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Stability of Clavulanic Acid in Aqueous Solutions

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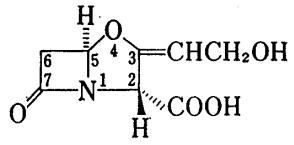
The stability of clavulanic acid in aqueous solutions has been investigated over a pH range of 3.15 to 10.10 at 35°C and at an ionic strength of 0.5. The changes in the concentration of intact clavulanic acid in buffer solutions were determined by reversed phase HPLC with UV-detection using a mobile phase containing tetrabutylammonium bromide. The observed degradation rates at various pH's were found to follow pseudo-first-order kinetics, and were significantly affected by catalysis due to buffer salts. The catalytic rate constants were estimated at three different concentrations of buffer systems. The pH *vs.* rate profiles obtained from non-buffer-catalyzed rate constants, k_{pH}, revealed that the degradation in alkaline solutions proceeded, as a whole, about 10 times faster than in acidic media, and maximal stability was attained at pH 6.39. The Arrhenius activation energies at pH 3.94, 6.67, and 8.74 were estimated as 19.0, 14.7, and 18.3 kcal/mol, respectively.

Keywords——clavulanic acid; high performance liquid chromatography; stability in aqueous solutions; pH effect on stability; salt effect on stability; degradation kinetics

Introduction

Clavulanic acid, z-(2*R*,5*R*)-3-(β-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo-[3.2.0]-heptane-2-carboxylic acid (I), is a novel fused β-lactam produced by *Streptomyces clavuligerus* ATCC 27064.[1,2] It has a potent inhibitory activity against β-lactamases from a variety of Gram-positive and Gram-negative bacteria,[3] but exhibits only weak antibacterial activity itself, therefore being unsuitable for use alone. It is known from a number of *in vitro* microbial experiments that the combined use of clavulanic acid with certain penicillins or cephalosporins gives rise to a marked reduction in MIC against various β-lactamase-producing clinical isolates,[3] since clavulanic acid is irreversibly bound to β-lactamases.[4–7] Therefore it is expected from the view point of clinical chemotherapy that clavulanic acid may be able to potentiate the activity of β-lactam antibiotics which show poor efficacy against β-lactamase-producing bacteria.



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A limited number of papers have described *in vivo* activities[8–10] and biosynthesis[11,12] of clavulanic acid, and kinetic[6] and chemical[7] features of the inactivation of β-lactamases by clavulanic acid, but none referred to the stability of this drug in aqueous solutions. The present investigation deals with kinetic aspects of the degradation of clavulanic acid in buffer solutions of various pH's.

Experimental

Reagents and Materials——Clavulanic acid (potassium salt) was supplied by Beecham Yakuhin Co. Ltd. (Tokyo, Japan). Tetrabutylammonium bromide (TBAB) and buffer salts used (citrate, acetate, phosphate, borate, and carbonate) were commercial products of reagent grade. Deionized distilled water was used for the preparations of buffer solutions and the mobile phase of HPLC.

Chromatography——A high performance liquid chromatograph (TWINCLE, Jasco, Tokyo) equipped with two variable-wavelength UV-detectors (UVIDEC-100-III, Jasco) was used. The detectors were connected

in series and the wavelengths were set at 230 nm and 276 nm. The stationary phase used was LiChrosorb RP-18 (10 μm, E. Merck, West Germany) packed in 25 cm × 4.6 mm i.d. stainless steel tubing, and the mobile phase was a mixture of aqueous 5 mM TBAB+2.5 mM NaH_2PO_4+2.5 mM Na_2HPO_4 solution/methanol= 5/1 (v/v) (final pH 7.25), whose flow rate was maintained at 1.0 ml/min (60 kg/cm²). A short column (5 cm × 4.6 mm i.d.) packed with LiChrosorb RP-2 (E. Merck) was used to guard the main column. All chromatographic operations were carried out under ambient conditions.

Kinetic Procedure——The buffer systems used were citrate buffer for pH 3.15, acetate buffers for pH 3.58, 3.94, 4.41, 4.79, 4.99, phosphate buffers for pH 5.63, 6.28, 6.67, 7.12, 7.74, 7.96, borate buffers for pH 8.30, 8.52, 8.74, 9.09, and carbonate buffers for pH 9.45 and 10.10. The ionic strengths of all buffer solutions were adjusted to 0.5 with potassium chloride, if necessary. The pH values were measured on a pH meter (HM-20E, Toa Electronics Ltd., Tokyo, Japan) and corrected to constant temperature (35°C). Weighed amounts of clavulanic acid were dissolved in the buffer solutions to make concentrations of 2 to 3 mM. Aliquots (1 ml) of the solutions were each sealed in a small glass vial and kept at 35±0.1°C in a thermostated water bath. Aliquots of 10 to 20 μl of solution were accurately withdrawn into a microsyringe at appropriate time intervals until the reaction time reached at most five times the half-life, and were subjected to HPLC analysis. The change in the concentration of remaining clavulanic acid was determined by using a data analyzer (Chromatopac C-R1A, Shimadzu, Kyoto, Japan) from the absorbance values at 230 nm. The rate constants were estimated by the least-squares method from the slope of time *vs.* log (remaining %) plots.

Results

HPLC Observations of Clavulanic Acid

Prior to the kinetic investigations, the degradation profiles were preliminarily determined through UV-absorption and HPLC analysis of aqueous clavulanic acid solutions of various pH values. Fig. 1 shows UV-absorption spectra of clavulanic acid before and after degradation in phosphate buffer solution of pH 7.83 at 35°C. The intensity of the band at λ_{max}=204 nm decreased with reaction time, with a concomitant increase in absorbance at 276 nm. This suggested that the band at 276 nm might be due to degradation product(s) of clavulanic acid. HPLC analysis of the reaction solution (pH 7.83, 35±0.1°C) gave the chromatogram shown in Fig. 2; the effluents were monitored at 230 nm for detection of intact clavulanic acid and simultaneously at 276 nm for degradation products. The sensitivity at the latter wavelength was set at twice that at the former. Fig. 2(a) indicates that about 15% of clavulanic acid (peak 3) was degraded to yield a product (peak 2) at 1 h after the start of the reaction, and the formation of additional products (peaks 1 and 4 in Fig. 2(b)) was observed by 7 h, when about 50% of clavulanic acid was degraded. When the reaction was continued until 24 h (Fig. 2(c)) about 90% of clavulanic acid disappeared and the initial product (peak 2) was further degraded with simultaneous formation of another product (peak 5) along with many minor products, while the degradation products of peaks 1 and 4 continued to increase. Fig. 3 shows the chromatograms corresponding to about 40% and 50% degradation of clavulanic acid at pH 5.00 and 3.24, respectively. The degradation in neutral and acidic solutions differs from that in alkaline solution; the pH 5.00 solution gave no peaks assignable to degradation products (Fig. 3(a)), indicating that clavulanic acid was converted to non-UV-absorbing substances, whereas

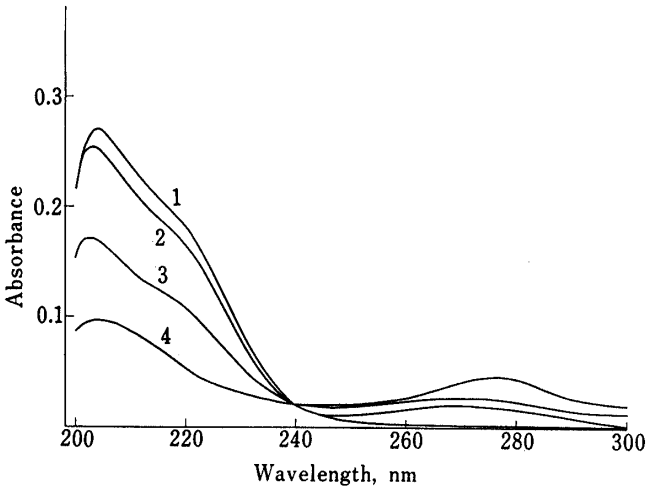

Fig. 1. UV–Absorption Spectra of Clavulanic Acid at Various Reaction Times

Initial concentration of clavulanic acid, 150 μg/ml in phosphate buffer (pH 7.83, μ=0.5). Reaction temperature: 35±0.1°C. Reaction times: 1; 0 h, 2; 1 h, 3; 7 h, 4; 24 h.

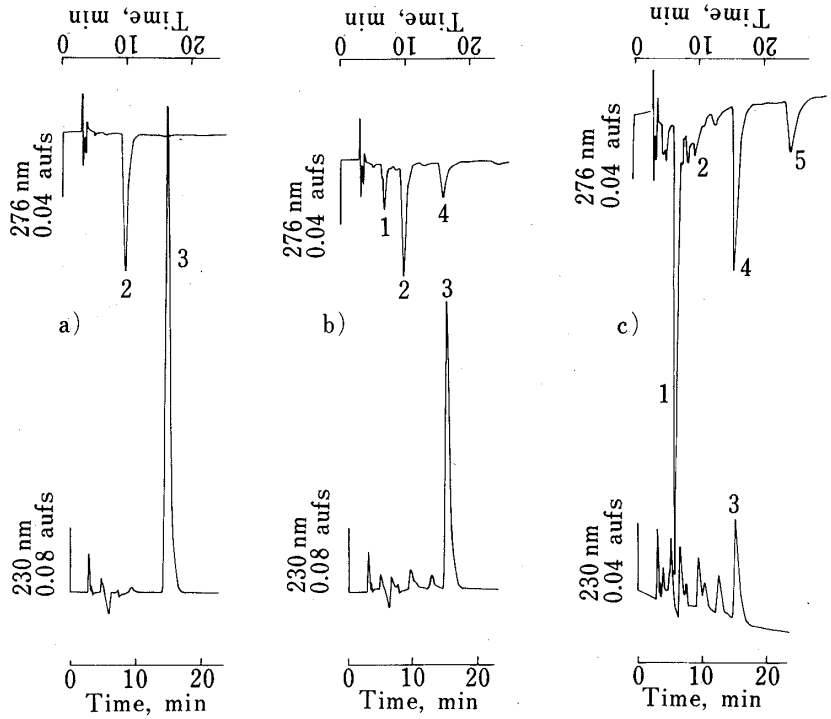

Fig. 2. Chromatograms of Clavulanic Acid (peak 3) and Degradation Products (peaks 1, 2, 4, and 5)

Clavulanic acid (initial concentration, 750 μg/ml in pH 7.83 phosphate buffer) was degraded at 35±0.1°C and μ=0.5 for (a) 1 h, (b) 7 h, (c) 24 h. Sample sizes: (a) 10 μl, (b) 10 μl, (c) 20 μl.

acidic degradation at pH 3.24 (Fig. 3(b)) yielded relatively hydrophilic products having UV-absorption at around 230 nm.

Although the nature of the degradation products is unknown, the above observations suggest that clavulanic acid undergoes a complicated pH-dependent degradation. The rate profiles are discussed below.

Stability of Clavulanic Acid

The degradation kinetics of clavulanic acid were investigated at various pH values and buffer concentrations at constant temperature (35°C) and ionic strength (μ=0.5). The time *vs.* log (residual %) plots in 0.3 M buffer solutions, given in Figs. 4, 5, and 6, indicate that the degradation in the pH range examined followed apparent pseudo-first-order kinetics with respect to the concentration of clavulanic acid. The observed rate constants, k_{obs}, were estimated by means of the least-squares method from the slopes of the plots. The catalytic effects of buffer species were investigated by measuring the rate constants at three different concentrations of each buffer system at constant pH and ionic strength. The results are given in Table I.

Typical plots of k_{obs} *vs.* phosphate buffer concentration over a pH range of 5.63 to 7.96 are shown in Fig. 7, which indicates a linear catalytic effect of phosphate ion on the degradation rate constants. The extrapolation of each line to zero concentration of phosphate buffer provides, as the intercept, the value of the pseudo-first-order rate constant, k_{pH}, corresponding to non-buffer-catalyzed degradation. The k_{pH} values listed in Table I are those thus estimated for all the buffer systems used. In a particular pH region (5.63—7.96) only $H_2PO_4^-$ and HPO_4^{2-} ions appear to be effective catalysts. Taking the pK_a value of $H_2PO_4^-$ ion at 35°C (μ=0.5) to be 6.58,[13] it is expected from Fig. 7 that the catalytic effect of HPO_4^{2-} may be predominant over that of $H_2PO_4^-$ in accelerating the degradation of clavulanic acid within

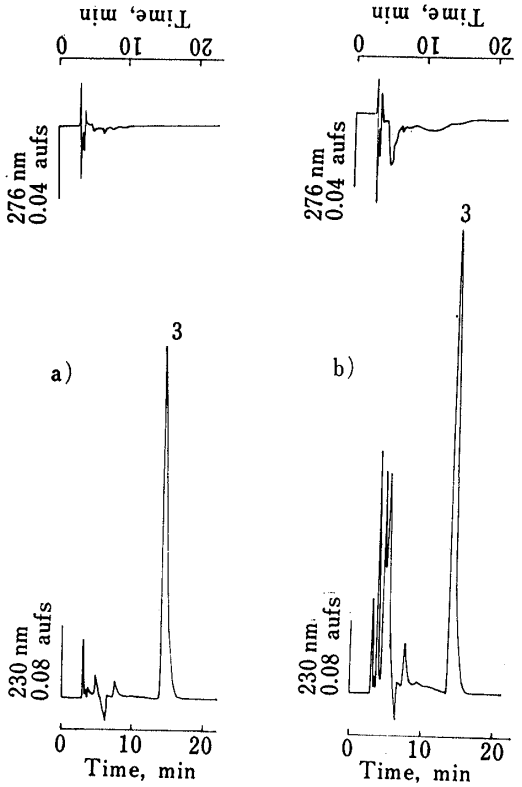

Fig. 3. Chromatograms of Clavulanic Acid (peak 3) and Degradation Products

Clavulanic acid (initial concentration 750 μg/ml) was degraded at 35±0.1°C and μ=0.5 (a) in pH 5.00 phosphate buffer solution for 24 h, (b) in pH 3.24 phosphate buffer solution for 4 h. Sample sizes: (a) 10 μl, (b) 20 μl.

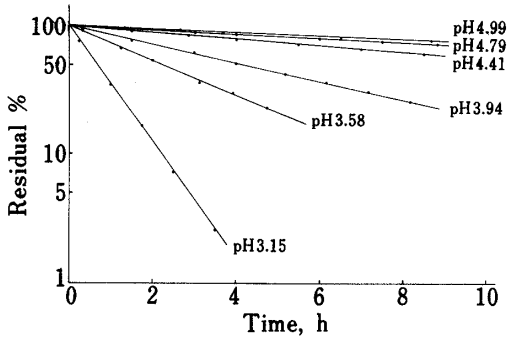

Fig. 4. Apparent First-Order Plots for the Degradation of Clavulanic Acid at Various pH Values in the Acidic Region at 35 ± 0.1 °C, μ=0.5, and Total Buffer Concentration=0.3 M

For buffer salts used, see "Experimental."

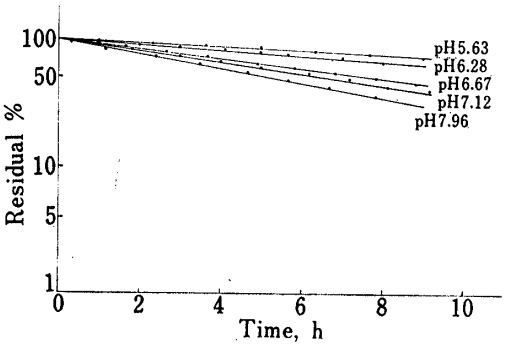

Fig. 5. Apparent First-Order Plots for the Degradation of Clavulanic Acid at Various pH Values in the Neutral Region at 35±0.1°C and μ=0.5

Total buffer concentration=0.15 M for pH 7.96, 0.14 M for pH 7.12, 0.13 M for pH 6.67, 0.1 M for pH 6.28 and 5.63. For buffer salts used, see "Experimental."

the phosphate buffer pH region. Thus, the contributions of these phosphate ions were estimated as follows:

$$k_{obs} = k_{pH} + (k_1 f_1 + k_2 f_2) P_t, \quad (1)$$

where k_1 and k_2 denote the catalytic rate constants due to $H_2PO_4^-$ and HPO_4^{2-}, respectively, and f_1 and f_2 represent the fractions of $H_2PO_4^-$ and HPO_4^{2-} in total phosphate concentration (P_t); therefore $f_1+f_2=1$ and $pK_a=pf_2-pf_1+pH$. The values of k_1 and k_2 thus calculated are listed in Table II together with those for other buffer systems obtained in a similar manner.

The k_{pH} *vs.* pH profile is shown in Fig. 8, where the closed circles indicate experimental values and the curve illustrates the best fit of the equation,

$$k_{pH} = k_{H,O} + k_H a_H + k_{OH}(K_W/a_H) \quad (2)$$

to the data points. In Eq. 2, k_H and k_{OH} represent second-order rate constants of proton- and hydroxide ion-catalyzed degradations, respectively, $k_{H,O}$ is the rate constant of spontaneous or water-catalyzed degradation, a_H is the proton activity as measured with a glass electrode, and $K_W=2.09\times10^{-14}$ at 35°C, μ=0.5.[14] Eq. 2 was used in this simulation,

TABLE I. Rate Constants for Degradation of Clavulanic Acid at 35°C and $\mu=0.5$

Buffer	pH	k_{obs}, h^{-1}			k_{pH}, h^{-1}
		0.30 M	0.20 M	0.10 M	
Citrate					
	3.15	1.04	0.849	0.655	0.462
Acetate					
	3.58	0.308	0.289	0.265	0.244
	3.94	0.163	0.158	0.151	0.145
	4.41	0.059	0.054	0.044	0.038
	4.79	0.036	0.033	0.028	0.025
	4.99	0.029	0.025	0.020	0.016
Phosphate					
	5.63	0.055	0.042	0.026	0.012
	6.28	0.112	0.080	0.045	0.012
	6.67	0.149[a]	0.080[b]	0.057[c]	0.011
	7.12	0.138[d]	0.100[e]	0.053[f]	0.012
	7.74	0.143[g]	0.104[h]	0.058[i]	0.016
	7.96	0.130[j]	0.100[k]	0.057[l]	0.023
Borate					
	8.30	0.117	0.111	0.097	0.088
	8.52	0.180	0.165	0.144	0.127
	8.74	0.248	0.212	0.172	0.134
	9.09	0.400	0.350	0.268	0.208
Carbonate					
	9.45	0.472	0.424	0.397	0.356
	10.10	1.43[a]	1.34[m]	1.24[c]	1.12

Buffer concentration (M): *a*) 0.25, *b*) 0.13, *c*) 0.08, *d*) 0.21, *e*) 0.14, *f*) 0.07, *g*) 0.18, *h*) 0.12, *i*) 0.06, *j*) 0.15, *k*) 0.10, *l*) 0.05, *m*) 0.17.
The ionic strengths of all buffer solutions were adjusted to 0.5 by addition of KCl, if necessary.

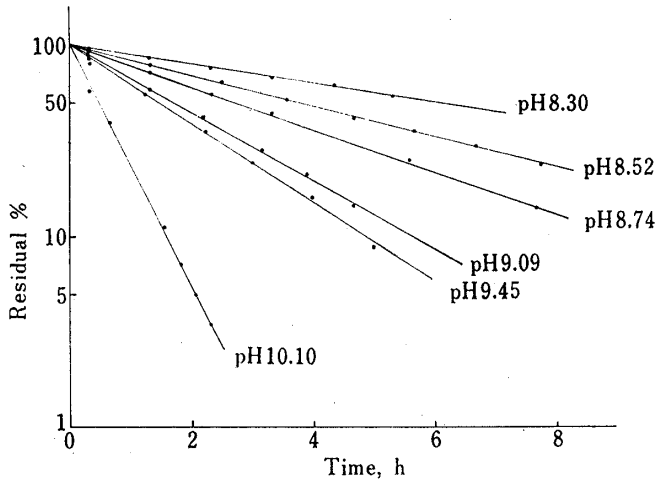

Fig. 6. Apparent First-Order Plots for the Degradation of Clavulanic Acid at Various pH Values in the Alkaline Region at 35±0.1°C and $\mu=0.5$

Total buffer concentration=0.3 M for pH 8.30, 8.52, 8.74, 9.09, 9.45 and 0.25 M for pH 10.10. For buffer salts used, see "Experimental."

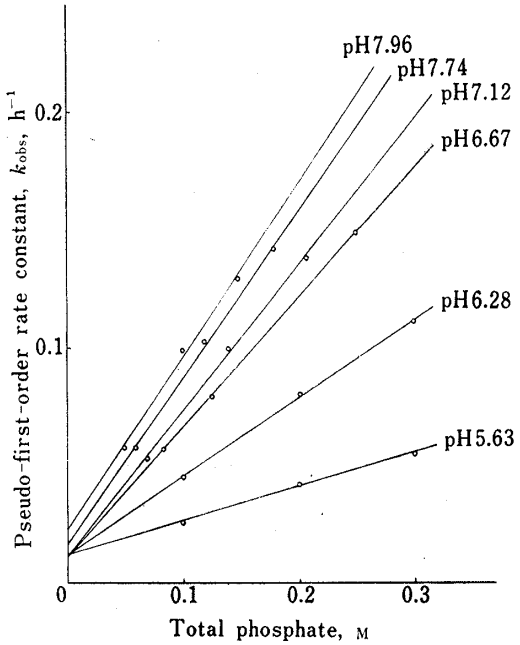

Fig. 7. Dependence of the Pseudo-First-Order Rate Constant, k_{obs}, on Total Phosphate Buffer Concentration at Various pH Values at 35±0.1°C and $\mu=0.5$

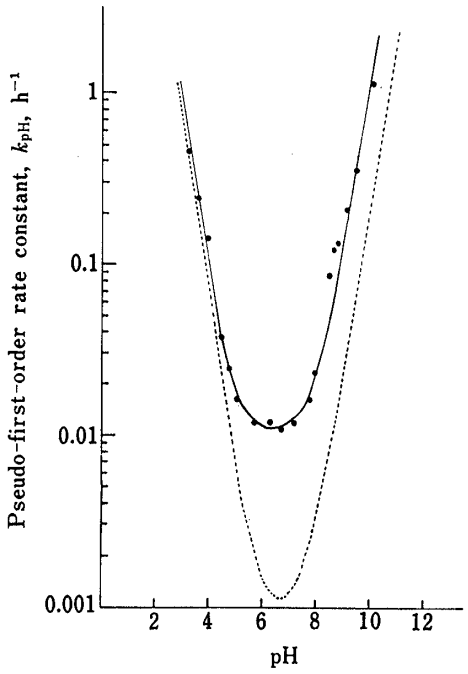

Fig. 8. Log k_{pH} *vs.* pH Profile of the Degradation of Clavulanic Acid at 35 ± 0.1°C and $\mu = 0.5$

The closed circles indicate experimental values, the solid line shows the theoretical curve according to Eq. 2, and the dashed line represents the curve for penicillin G at 35°C and $\mu = 0.5$.[18]

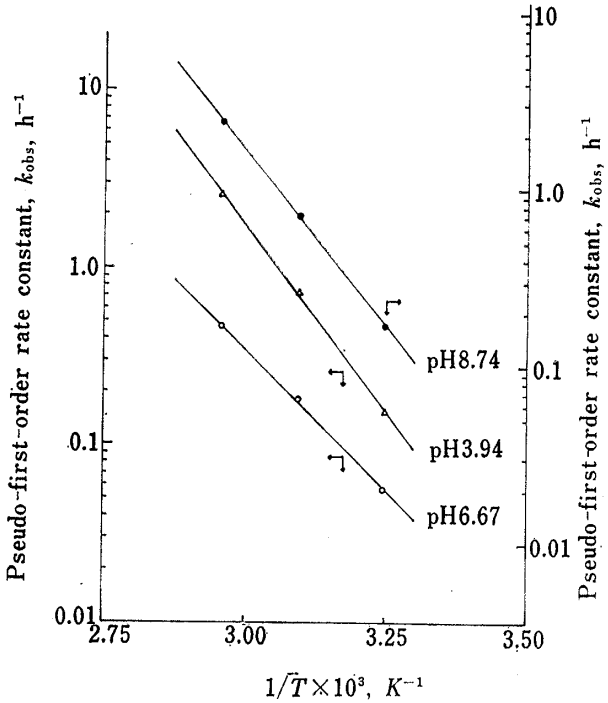

Fig. 9. Arrhenius Plots of the Degradation of Clavulanic Acid at pH 3.94 (Acetate Buffer), pH 6.67 (Phosphate Buffer), and pH 8.74 (Borate Buffer)

TABLB II. Catalytic Rate Constants of Phosphate, Acetate, and Borate Buffer Ions ($\mu = 0.5$)

Buffer	k_1	k_2
Phosphate[a]	0.112	0.770
Acetate[b]	0.182	0.010
Borate[c]	0.025	1.12

a) k_1; $H_2PO_4^-$, k_2; HPO_4^{2-}.
b) k_1; CH_3COOH, k_2; CH_3COO^-.
c) k_1; H_3BO_3, k_2; $H_4BO_4^-$.

TABLE III. Rate Constants and Arrhenius Activation Parameters for Degradation of Clavulanic Acid at $\mu = 0.5$

pH	T, °C	k_{obs}, h^{-1}	E_a, kcal/mol	log A, h^{-1}
3.94	35	0.151	19.0	12.7
	50	0.733		
	65	2.55		
6.67	35	0.057	14.7	9.17
	50	0.181		
	65	0.479		
8.74	35	0.172	18.3	12.3
	50	0.728		
	65	2.45		

because the observed rate profile in the pH range of 3.15 to 10.10 seemed unlikely to involve a change in the species of clavulanic acid. The species is obviously an ionized form in this pH region, since pK_a of clavulanic acid is 2.4.[15] The k_{H_2O}, k_H, and k_{OH} values thus computed by means of a weighted least-squares method were 1.07×10^{-2} (h^{-1}), 7.64×10^{2} ($mol^{-1}\,h^{-1}$), and 6.14×10^{3} ($mol^{-1}\,h^{-1}$), respectively. The pH for maximal stability of clavulanic acid, which is given by

$$pH_{min} = \frac{1}{2}(pk_{OH} - pk_H + pK_W) \quad (3)$$

is estimated to be 6.39.

The temperature effect on the degradation of clavulanic acid was determined at three different pH values in the acidic, neutral and alkaline regions with $\mu=0.5$. The observed rate constants at 35°C, 50°C, and 65°C are given in Table III and Arrhenius plots of the data at pH 3.94, 6.67, and 8.74 are shown in Fig. 9. The apparent activation energies (E_a) estimated from the lines in Fig. 9 are also listed in Table III together with the values of frequency factor (log A), indicating that the Arrhenius parameters for the degradation at pH 3.94 and 8.74 are very similar.

Discussion

The assays of clavulanic acid employed in previous studies were based on the microbiological method. No spectrometric procedures have been available, presumably because clavulanic acid has poor UV-absorption above 210 nm,[2] and neither iodometric nor hydroxamate assay methods are suitable for quantitative determination.[7] In the previous paper[16] we investigated the solvent effects on the bathochromic shifts of λ_{max} of clavulanic acid solution, and established a reversed phase HPLC method with UV detection for the determination of clavulanic acid in urine. The established method is employed in the present work with a slight modification.

There have been a number of investigations dealing with the degradation of β-lactam antibiotics in aqueous solutions.[17,18] It is well known that penicillins are hydrolyzed to penicilloic acids in neutral and alkaline solutions, and are converted to penicillenic acids in acidic media, whereas cephalosporins, unlike penicillins, undergo complicated degradation reactions and do not yield the corresponding cephalosporoic acids as stable products. The kinetic investigations of such degradation reactions have revealed that the shoulder-type breaks of the log k_{pH} *vs.* pH curves appearing in the low pH region were due to the difference in proton-catalyzed degradation rate between free and ionized species of penicillins, and their rates were dependent on the structures of side-chain substituents. If there is another ionizable group such as an amino group on the side-chain, increasing numbers of species become involved in the kinetic processes, resulting in complicated pH-rate profiles. From a comparison of the present results with those reported previously, it is clear that the shape of the log k_{pH} *vs.* pH curve of clavulanic acid is similar to that of penicillin G, exhibiting a steeper slope and narrower range of pH-independent (spontaneous or water catalyzed) degradation than other penicillins or cephalosporins. The values of k_{H_2O}, k_H, and k_{OH} for penicillin G, for reference, have been given as 0.90×10^{-3} (h^{-1}), 6.0×10^{2} ($mol^{-1}\,h^{-1}$), and 1.19×10^{3} ($mol^{-1}\,h^{-1}$), respectively.[18] This means that the degradation of clavulanic acid proceeds about 10 times faster at pH_{min} and about 5 times faster in the alkaline region than that of penicillin G, whereas they are comparable in the acidic region.

It is interesting to compare the rate profile of clavulanic acid with that of amoxicillin, since they are formulated as a combined tablet. The kinetics of amoxicillin degradation in aqueous solutions have been reported by Zia *et al.*,[19] and the log k_{pH} *vs.* pH curve exhibits a shoulder-type break with maximal stability at around 5.8 to 6.5. The degradation rate

constant thereat is about 10 times smaller than that of clavulanic acid. Although the mechanisms of degradation of clavulanic acid are unknown at present, it is tentatively suggested from the present results that clavulanic acid undergoes complicated acid–base catalyzed reactions depending on pH and buffer salts, which may involve β-lactam ring opening and/or cleavage of the oxapenam ring.

The present results should be useful in the development of pharmaceutical preparations, in the establishment of analytical procedures, and in achieving an understanding of the pharmacokinetic properties of clavulanic acid.

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