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A kinetic study on the degradation of erythromycin A in aqueous solution

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Abstract

The pH is a critical factor determining the rate of the degradation of erythromycin A in aqueous solutions. However, the kinetics of the acid- and base-catalyzed degradation is still uncertain. This study used a sensitive coulometric detection method to determine concentrations of erythromycin A and its degradation products. To determine the buffer-independent rate constants, sodium acetate (0.05–0.2 M) and Tris–HCl (0.1–0.5 M) were used in a pH range of 3.5–5.5 and 7.0–9.0, respectively. In acidic conditions, anhydroerythromycin A appeared to be produced directly through an internal dehydration of erythromycin A-6,9-hemiketal which simultaneously established an equilibrium with erythromycin A enol ether on the other hand. In weakly alkaline conditions, hydroxide ion appeared to catalyze the hydrolysis of the lactonyl ester bond of erythromycin A-6,9-hemiketal by the pseudo-first-order kinetics, and the C13 → C11 translactonization and internal dehydration reactions subsequently occurred to form pseudoerythromycin A enol ether. We suggest here a predictive model for reasonable interpretation of the kinetics of erythromycin A degradation in aqueous solutions, in which the observed rate constant was expressed by the sum of the partial reaction rate constants for the acid- and base-catalyzed degradation of erythromycin A-6,9-hemiketal as a function of pH in a range of 3.0–10.0.

Keywords: Acid-catalysis; Base-catalysis; Erythromycin; Kinetics

1. Introduction

Erythromycin A is widely used not only in humans, but also in food-producing animals to control bacterial diseases and promote animal growth (Pothuluri et al., 1998). Among 21 veterinary and human antibiotics, erythromycin A is most frequently detected from 139 US stream sites considered susceptible to contamination from human, industrial, and wastewater (Kolpin et al., 2002). Erythromycin A is unstable in acidic and alkaline solutions. The decomposition through an internal dehydration reaction results in the formation of anhydroerythromycin A, erythromycin A enol ether and pseudoerythromycin A enol ether with a low biological activity (Kibwage et al., 1985, 1987a; Fiese and Steffen, 1990; Flynn et al., 1955).

With decreasing the pH of aqueous solutions, the solubility of erythromycin A increases, and the rates of degradation of erythromycin A and production of anhydroerythromycin A are enhanced by the (pseudo)-

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first-order kinetics (Nakagawa et al., 1992; Volmer and Hui, 1998). In the previous studies of the acid-catalyzed erythromycin A degradation, two controversial pathways have been proposed. On one side, erythromycin A enol ether is regarded as an intermediate in the conversion of erythromycin A to anhydroerythromycin A (Atkins et al., 1986; Alam et al., 1995). On the other side, anhydroerythromycin A is produced directly through an internal dehydration of transient erythromycin A-6,9-hemiketal which simultaneously establishes an equilibrium with erythromycin A enol ether (Cachet et al., 1989).

In slightly alkaline conditions, pseudoerythromycins are produced by the base-catalyzed hydrolysis of erythromycin A-6,9-hemiketal and followed by the subsequent dehydration reactions in the rearrangement of the lactonyl ester bond from C13 to C11 and the formation of pseudoerythromycin A enol ether (Kibwage et al., 1987a,b; Cachet et al., 1987, 1989). However, little is known about the kinetics of the base-catalyzed degradation in aqueous solutions.

From the previous kinetic studies, concentrations of erythromycin A and its degradation products have been determined by spectrophotometry (Atkins et al., 1986; Nakagawa et al., 1992), liquid chromatography/tandem mass spectrometry (Alam et al., 1995; Volmer and Hui, 1998), high performance liquid chromatography (HPLC) with UV detection (Cachet et al., 1989, 1991), or densitometry of TLC plates (Lazarevski et al., 1978). These methods are inappropriate for the robust and high throughput analyses. In this study, we used a reversed-phased HPLC with electrochemical detection, which is known as the most selective and sensitive method for the determination of macrolides containing the tertiary amines in pharmacokinetic and bioavailability investigations (Marzo and Dal Bo, 1998). The partial degradation rates were determined with individual erythromycins A including erythromycin A, anhydroerythromycin A, erythromycin A enol ether and pseudoerythromycin A enol ether at various pH conditions. Because the degradation rates could be influenced by the types and the concentrations of buffer components (Atkins et al., 1986; Cachet et al., 1989), the buffer concentrations varied to determine the buffer-independent rate constants and the buffer-catalytic coefficients by the extrapolation of the plots of observed rate constant versus buffer concentration.

2. Materials and methods

2.1. Standard and sample preparations

Standard solutions of each 5 mg of anhydroerythromycin A, erythromycin A, erythromycin A enol ether and pseudoerythromycin A enol ether were prepared in 1 ml of acetonitrile, and stored at −20 °C. Solvents and chemicals used in preparations of buffers and HPLC mobile phases were of the analytical grades, J.T. Bakers (Philipsburg, NJ), and water used was deionized (>18 MΩ) using a Super-Q Plus water purification system (Millipore, Bedford, MA).

Fifty microliters of individual erythromycins A dissolved in acetonitrile were dispensed to 10 ml-test tubes, and the acetonitrile solvent was evaporated before addition of 5 ml of aqueous buffer to make the final concentration of 50 μg ml^{-1}. Buffers consisted of sodium acetate in a pH range of 3.5–7.0 and Tris–HCl in a pH range of 7.0–9.0. Concentrations of sodium acetate buffers were 0.05, 0.1, 0.15 and 0.2 M, and Tris–HCl buffers were 0.1, 0.2, 0.3, 0.4 and 0.5 M. The test tubes were tightly closed with flanged polypropylene plugs, and were incubated at 25 °C on rotary shaker (150 rpm). Aliquots (100 μl) of the samples taken at 0, 20, 40, 60, 120, 1080 and 2520 min were immediately mixed with three volumes of cold 0.25 M ammonium acetate (pH 7.0)–acetonitrile–methanol (40:50:10, v/v/v). Concentrations of erythromycin A, anhydroerythromycin A, erythromycin A enol ether and pseudoerythromycin A enol ether were determined in the presence of an internal standard, 2-hydroxycarbazole (final concentration, 2 μg ml^{-1}), as described below in the HPLC-coulometric assay. The observed rate constants (k_{obs}, min^{-1}) for the degradation of individual erythromycins A were determined from non-linear regression by the first-order decay kinetics, $C_t = C_0 \exp(-k_{obs} \times t)$, in which C_t and C_0 are the compound concentrations at time t and 0, respectively. According to the method of Atkins et al. (1986), the buffer-independent rate constant (k_0, min^{-1}) and the buffer-catalytic coefficient (k_{buffer}, M^{-1} min^{-1}) were determined from the plot of the observed rate constants versus molar concentrations of buffer (C_{buffer}), $k_{obs} = k_0 + k_{buffer} \times C_{buffer}$. The statistics were reported as the standard errors of estimates.

2.2. HPLC with electrochemical detection

Concentrations of individual erythromycins A were determined by a reversed-phase HPLC with electrochemical detection in the presence of an internal standard, 2-hydroxycarbazole (final concentration, 2 μg ml^{-1}). The chromatographic system consisted of an ESA solvent delivery module 581, a guard column packed with C_{18}-μBondapak (Alltech Associate Inc., IL) and a reversed-phase Radial-Pak Resolve Silica cartridge, 5 μm, 0.8 cm × 10 cm (Millipore, Waters Chromatography) fixed in a Waters Radial Compression Module. ESA Coulochem® II-500 electrochemical detector was equipped with an ESA Model 5020 guard cell and an ESA Model 5010 dual electrode cell (ESA Inc., MA). A 20 μl of sample was injected with the ESA Model 545 Autosampler equipped with a Rheodyne 7125 injector and a 20 μl loop. Potentials of the guard cell, and the screening and working electrodes were set at 900, 650 and 850 mV, respectively. The isocratic mobile phase consisted of acetonitrile–methanol–0.25 M ammonium acetate (50:10:40, v/v/v; pH 7.0), pumped at a flow rate of 1 ml min^{-1}. Under these operation conditions, the compound peaks of erythromycin A, anhydroerythromycin A, erythromycin A enol ether and pseudoerythromycin A enol ether were detected at 5.5 ± 0.01 min, 6.5 ± 0.10 min, 8.50 ± 0.05 min and 11.5 ± 0.10 min, respectively, and all of the standard curves constructed with five data points in a range of 0.5–100 μg ml^{-1} showed a good linearity ($r^2 > 0.99$).

2.3. Spectrometry

Analyses of erythromycin A and its degradation products were performed on an HP 1090L/M HPLC system (Hewlett-Packard, Palo Alto, CA) and an HP 5989B quadrupole mass spectrometer operated in the positive-ion electrospray mode. Full scans were acquired from *m/z* 50 to 900 at 0.92 scans per second. Components were resolved using a Prodigy ODS3 column, 5 μm, 2.0 mm × 250 mm (Phenomenex, Torrance, CA). The mobile phase was delivered at 0.2 ml min^{-1} by a 45 min-linear gradient of acetonitrile/water from 20%/80% (v/v) to 80%/20% (v/v) with constant 3 mM ammonium formate. The molecular weight was confirmed as protonated molecules at a capillary exit voltage of +100 V, and samples were reanalyzed with a capillary exit voltage of +200 V for the fragmentation by in-source collision-induced dissociation (CID).

The UV and visible absorption spectrometry was performed using an Hewlett Packard 8453 UV-Vis spectroscopy system (Waldbronn, Germany), and the absorption maxima and the molar extinction coefficients (M^{-1} cm^{-1}) of erythromycin compounds were determined in methanol (λ_{max}^{MeOH}).

The ^{13}C NMR spectra of erythromycins A were recorded on a 300.075 MHz Varian NMR spectrometer (Palo Alto, CA) in $CDCl_3$ employing proton decoupling during data acquisition. Parameters were as follows: spectral width, 20,000 Hz; 90° pulse, 13 μs; relaxation delay, 1 s; relaxation time, 1.49 s.

2.3.1. Erythromycin A (*I*)

Erythromycin A (purity 98.5%) was purchased from Sigma (St. Louis, MO). One gram of this compound was washed with 20 ml of cold water. The white powder was filtered, and dried in vacuo.

2.3.1.1. Anal. The UV-spectrum showed a single absorption maximum (λ_{max}^{MeOH}) at 289 nm (ε = 5.9 M^{-1} cm^{-1}). The ESI-mass spectrum with CID showed significant ions at *m/z* 734 [MH$^+$] (base peak), 576, 558, 158, 116 and 83. The ^{13}C NMR spectrum in $CDCl_3$ showed chemical shifts (ppm) at 217.16, 170.92, 78.67, 74.99, 73.00, 72.54, 72.11, 70.00, 69.70, 67.71, 65.96, 63.97, 60.71, 60.60, 44.57, 40.25, 39.97, 35.38, 34.48, 33.55, 32.88, 30.00, 23.94, 21.98, 16.57, 16. 45, 16.16, 13.68, 13.35, 11.25, 11.04, 7.11, 5.74 and 4.24.

2.3.2. Erythromycin A enol ether (*III*)

One gram of erythromycin A was dissolved in 10 ml of glacial acetic acid, and treated by a method modified from Kurath et al. (1971). The 2-h reaction mixture was neutralized with 0.5 M sodium bicarbonate, and extracted three times with equal volumes of ethyl acetate. Evaporation gave 1.05 g of residue. Residue was dissolved in 2 ml of methanol, and placed on ice. The product was recrystallized with drops of water. The product was recovered by centrifugation at 4 °C and 12,000 × *g* for 15 min, and dried in vacuo.

2.3.2.1. Anal. The UV-spectrum showed a single absorption maximum (λ_{max}^{MeOH}) at 207 nm (ε =

6950 M^{-1} cm^{-1}). The ESI-mass spectrum with CID showed significant ions at *m/z* 716 [MH^+], 558 (base peak), 158, 116 and 83. The ^{13}C NMR spectrum in $CDCl_3$ showed chemical shifts (ppm) at 174.41, 146.79, 97.78, 96.63, 89.80, 80.56, 75.46, 73.36, 71.62, 70.46, 68.15, 65.86, 65.43, 63.74, 60.97, 60.75, 60.70 (?), 44.64, 39.90, 38.27, 37.72, 35.52, 35.41, 29.75, 25.52, 21.36, 16.68, 16.36, 16.13, 11.24, 8.68, 7.02, 6.01 and 3.96.

*2.3.3. Anhydroerythromycin A (**IV**)*

One gram of erythromycin A was dissolved in 100 ml of dilute acetic acid (pH 3.5), and treated by a method modified from Wiley et al. (1957). The overnight reaction mixture was neutralized with ammonium hydroxide, and extracted three times with equal volumes of ethyl acetate. Evaporation gave 0.9 g of residue. Residue was dissolved in 2 ml of methanol, and placed on ice. The product was recrystallized with drops of water. The product was recovered by centrifugation at 4 °C and 12,000 × *g* for 15 min, and dried in vacuo.

2.3.3.1. Anal. The UV-spectrum showed a single absorption maximum (λ_{max}^{MeOH}) at 276 nm (ε = 10.6 M^{-1} cm^{-1}). The ESI-mass spectrum with CID showed significant ions at *m/z* 716 [MH^+], 558 (base peak), 540, 522, 158, 116 and 83. The ^{13}C NMR spectrum in $CDCl_3$ showed chemical shifts (ppm) at 174.7, 111.11, 97.93, 89.96, 82.50, 81.42, 77.53, 76.41, 73.34, 72.81, 72.71, 67.89, 64.61, 64.29, 60.88, 60.17, 46.66, 44.44, 40.99, 38.13, 36.44, 35.59, 35.47, 29.67, 24.35, 23.08, 20.10, 19.48, 16.75, 16.31, 16.31, 12.90, 11.85, 9.56, 8.84, 7.39 and 6.02.

*2.3.4. Pseudoerythromycin A enol ether (**VII**)*

One gram of erythromycin A was suspended in 50 ml of 0.1 M ammonium hydroxide, which had been adjusted to pH 8.5–9.5 with acetic acid. The suspension was incubated at 60 °C until erythromycin A completely disappeared during the stirring. The reaction mixture was neutralized with acetic acid, and extracted three times with equal volumes of ethyl acetate. Evaporation gave 0.88 g of residue. The residue was dissolved in 2 ml of methanol, and drops of water were added until the solution became amorphous. The amorphous product was centrifuged at 4 °C and 12,000 × *g* for 15 min, and dried in vacuo.

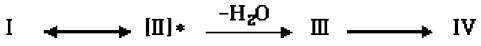
2.3.4.1. Anal. The UV-spectrum showed a single absorption maximum (λ_{max}^{MeOH}) at 208–209 nm (ε = 5627 M^{-1} cm^{-1}), which was characteristic of a double bond. The ESI mass spectrum with CID showed significant ions at *m/z* 716 [MH^+] (base peak), 558, 158, 116 and 83. The ^{13}C NMR spectrum in $CDCl_3$ showed chemical shifts (ppm) at 171.13, 144.67, 98.84, 96.52, 92.56, 81.06, 76.86, 75.49, 73.16, 72.91, 72.62, 71.52, 67.51, 66.02, 63.97, 60.48, 60.43, 44.48, 41.89, 38.45, 35.47, 33.83, 30.30, 26.74, 21.83, 17.57, 16.62, 16.56, 16.28, 13.48, 11.55, 10.39, 7.07, 6.39, 6.16 and 4.58.

3. Results and discussion

3.1. Degradation products of erythromycins

Erythromycin A (**I**) which had been washed with cold water showed two separated compound peaks by the LC/ESI-mass analysis using a Prodigy ODS3 HPLC column. However, it was difficult to separate the two peaks **I** and **II** by the used HPLC-coulometric method. A possible reason is either that only one of the two molecule structures mainly exists under the analytical conditions, or that the two compound peaks completely overlap in a single peak (**I** + **II**) owing to little difference between their retention times.

By the mass analyses with CID at +200 V, the +ESI-mass spectrum of the small peak at 18.2 min had significant ions (%RA, percent relative abundance) at *m/z* 734 (100, $[M+H]^+$), 576 (20), 558 (2), 158 (21), 116 (1) and 83 (1), and the large peak at 18.7 min had the major ions at *m/z* 734 (100, $[M+H]^+$), 576 (41), 558 (4), 158 (10), 116 (2) and 83 (3). The mass spectra indicated that the large peak was erythromycin A-6,9-hemiketal (**II**) with higher %RA values at *m/z* 576 (dehydrocladinose, $[M+H]^+$-158, 41% of the base peak) and *m/z* 558 for the loss of water ($[M+H]^+$-158-18, 4% of the base peak) compared with those of the small peak. The relative areas of the small and large peaks were accounted for 17.4 and 82.6%, respectively, and the equilibrium constant ($K_{eq} = k_1/k_{-1}$) was tentatively calculated at 4.75 from the ratio of the relative peak areas. The synopsis of the chromatographic and mass spectral data of the compounds **I** and **II** indicated that the 6,9-hemiketal (**II**) structure was rapidly formed in aqueous solution. This is con-

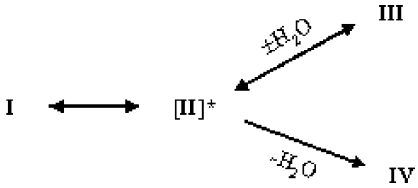


Scheme 1. Formation of anhydroerythromycin A (**IV**) from the acid-catalyzed degradation of erythromycin A (**I**) *via* erythromycin A enol ether (**III**) (Atkins et al., 1986; Alam et al., 1995).

sistent with the studies of Perun (1967) that the aglycone of erythromycin B was rapidly converted to the 6,9-hemiketal structure with a half-life of less than 4 min in 10^{-4} M methanolic hydrochloric acid, and that the establishment of equilibrium between erythromycin and the 6,9-hemiketal relies upon the concentration of water. Perun et al. (1970) suggested that a facile reaction between erythronolide B and erythronolide B-6,9-hemiketal occurs even in weakly basic solutions as well as in aqueous and methanolic solutions. The conformation studies with rigid 14-membered macrolides proved that this reaction occurs readily owing to the proximate relationship between the C6-hydroxyl group and the C9-carbonyl group (Perun et al., 1970; Perun, 1971; Egan et al., 1973). Therefore, it was assumed that there was always equilibrium between erythromycin A (**I**) and erythromycin A-6,9-hemiketal (**II**) in the acid- and base-catalyzed degradation.

Alam et al. (1995) suggested that erythromycin A in D_2O is converted *via* the enol ether intermediate (**III**) to the spiroketal form (**IV**) by one- and two-dimensional NMR techniques. Atkins et al. (1986) suggested that the tertiary alcohol of erythromycin A-6,9-hemiketal (**II**) forms a labile carbenium ion at the C9, and this ion dehydrates readily to form erythromycin A enol ether (**III**). The same authors suggested that the enol ether (**III**) structure rearranges easily to form the 9,12-spiroketal (**IV**) without build-up of an observable intermediate, since the conformation of erythromycin A (**I**) has a proximity between the C12-hydroxyl group and the C9-carbon atom (Egan et al., 1973; Ogura et al., 1981). If the internal dehydration reaction of compound **II** to form compound **III** and the subsequent structure rearrangement to form compound **IV** occur rapidly and irreversibly, only a small amount of erythromycin A enol ether (**III**) remains in acidic solutions. The proposed reaction pathways are schematized as in Scheme 1.

In contrast, Cachet et al. (1989) suggested that the acid-catalyzed degradation of erythromycin A (**I**) involved two different pathways: i.e. erythromycin



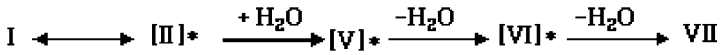
Scheme 2. Simultaneous formation of erythromycin A enol ether (**III**) and anhydroerythromycin A (**IV**) by the acid-catalyzed degradation of erythromycin A (**I**) (Cachet et al., 1989).

A-6,9-hemiketal (**II**) establishes an equilibrium with erythromycin A enol ether (**III**) through reversible dehydration–hydration reactions in one hand, and is simultaneously converted to anhydroerythromycin A (**IV**) by an irreversible dehydration reaction on the other hand (Scheme 2).

Additionally, transient erythromycin A-6,9-hemiketal (**II**) appears to undergo the base-catalyzed degradation, resulting in the formation of pseudoerythromycin A-6,9-hemiketal (**VI**) and pseudoerythromycin A enol ether (**VII**) (Kibwage et al., 1987a,b; Cachet et al., 1989). Volmer and Hui (1998) found that additional peaks at *m*/*z* 734 and *m*/*z* 752 were detected at high pH, while these peaks were not obtained from the acid-catalyzed degradation of erythromycin A. The same authors suggested that these compounds resulted from the hydrolysis of the lactonyl ester bond of erythromycin A (**I**) and the C13 → C11 translactionization at slightly alkaline conditions below pH 10. By the base-catalysis, it is assumed that the lactonyl ester bond of compound **II** is hydrolyzed to give the carboxylic acid (**V**) at the C1-position. The carboxylic acid is readily to esterify with the hydroxyl group at C11 to form pseudoerythromycin A-6,9-hemiketal (**VI**), since the C11-hydroxyl group is the only axial hydroxyl group adjacent to the C1-carbonyl group (Perun, 1971; Ogura et al., 1978). The unstable hemiketal (**VI**) might be easily converted to pseudoerythromycin A enol ether (**VII**) through an internal dehydration (Scheme 3).

Provided that in alkaline conditions the C13 → C11 translactonization and the internal dehydration reactions occur rapidly and irreversibly, it is difficult to detect the intermediates **V** and **VI** during erythromycin A (**I**) degradation.

Summarized with the degradation of erythromycin A in acidic and slightly alkaline conditions, ery-



Scheme 3. Proposed pathway of the base-catalyzed degradation of erythromycin A (**I**) to form pseudoerythromycin A enol ether (**VII**) (Kibwage et al., 1987a,b).

thromycin A (**I**) is converted *via* the 6,9-hemiketal (**II**) to erythromycin A enol ether (**III**), anhydroerythromycin A (**IV**), and pseudoerythromycin A enol ether (**VII**), which are isobaric compounds of *m/z* 716. The mass spectra of pure samples of erythromycin A (**I**) and the isobaric compounds of *m/z* 716 are given in Fig. 1. The loss of 18 indicates the occurrence of an internal dehydration reaction, $[MH^+ - H_2O]$. The structures of erythromycin A (**I**) and its acid- and base-catalyzed degradation products are given in Fig. 2.

3.2. Acid-catalyzed erythromycin A degradation

When acetic acid was used as a Bronsted acid (HA) donating hydrogen ion, an internal dehydration of erythromycin A-6,9-hemiketal (**II**) to form anhydroerythromycin A (**IV**) was catalyzed by the (pseudo)-first-order kinetic reaction with hydrogen ion. In this condition, the production of pseudoerythromycin A enol ether (**VII**) was negligibly small. From the acid-catalyzed degradation of erythromycin A (**I**) and erythromycin A enol ether (**III**), the plots of the observed degradation rate constants (k_{obs}) versus acid concentrations (C_{HA}) were analyzed by linear regressions using the equation, $k_{obs} = k_0 + k_{HA} \times C_{HA}$. The calculated buffer-independent rate constant (k_0, min^{-1}) and the buffer-catalytic coefficient (k_{HA}, $M^{-1}\,min^{-1}$) are given in Table 1. In the given pH range, the observed rate constants for the degradation of anhydroerythromycin A were constant ($2.5 \times 10^{-5}\,min^{-1}$), and were not influenced by the buffer concentrations (data not shown). Anhydroerythromycin A may be degraded to erythralosamine (5-*O*-desosaminyl-10,11-anhydroerythronolide A-6,9;9,12-spiroketal) by the cleavage of dehydrocladinose and an internal dehydration reaction (Flynn et al., 1954).

With decreasing one unit of the pH, the rate of erythromycin A (**I**) degradation was enhanced by about one order of the magnitude, indicating a (pseudo)-first-order in the molar concentration of hydrogen ion. The acid-catalyzed degradation of erythromycin A (**I**) resulted in the rapid production of anhydroerythromycin A (**IV**) as the major product (Fig. 3A), whereas the degradation of erythromycin A enol ether (**III**) resulted in the simultaneous formation of erythromycin A (**I**) and anhydroerythromycin A (**IV**) under the same condition (Fig. 3B). It seemed that anhydroerythromycin A (**IV**) was formed directly through an internal dehydration reaction of erythromycin A-6,9-hemiketal (**II**) which established an equilibrium with the enol ether (**III**), as suggested by Cachet et al. (1989). The acid-catalyzed degradation of compound **I** can be simplified with the con-

Table 1
Buffer-independent rate constants (k_0)[a] and the buffer-catalytic coefficients (k_{HA}) for the acid-catalyzed degradation of erythromycin A and erythromycin A enol ether in 0.05–0.2 M sodium acetate buffers at 25 °C

Buffer pH[b]	Erythromycin A		Erythromycin A enol ether	
	$10^3 \times k_0$ (min^{-1})	$10^3 \times k_{HA}$ ($M^{-1}\,min^{-1}$)	$10^3 \times k_0$ (min^{-1})	$10^3 \times k_{HA}$ ($M^{-1}\,min^{-1}$)
3.5	11.1 ± 1.60	32.8 ± 12.0	4.70 ± 0.45	29.0 ± 3.20
4.0	6.23 ± 1.02	10.8 ± 7.5	0.55 ± 0.22	25.0 ± 1.60
4.5	1.29 ± 0.69	22.4 ± 5.07	0.82 ± 0.22	41.0 ± 1.60
5.0	0.45 ± 0.039	1.91 ± 0.29	0.28 ± 0.22	28.0 ± 0.89
5.5	0.072 ± 0.013	0.50 ± 0.092	0.30 ± 0.057	12.0 ± 0.41

[a] The buffer-independent rate constant (k_0) and the buffer-catalytic coefficient (k_{HA}) were estimated from the linear regression of the plots of observed rate constants (k_{obs}) vs. molar concentrations of acetic acid buffer (C_{HA}) in the relationship of $k_{obs} = k_0 + k_{HA} \times C_{HA}$. The statistics are reported as the standard errors of the estimates.

[b] The pH of acetic acid was adjusted to a desired point with 1 M sodium hydroxide.

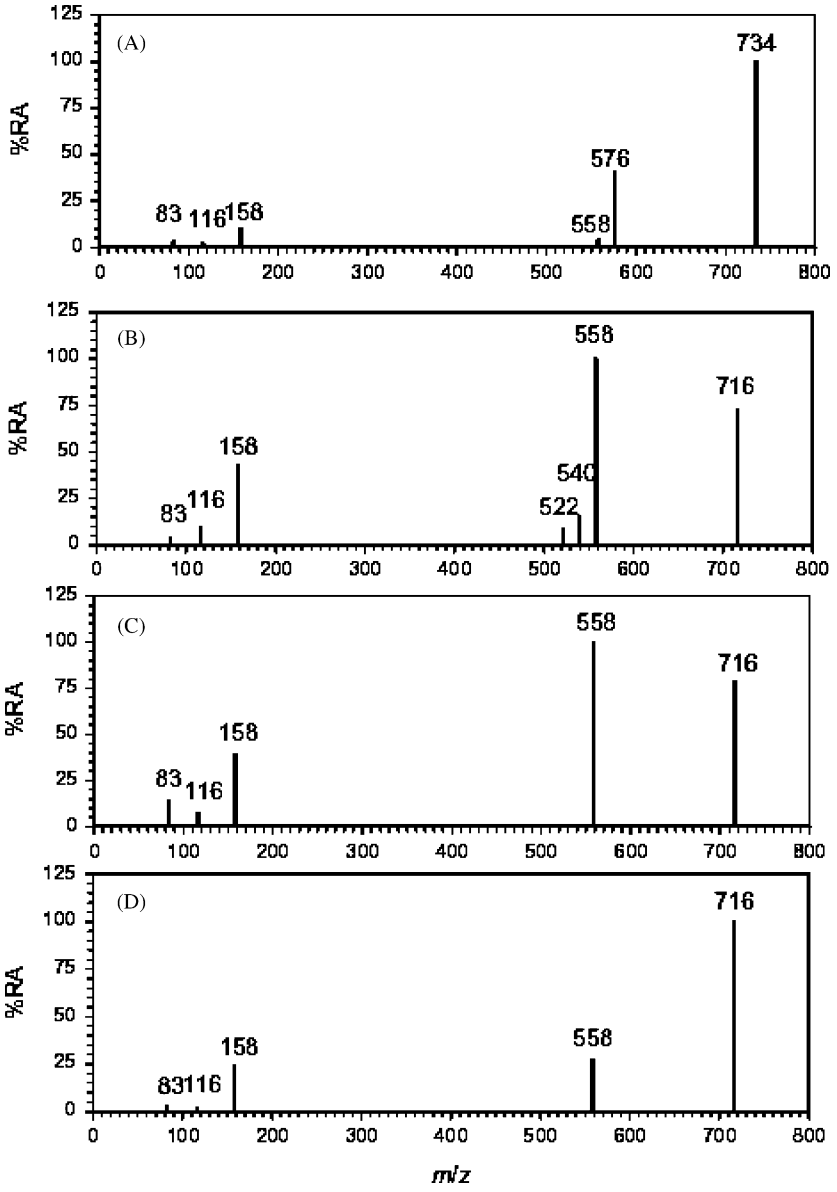
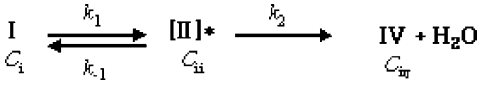


Fig. 1. Mass spectra of pure samples of (A) erythromycin A, (B) anhydroerythromycin A, (C) erythromycin A enol ether and (D) pseudoerythromycin A enol ether with ion-source collision-induced dissociation at +200 V.

centrations of **I**, **II**and **IV** at time *t*, as follows.With

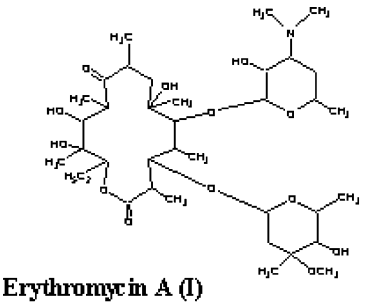


those concentrations defined under the equations, the application of elementary kinetic laws leads to the following differential equations.

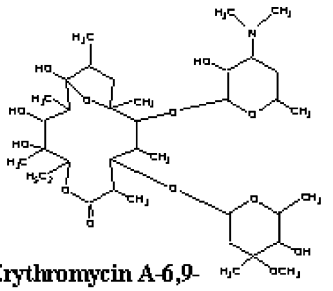
$$-\frac{\partial C_{\mathbf{I}}}{\partial t} = k_1 \times C_{\mathbf{I}} - k_{-1} \times C_{\mathbf{II}} \quad (1)$$

$$\frac{\partial C_{\mathbf{II}}}{\partial t} = k_1 \times C_{\mathbf{I}} - k_{-1} \times C_{\mathbf{II}} - k_2 \times C_{\mathbf{II}} \quad (2)$$

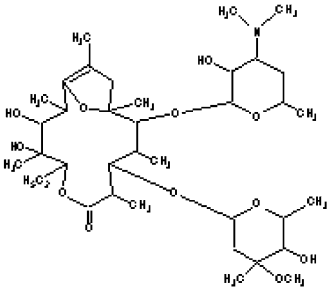
$$\frac{\partial C_{\mathbf{IV}}}{\partial t} = k_2 \times C_{\mathbf{II}} \quad (3)$$



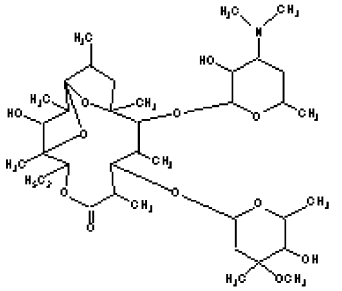
Erythromycin A (I)



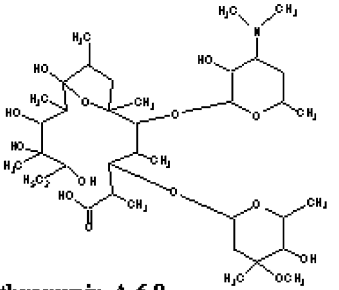
Erythromycin A-6,9-hemiketal (II)



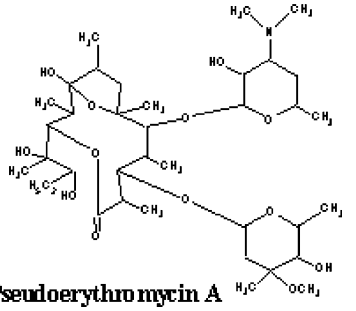
Erythromycin A enol ether (III)



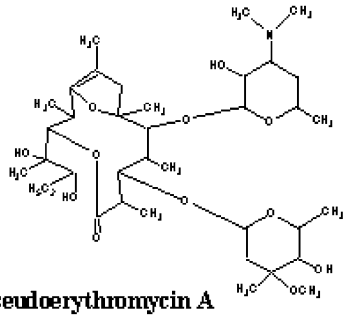
Anhydroerythromycin A (IV)



Erythromycin A-6,9-hemiketal carboxylic acid (V)



Pseudoerythromycin A 6,9-hemiketal (VI)



Pseudoerythromycin A enol ether (VII)

Fig. 2. Structures of erythromycin A and the degradants in aqueous solutions.

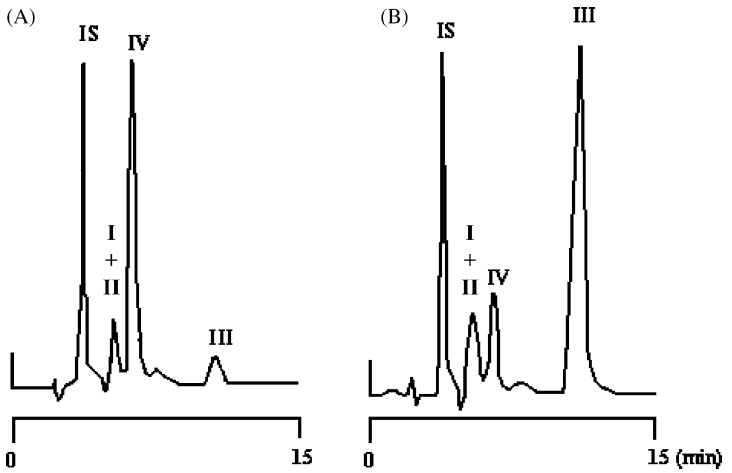


Fig. 3. HPLC profiles for acid-catalyzed degradation of (A) erythromycin A and (B) erythromycin A enol ether after 1 h of the incubation in 0.1 M sodium acetate buffer (pH 3.5) at 25 °C. The compound peaks are quantified in the presence of an internal standard (IS), 2-hydroxycarbazole (final concentration, 2 μg ml^{-1}), and are identified with the roman numerals shown in Fig. 2.

By solving the simultaneous equations, the sum of partial reaction rates for the degradation of compounds **I** and **II** in equilibrium is equal to the rate of the production of anhydroerythromycin A (**IV**) with the rate constant, k_2:

$$-\left(\frac{\partial C_{\mathbf{I}}}{\partial t}+\frac{\partial C_{\mathbf{II}}}{\partial t}\right)=\frac{\partial C_{\mathbf{IV}}}{\partial t}=k_2\times C_{\mathbf{II}} \quad (4)$$

Provided that there is a facile reaction for the establishment of equilibrium between compounds **I** and **II**, and that a large quantity of compound **II** is present throughout the course of reaction, the rate of the acid-catalyzed degradation of erythromycin A (**I**) is approximately equal to the rate of formation of anhydroerythromycin A (**IV**).

$$-\frac{\partial C_{\mathbf{I}}}{\partial t}=\frac{\partial C_{\mathbf{IV}}}{\partial t}=k_2\times C_{\mathbf{II}}=k_2\times\frac{k_1}{k_{-1}}\times C_{\mathbf{I}} \quad (5)$$

In this case, the rate-limiting step is the production of anhydroerythromycin A (**IV**) through an internal dehydration reaction of the 6,9-hemiketal (**II**).

For estimation of the observed rate constants (k_{obs}, min^{-1}) for erythromycin A (**I**) degradation, the standard curve was constructed with measuring the single peak area at 5.5 min using erythromycin A (**I**) standards (0.5–100 μg ml^{-1}). Although the large amounts were rapidly converted to erythromycin A-6,9-hemiketal (**II**), the estimation of the observed rate was not affected by the quantification method under the assumption that there was always equilibrium between compounds **I** and **II** in a ratio of $1{:}k_1/k_{-1}$. When the pH of 0.1 M sodium acetate buffers varied from 3.5 to 7.0, the observed rate constants for the acid-catalyzed degradation of erythromycin A (**I**) decreased logarithmically (Table 2). The observed rate constants can be expressed in terms of molar

Table 2
Observed rate constants, k_{obs}, for the degradation of erythromycin A at 25 °C

0.1 M Sodium acetate[a]		0.1 M Tris–HCl[b]	
pH	$10^3 \times k_{obs}$ (min^{-1})	pH	$10^3 \times k_{obs}$ (min^{-1})
3.5	14.4[c]	7.0	0.015 ± 0.002
4.0	7.31[c]	7.5	0.019 ± 0.003
4.5	3.53[c]	8.0	0.032 ± 0.010
5.0	0.64[c]	8.5	0.062 ± 0.024
5.5	0.12[c]	9.0	0.077 ± 0.007
6.0	0.080 ± 0.001		
6.5	0.049 ± 0.010		
7.0	0.020 ± 0.006		

[a] The pH values of 0.1 M acetic acid buffer were adjusted with 1 M NaOH.

[b] The pH values of 0.1 M Tris buffer were adjusted with 1 M HCl.

[c] At pH 3.5–5.5, the observed rate constants for the acidic degradation of erythromycin A were calculated by the equation, $k_{obs} = k_0 + (0.1 \times k_{HA})$, using buffer-independent rate constants (k_0) and the buffer-catalytic coefficients (k_{HA}) given in Table 1.

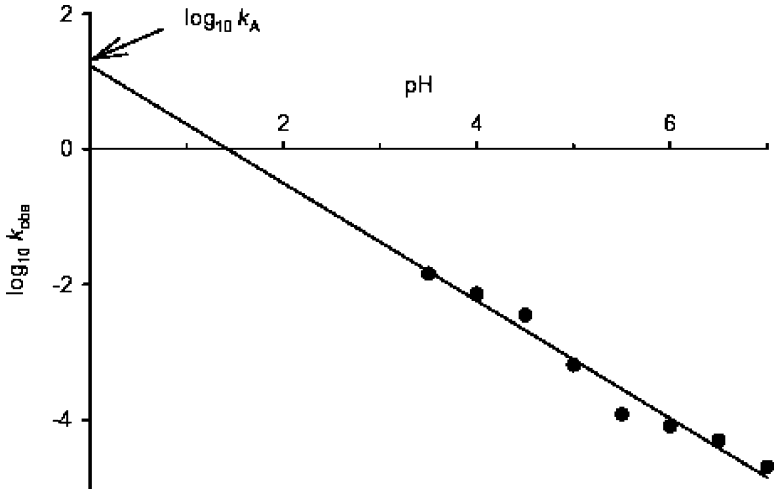


Fig. 4. A logarithmic plot of k_{obs} vs. $[H^+]$ for the determination of the acid-catalytic coefficient, k_A, and the reaction order of hydrogen ion, a, from acid-catalyzed degradation of erythromycin A to form anhydroerythromycin A at pH 3.5–7.0.

concentration of hydrogen ion:

$$k_{obs} = k_2 \times \frac{k_1}{k_{-1}} = k_A \times [H^+]^a \quad (6)$$

where k_A is the acid-catalytic coefficient, and a is the reaction order of hydrogen ion. As the reaction order, a, is close to one, hydrogen ion catalyzes the internal dehydration reaction of erythromycin A-6,9-hemiketal (**II**) to form anhydroerythromycin A (**IV**) by the first-order kinetics. In this case, the acid-catalytic coefficient, k_A, corresponds to the catalytic rate coefficient of hydrogen ion proposed by Atkins et al. (1986). Fig. 4 shows the logarithmic plot of the observed rate constants versus molar concentrations of hydrogen ion. By the extrapolation of the linear curve, the reaction order, a, and the acid-catalytic coefficient, k_A, were estimated at 0.87 ± 0.064 and 17.2 ± 2.2, respectively.

3.3. Base-catalyzed erythromycin A degradation

When the pH of Tris–HCl buffers increased from 7.0 to 9.0, the rates of the production of erythromycin A enol ether (**III**) and anhydroerythromycin A (**IV**) became negligibly small, whereas the rates of pseudoerythromycin A enol ether (**VII**) production gradually increased (Fig. 5). The degradation of compound **VII** was negligibly small during the same period. Concentrations of Tris–HCl buffers in a range of 0.1–0.5 M had no significant influence on the observed rate constants for the base-catalyzed degradation of erythromycin A (**I**). In this study, neither erythromycin A-6,9-hemiketal carboxylic acid (**V**) nor pseudoerythromycin A-6,9-hemiketal (**VI**) was detected, probably due to the fast reactions to produce pseudoerythromycin A enol ether (**VII**). Accordingly, the base-catalyzed degradation of erythromycin A (**I**) can

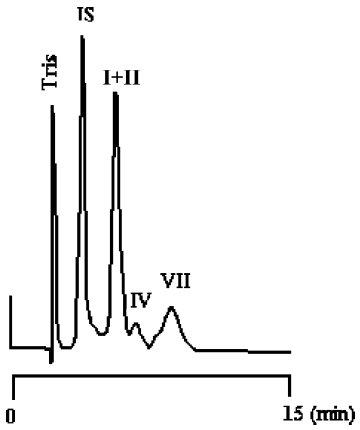
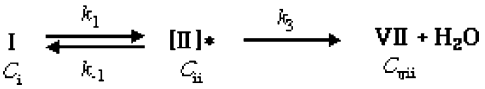


Fig. 5. An HPLC profile for base-catalyzed degradation of erythromycin A after 136 h of the incubation in 0.1 M Tris–HCl (pH 9.0) at 25 °C. The quantification and identification of the compound peaks are done as described in Fig. 3.

be simplified with the concentrations of **I**, **II** and **VII** at time *t*.



where k_3 implies a rate-limiting step of at least three reaction steps proposed in Scheme 3. The C13 → C11 translactonization involves the two reactions for the base-catalyzed hydrolysis of the lactonyl ester bond of erythromycin A-6,9-hermiketal (**II**) at C13 and the subsequent esterification of the carboxylic acid (**V**) with the C11-hydroxyl group, resulting in the formation of pseudoerythromycin A-6,9-hemiketal (**VI**). Pseudoerythromycin A enol ether (**VII**) resulted from the internal dehydration of transient intermediate **VI**. However, it was difficult to determine the rate-limiting step from the base-catalyzed degradation of erythromycin A (**I**) in this study.

In a similar manner as described in the acid-catalyzed degradation, the rate of the base-catalyzed degradation of erythromycin A (**I**) can be expressed on an equality with the rate of the production of pseudoerythromycin A enol ether (**VII**):

$$-\frac{\partial C_{\mathbf{I}}}{\partial t} = \frac{\partial C_{\mathbf{VII}}}{\partial t} = k_3 \times C_{\mathbf{II}} = k_3 \times \frac{k_1}{k_{-1}} \times C_{\mathbf{I}} \qquad (7)$$

When the pH of 0.1 M Tris–HCl buffers varied from 7.0 to 9.0, erythromycin A (**I**) was slowly converted to pseudoerythromycin A enol ether (**VII**) by the pseudo-first-order kinetics (Table 2). The observed rate constants (k_{obs}, min^{-1}) for the base-catalyzed degradation of erythromycin A (**I**) can be expressed in terms of the base-catalytic coefficient (k_B) and the reaction order in the molar concentration of hydroxide ion, *b*:

$$k_{obs} = k_3 \times \frac{k_1}{k_{-1}} = k_B[OH^-]^b \qquad (8)$$

Fig. 6 shows a logarithmic plot of the observed rate constants versus molar concentrations of hydroxide ion. By the extrapolation of the linear curve, the reaction order, *b*, and the base-catalytic coefficient, k_B, were calculated at 0.387 ± 0.038 and 7.06×10^{-3}, respectively. The pseudo-first-order reaction kinetics with the reaction order (*b*) of less than 1 may be due to the fact that water molecule participates in the base-catalyzed hydrolysis of the lactonyl ester bond of erythromycin A-6,9-hemiketal (**II**) at C13.

3.4. Prediction of erythromycin A degradation in terms of the pH of aqueous solutions

The purpose of this study is to present a proper kinetic model for prediction of the rates of erythromycin A (**I**) degradation in aqueous solutions. When the rate of erythromycin A (**I**) degradation is approximately equal to the sum of partial reaction rates for the production of anhydroerythromycin A (**VI**) and pseudo-

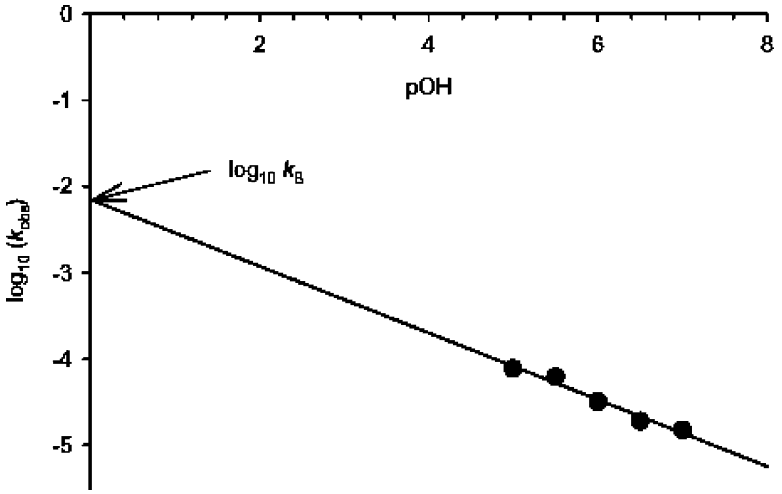


Fig. 6. A logarithmic plot of k_{obs} vs. [OH^-] for the determination of the base-catalytic coefficient, k_B, and the reaction order of hydroxide ion, *b*, from the base-catalyzed degradation of erythromycin A to form pseudoerythromycin A enol ether at pH 7.0–9.0.

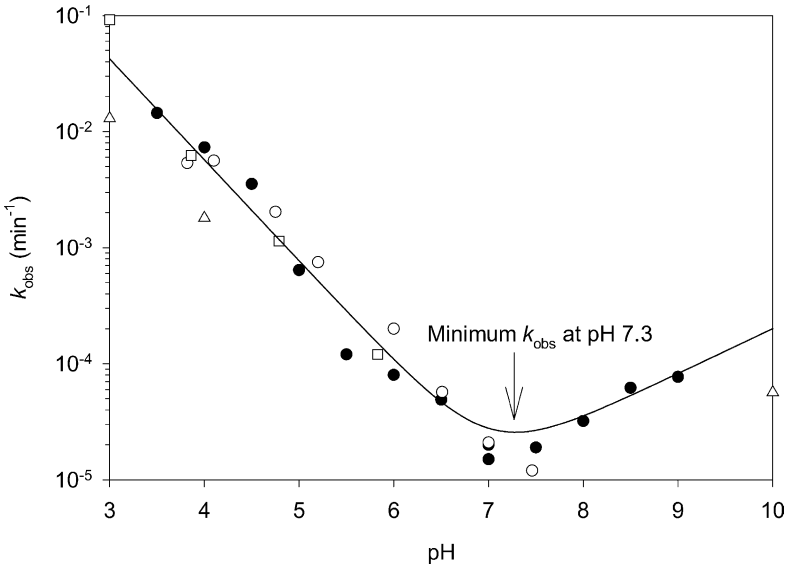


Fig. 7. Prediction of erythromycin A degradation in terms of the pH of aqueous solutions by the proposed Eq. (10). Measured data points were obtained from this study (●), Atkins et al. (1986, ○), Cachet et al. (1989, △) and Volmer and Hui (1998, □).

erythromycin A enol ether (**VII**), the predictive kinetic model can be expressed by the sum of the partial differential Eqs. (5) and (7) in terms of molar concentration of hydrogen ion in aqueous solution:

$$-\frac{dC_{\mathbf{I}}}{dt} = \frac{\partial C_{\mathbf{IV}}}{\partial t} + \frac{\partial C_{\mathbf{VII}}}{\partial t} \Rightarrow k_{obs} = k_A \times [H^+]^a + k_B \times \left(\frac{K_w}{[H^+]}\right)^b \quad (9)$$

where K_w is the ion product constant of water, i.e. $K_w = [OH^-][H^+] = 10^{-14}$.

The acid- and base-catalytic coefficients (k_A and k_B, respectively) and the reaction orders of hydrogen ion and hydroxide ion (a and b, respectively) can be obtained from the pH-controlled experiments, as seen in Figs. 4 and 6. When the data for the observed rate constants for the degradation of erythromycin A (**I**) in aqueous solutions were obtained from this study and other studies of Atkins et al. (1986), Cachet et al. (1989) and Volmer and Hui (1998) under similar conditions, the model Eq. (10) provided a good interpretation of the kinetics of the degradation of erythromycin A (**I**) in aqueous solutions as a function of pH in a range of 3.0–10.0 (Fig. 7).

$$k_{obs} = 17.2 \times 10^{(-0.870 \times pH)} + (2.70 \times 10^{-8}) \times 10^{(0.387 \times pH)} \quad (10)$$

By Eq. (10), it is calculated that erythromycin A (**I**) in aqueous solution is most stable at pH 7.3.

4. Conclusion

The results obtained from this study demonstrate distinct three pathways for the degradation of erythromycin A (**I**) which is likely to establish an equilibrium with erythromycin A-6,9-hemiketal (**II**) in aqueous solution. In acidic conditions, the 6,9-hemiketal (**II**) seemed to be converted to anhydroerythromycin A (**IV**) through an internal dehydration reaction, and on the other hand there was a reversible reaction of the 6,9-hemiketal (**II**) to form erythromycin A enol ether (**III**). In slightly alkaline conditions, erythromycin A (**I**) was decomposed to pseudoerythromycin A enol ether (**VII**). The lactonyl ester bond of erythromycin A-6,9-hemiketal (**II**) appeared to be hydrolyzed by the base-catalysis of hydroxide ion, and the reaction was followed by the re-esterification of the carboxylic acid (**V**) with the C11-hydroxyl group and an internal dehydration reaction of pseudoerythromycin A-6,9-hemiketal (**VI**) to form pseudoerythromycin A enol ether (**VII**). This study suggests that the sum of partial reaction rates for the

acid- and base-catalyzed degradation of erythromycin A (**I**) *via* the 6,9-hemiketal (**II**) to produce anhydroerythromycin A (**IV**) and pseudoerythromycin A enol ether (**VII**) determines the kinetics of erythromycin A (**I**) degradation as a function of pH in aqueous solutions.

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References

Alam, P., Buxton, P.C., Parkinson, J.A., Barber, J., 1995. Structural studies on erythromycin A enol ether: full assignments of the ^{1}H and ^{13}C NMR spectra. J. Chem. Soc. Perkin Trans. 2, 1163–1167.

Atkins, P.J., Herbert, T.O., Jones, N.B., 1986. Kinetic studies on the decomposition of erythromycin A in aqueous acid and neutral buffers. Int. J. Pharm. 30, 199–207.

Cachet, Th., de Turck, K., Roets, E., Hoogmartens, J., 1991. Quantitative analysis of erythromycin by reversed-phase liquid chromatography using column-switching. J. Pharm. Biomed. Anal. 9, 547–555.

Cachet, Th., Roets, E., Hoogmartens, J., Vanderhaeghe, H., 1987. Separation of novel derivatives from commercial erythromycin samples by thin-layer chromatography. J. Chromatogr. 403, 343–349.

Cachet, Th., Van den Mooter, G., Hauchecorne, R., Vinckier, C., Hoogmartens, J., 1989. Decomposition kinetics of erythromycin A in acidic aqueous solutions. Int. J. Pharm. 55, 59–65.

Egan, R.S., Perun, T.J., Martin, J.R., Mitscher, L.A., 1973. The conformation of erythronolide, the 14-membered aglycone ring of erythromycin antibiotics. Tetrahedron 29, 2525–2538.

Fiese, E.F., Steffen, S.H., 1990. Comparison of the acid stability of azithromycin and erythromycin A. J. Antimicrob. Chemother. 25, 39–47.

Flynn, E.H., Murphy, H.W., McMahon, R.E., 1955. Erythromycin. II. Des-*N*-methylerythromycin and *N*-methyl-^{14}C-erythromycin. J. Am. Chem. Soc. 77, 3104–3106.

Flynn, E.H., Sigal Jr., M.V., Wiley, P.F., Gerzon, K., 1954. Erythromycin. I. Properties and degradation studies. J. Am. Chem. Soc. 76, 3121–3131.

Kibwage, I.O., Busson, R., Janssen, G., Hoogmartens, J., Vanderhaege, H., Bracke, J., 1987a. Translactonization in erythromycin. J. Org. Chem. 52, 990–996.

Kibwage, I.O., Hoogmartens, J., Roets, E., Vanderhaeghe, H., Verbist, L., Dubost, M., Pascal, C., Petitjean, P., Levol, G., 1985. Antibacterial activities of erythromycin A, B, C, and D, and some of their derivatives. Antimicrob. Agents Chemother. 28, 630–633.

Kibwage, I.O., Janssen, G., Busson, R., Hoogmartens, J., Vanderhaeghe, H., Verbist, L., 1987b. Identification of novel erythromycin derivatives in mother liquor concentrates of *Streptomyces erythraeus*. J. Antibiot. 40, 1–6.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000: a national reconnaissance. Environ. Sci. Technol. 36, 1202–1211.

Kurath, P., Jones, P.H., Egan, R.S., Perun, T.J., 1971. Acid degradation of erythromycin A and erythromycin B. Experientia 27, 362–363.

Lazarevski, T., Radobolja, G., Djokic, S., 1978. Erythromycin VI: kinetics of acid-catalyzed hydrolysis of erythromycin oxime and erythromycylamine. J. Pharm. Sci. 67, 1031–1033.

Marzo, A., Dal Bo, L., 1998. Chromatography as an analytical tool for selected antibiotic classes: a reappraisal addressed to pharmacokinetic applications. J. Chromatogr. A 812, 17–34.

Nakagawa, Y., Itai, S., Yoshida, T., Nagai, T., 1992. Physicochemical properties and stability in the acidic solution of a new macrolide antibiotic, clarithromycin, in comparison with erythromycin. Chem. Pharm. Bull. 40, 725–728.

Ogura, H., Furuhata, K., Harada, Y., Iitaka, Y., 1978. Stereochemistry of macrolides. 3. X-ray crystal structure analysis of 11, 4′′-bis[*O*-(*p*-bromobenzoyl)]oleandomycin. J. Am. Chem. Soc. 100, 6733–6737.

Ogura, H., Furuhata, K., Kuwano, H., Suzuki, M., 1981. Stereochemistry of macrolides: IV. Conformational studies on 14-membered macrolide–“diamond lattice” conformation models. Tetrahedron 37, 165–173.

Perun, T.J., Egan, R.S., Jones, P.H., Martin, J.R., Mitscher, L.A., Slater, B.J., 1970. Conformation of macrolide antibiotics. IV. Nuclear magnetic resonance, circular dichroism, and chemical studies of erythromycin derivatives. Antomicrob. Agents Chemother. 1969, 116–122.

Perun, T.J., 1967. Chemistry of erythronolide B. Acid-catalyzed transformations of the aglycone of erythromycin B. J. Org. Chem. 32, 2324–2330.

Perun, T.J., 1971. The chemistry and conformation of erythromycin. In: Mitsuhashi, S. (Ed.), Drug Action and Drug Resistance of Bacteria. University of Tokyo, Tokyo, pp. 123–152.

Pothuluri, J.V., Nawaz, M., Cerniglia, C.E., 1998. Environmental fate of antibiotics used in aquaculture. In: Sikdar, S.K., Irvine, R.L. (Eds.), Bioremediation: Principles and Practice, vol. II, Biodegradation Technology Developments. Technomic Publishing Co., Lancaster-Basel, pp. 221–248.

Volmer, D.A., Hui, J.P.M., 1998. Study of erythromycin A decomposition products in aqueous solution by solid-phase microextraction/liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass Spectrom. 12, 123–129.

Wiley, P.F., Gerzon, K., Flynn, E.H., Sigal Jr., M.V., Weaver, O., Quarck, U.C., Chauvette, R.R., Monahan, R., 1957. Erythromycin. X. Structure of erythromycin. J. Am. Chem. Soc. 79, 6062–6070.