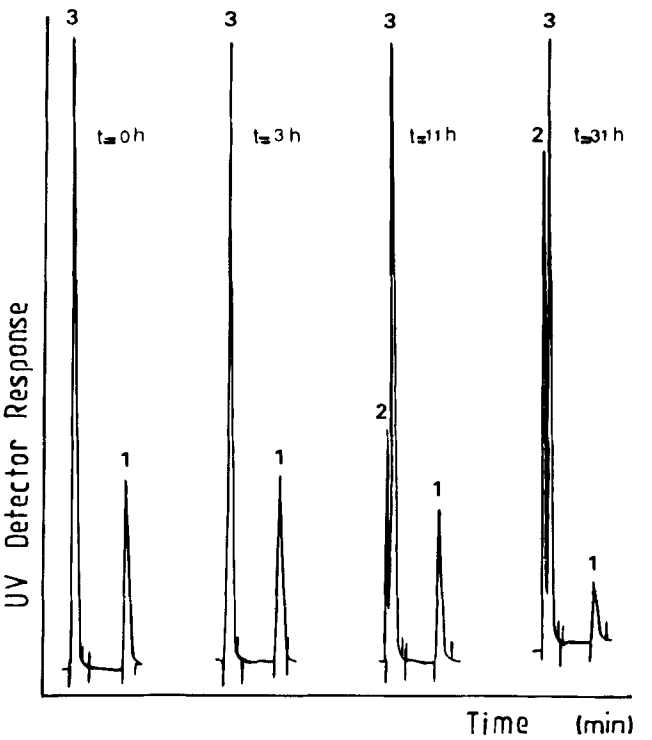


Figure 1—HPLC chromatograms of 1 in pH 1.25 buffer solution.

Table 3

compd	t_R (min)
1	7.09
2	1.45
3	1.87

Degradation Kinetics—Representative sets of stability profiles for **1** in different pH buffer solutions at 35 ± 0.1 °C are shown in Figure 2. The linear relationship between logarithmic residual concentration and storage time indicates pseudo-first-order degradation kinetics for **1** in aqueous solution. The degradation rate constant was determined from the slope of the line with the least-squares linear regression method. For every pH studied, the regression lines were linear with $r > 0.99$.

pH–Rate Profile—The effect of pH on the degradation of **1** in aqueous solution at 35 °C and constant μ (0.5) is shown in plots of log K_{obs} (overall observed rate constant) versus pH (Figure 3).

The pH–rate profile shows two important pH regions: one where a hydrogen ion-catalyzed reaction takes place (pH 0.65–3.50) and the other, a pH-independent region (pH 3.50–7.50). Since the slope of the straight line portion of the profile is close to −1, this region is associated with specific acid catalysis. In the pH-independent region, **1** was relatively stable with gradual decomposition to **2** mainly due to the effect of water on the neutral species of **1**.

Equation 2 is a general equation describing the degradation rate of **1** as a function of pH:

$$K_{obs} = k_H[H^+] + k_o \tag{2}$$

where K_{obs} is the overall observed rate constant, k_o is the spontaneous or water catalysis rate constant, and k_H is the second-order constant for the hydrogen ion-catalyzed degrada-

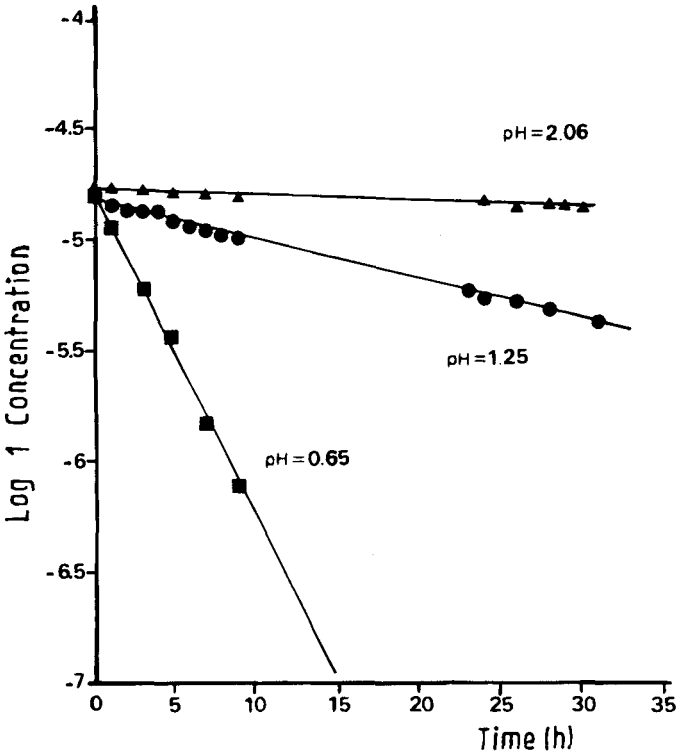


Figure 2—Pseudo-first-order degradation kinetics of **1** at various pH values, 35 °C, and μ = 0.5.

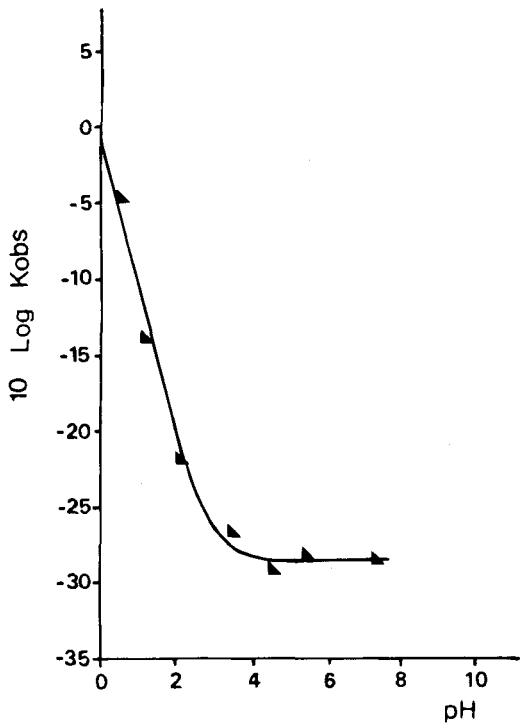
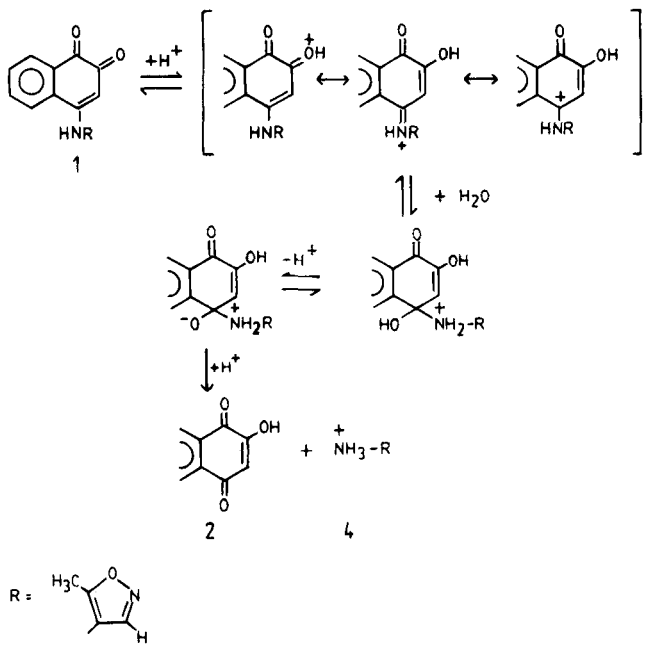


Figure 3—pH–rate profile of the degradation of **1** at 35 °C. The points are experimental values, and the solid line is the theoretical curve calculated from eq 2.

tion. The resulting rate constants were $k_H = 0.901\ M^{-1}\ h^{-1}$ and $k_o = 1.34 \times 10^{-3}\ h^{-1}$.

The buffer systems employed, observed rate constants, and t_{90} and $t_{1/2}$ values are given in Table 1.

The excellent agreement between the theoretical pH–rate profile and the experimental data indicates that eq 2 adequately describes the observed pH effect on the degradation kinetics of **1** and supports the proposed degradation process.



Scheme 2

Possible Degradation Mechanism—The mechanism for the acid catalytic degradation reaction of **1** can be postulated from previous studies[5,6] and the well-known hydrolysis of imines.[16–20] This process, shown in Scheme 2, involves a rapid pre-equilibrium protonation of substrate followed by attack of water on the protonated imine. The subsequent deamination involves cleavage of the carbon–4-nitrogen bond, leading to the stable degradation products compounds **2** and **4**.

Conclusions

Compound **1** is a 4-(isoxazolylamino)-1,2-naphthoquinone which undergoes irreversible acid hydrolysis on the carbon-4 of the naphthoquinone ring with the 4-amino-5-methylisoxazole as leaving group.

The development of a rapid, sensitive, accurate, and precise HPLC stability-indicating assay enabled us to determine simultaneously the precursor and its degradation product.

The pH–rate profile obtained indicates specific acid catalysis with a region of maximum stability between pH 3.50 and 7.50.

References and Notes

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