Kinetic Studies of the Hydrolysis and Lactonization of Camptothecin and Its Derivatives, CPT-11 and SN-38, in Aqueous Solution

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The effect of pH and temperature on the reaction rate of the hydrolysis and lactonization of camptothecin and its derivatives, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11) and 7-ethyl-10-hydroxycamptothecin (SN-38), in aqueous solution was studied by high-performance liquid chromatography or two-wavelength spectrophotometry. Hydrolysis and lactonization of each compound progressed according to pH- and temperature-dependent pseudo-first-order kinetics. The ratio of the lactone form of each compound to its hydroxy-acid form was determined mainly by the pH of the solution and was not influenced by temperature. Half-lives of the lactone and hydroxy-acid forms of CPT-11 at 37 °C and pH 7.4 were 13.7 min and 4.25 h, respectively.

Keywords camptothecin; lactone; kinetics; hydrolysis; lactonization; HPLC

7-Ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin (CPT-11) and 7-ethyl-10-hydroxycamptothecin (SN-38) are the derivatives of camptothecin, an antitumor alkaloid isolated from *Camptotheca acuminata*[1] and produced by Yakult Co., Ltd. (Tokyo, Japan).[2] SN-38 which has been synthesized by modifying the chemical structure of camptothecin possesses increased biological activities. The solubility in water of CPT-11, the prodrug of SN-38, is higher than that of SN-38. As shown in Fig. 1, the molecular structures of CPT-11, camptothecin and SN-38 all contain an α-hydroxy δ-lactone ring. The antitumor activities of camptothecin and its related compounds indicate that the existence and state of the lactone ring of each compound is important. We previously presented[3] a high performance liquid chromatography (HPLC) method for the simultaneous determination of lactone and hydroxy-acid forms of CPT-11, camptothecin and SN-38. Fassberg and Stella[4] minutely studied the hydrolysis and lactonization reactions of camptothecin and its analogs in aqueous solution at 25 °C. They also used an HPLC method with UV-detection. Yoshioka *et al.*[5] and Kaufman[6] investigated the interconversion of lactones by HPLC. Time needed for sample analysis limited the applicability of HPLC, showing that this method cannot measure rapid reactions.

We also investigated the rate of interconversion of the lactone and hydroxy-acid forms of these compounds in aqueous solution. An HPLC method could measure the rate of lactonization, but not the rate of hydrolysis, which is a faster reaction. Two-wavelength spectrophotometry, which is able to determine the concentration of each form continuously, was therefore used to measure the rate of hydrolysis of CPT-11.

Experimental

Materials CPT-11, Camptothecin and SN-38 were obtained from Yakult Co., Ltd. Sodium 1-heptanesulfonate for ion-pair chromatography (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) was used. Water was distilled and all other chemicals were of reagent grade. Britton–Robinson's buffer solutions were used with ionic strength adjusted by potassium chloride.

Determination of Lactone and Hydroxy-Acid Forms. Hydrolysis reactions of CPT-11 Absorbances of 354 and 390 nm were simultaneously monitored by a Hitachi 556 two-wavelength spectrophotometer. These data were collected and processed by a Fujitsu F-7740 computer. Concentrations of the lactone and hydroxy-acid forms, C_L and C_H, were calculated by the following equation:

$$C_L = (1461 \cdot A_{354} - 3329 \cdot A_{390})/42.83 \quad (1)$$

$$C_H = (3859 \cdot A_{390} - 407 \cdot A_{354})/42.83 \quad (2)$$

where A_{354} and A_{390} are the absorbances at 354 and 390 nm, respectively. The parameters in Eqs. 1 and 2 were determined by the absorption coefficients of CPT-11 at pH 4.0 and 10.0, as those of the lactone and hydroxy-acid forms, respectively.

Lactonization Reactions of CPT-11 The HPLC method used in this work was as reported in a previous paper,[3] excepting the detection method. A Hitachi 638 liquid chromatograph equipped with a variable-wavelength UV detector, UVLOG-5II (Oyobunko, Tokyo, Japan), and a Hitachi 638-08 auto sampler were used. The UV detector was set at 254 nm.

Hydrolysis and Lactonization Reactions of Camptothecin and SN-38 A previously reported[3] HPLC method was used. A Hitachi F1000 fluorescence detector was used, while the other HPLC apparatuses were as described above.

Preparation of Sample Solutions. Hydrolysis Reaction A buffer solution with a pH equal to the measurement condition was previously warmed to the measurement temperature. Acidic mother solutions (pH approx. 3) of suitable concentration were diluted 25 to 50 times with the buffer solution to give a sample solution, and the reaction was started. The sample solution was passed to an auto-sampler vial for HPLC or to a spectrophotometer quartz cell for two-wavelength spectrophotometry, and the measurement was started. Sample vials and quartz cells were maintained at measurement temperature.

Lactonization Reaction Alkaline mother solutions (pH approx. 10, prepared before use) of suitable concentrations were diluted 5 to 50 times with the previously warmed buffer solution, and the reaction was started. Measurements were started as described above.

Kinetic Study Conditions Hydrolysis and lactonization of CPT-11 at a concentration of 20 μg/ml were followed by two-wavelength spectrophotometry and HPLC, respectively. The hydrolysis and lactonization of camptothecin and SN-38 were followed by HPLC. The pH range for measurements of hydrolysis and lactonization was 6 to 9 and 4 to 6, respectively. All reactions were measured at 27, 32, 37 and 42 °C. The

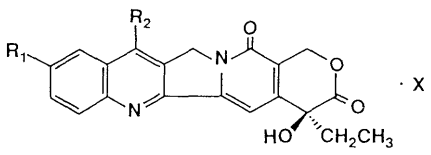

Fig. 1. Structures of Camptothecin, CPT-11 and SN-38

Camptothecin: $R_1 = R_2 = H$, X = none. CPT-11: $R_1 = OCONC_5H_9NC_5H_{10}$, $R_2 = C_2H_5$, $X = HCl.3H_2O$. SN-38: $R_1 = OH$, $R_2 = C_2H_5$, $X = H_2O$.

ratio of the lactone form at pH 4 to 9 of each compound was determined by HPLC. All studies were performed at an ionic strength of 0.2.

Ratio of Lactone Form in Equilibrium States Sample solutions at the correct pH and concentration for measurement were stored at a suitable temperature for 48 h and protected from light before measurement.

All kinetic studies were performed three times per experimental condition for CPT-11, and twice for camptothecin and SN-38. These data were treated as reversible reactions of hydrolysis and lactonization.

Results and Discussion

The CPT-11 molecule has two hydrolyzable sites (see Fig. 1), that is, a lactone group and a carbamyl group. Preliminary investigation showed the rate of hydrolysis of the carbamyl group to be much lower than that of lactone. It was therefore not necessary to consider the cleavage of carbamate when measuring the rate of hydrolysis and lactonization of the lactone of CPT-11 in this study.

Absorbances of CPT-11 at 354 and 390 nm changed as the hydrolysis reaction progressed. The concentrations of the lactone form and hydroxy-acid form were determined by Eqs. 1 and 2. Figure 2 shows the time–course of concentrations of the lactone form in hydrolysis reactions of CPT-11 at 37 °C with varying pH in a logarithmic scale. The lines drawn in Fig. 2 represent the lines of fit obtained from regression analysis, which fitted well to the plots. Since a linear relationship existed between time and logarithmic concentration, the hydrolysis of CPT-11 was understood to represent a pseudo-first-order reaction. Figure 3 shows the Arrhenius plots of the rate constants of hydrolysis of CPT-11 in buffer solutions of pH 6.0, 7.0, 8.0 and 9.0. The Arrhenius plots of each solution showed good linearity, and the activation energies and frequency factors were calculated from the plots. The first-order rate constants and other kinetic parameters of the hydrolysis of CPT-11 are summarized in Table I. The activation energies and frequency factors of hydrolysis decreased with increasing pH in the pH 7 to 9 region. The decrease in activation energies was stimulatory, but a decrease in frequency factors was inhibitory. The acceleration of the CPT-11 hydrolysis reaction with increasing pH was mainly explained by the contribution of activation energy.

We next discuss the lactonization of CPT-11. Figure 4 shows chromatograms of hydroxy-acid and lactone forms of CPT-11 in the lactonization reaction in a pH 4.0 buffer solution at 37 °C. As time progressed from the start of the lactonization reaction, the peaks of the hydroxy-acid and lactone forms became smaller and larger, respectively. These chromatograms clearly indicated that the hydroxy-acid form in the sample was converting into the lactone form. Figure 5 shows the time–course of hydroxy-acid form concentration in lactonization reactions of CPT-11 at 37 °C and at various pH in logarithmic scale and with lines of fit of regression analysis. Since a linear relationship existed between time and logarithmic concentration, the lactonization of CPT-11 was also understood to be a pseudo-first-order reaction. The kinetic parameters of the

Table I. Kinetic Parameters of Hydrolysis of CPT-11 in Aqueous Solution

pH	Rate constant (h^{-1})				E_a (kcal·mol^{-1})	A (h^{-1})
	27 °C	32 °C	37 °C	42 °C		
6.0	0.0629	0.101	0.115	0.257	16.3	4.63×10^{10}
7.0	0.461	0.716	1.14	1.60	15.8	1.47×10^{11}
8.0	4.65	5.79	8.54	10.5	10.6	2.58×10^{8}
9.0	28.3	35.2	47.8	51.9	8.00	1.94×10^{7}

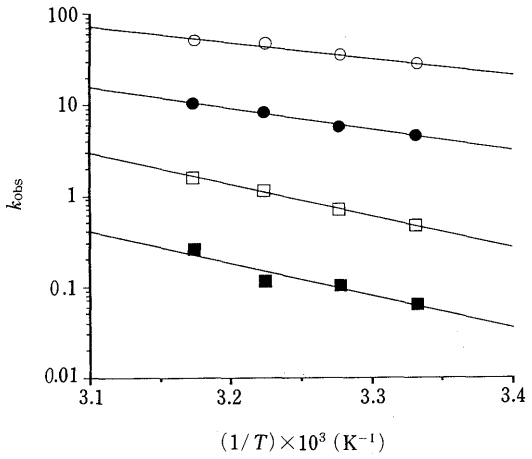

Fig. 3. Arrhenius Plots of Hydrolysis Reactions of CPT-11

■, pH 6.0; □, pH 7.0; ●, pH 8.0; ○, pH 9.0.

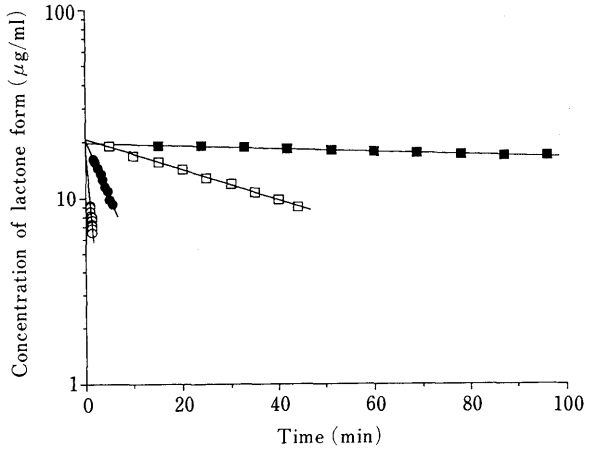

Fig. 2. Time–Courses of the Lactone Form of CPT-11 in Buffer Solutions with Varying pH at 37 °C

■, pH 6.0; □, pH 7.0; ●, pH 8.0; ○, pH 9.0.

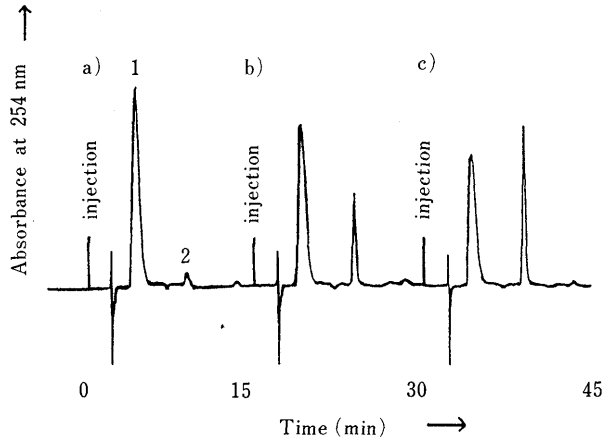

Fig. 4. Chromatograms of Hydroxy-Acid and Lactone Forms of CPT-11 in Buffer Solution of pH 4.0 at 37 °C

a) Soon after reaction started, b) after 15 min, c) after 30 min.

lactonization of CPT-11 are summarized in Table II. The acceleration of the lactonization reaction was mainly explained by the contribution of activation energy in a similar manner to the hydrolysis.

The ratios of lactone and hydroxy-acid forms in buffer solutions of various pH were constant at and after 48 h from the commencement of both the hydrolysis and lactonization reactions. Figure 6 shows the pH profiles of the amount of lactone form in equilibrium states. It shows that temperature and concentration have no effect on the percent of lactone form. The temperature and concentration of the sample did not affect the pH profile, and only pH determined the amount of the lactone form. These results show that temperature essentially had an equal effect, caused by the combination of activation energy and frequency factor, on the hydrolysis and lactonization reaction rate in the range of about 27 to 42 °C. This conclusion was confirmed by comparing the reaction rates in Tables I and II.

The interconversion reaction between the lactone and hydroxy-acid forms of SN-38 and camptothecin was also followed by HPLC. Analysis of the data showed that all these reactions progressed as pseudo-first-order reactions. The kinetic parameters of the hydrolysis and lactonization of SN-38 and camptothecin are shown in Table III.

These results led to the conclusion that the hydrolysis and lactonization of CPT-11, camptothecin and SN-38 obeyed the mechanisms of pseudo-first-order reaction. The rate constants of the hydrolysis and lactonization of these compounds at 37 °C, including values estimated by the relationship between the rate constants and the constants of equilibrium in reversible reactions, are showed in Fig. 7 and Table IV. Fassberg and Stella also examined the constants of the lactone/hydroxy-acid equilibrium of camptothecin.[4] Compared with the constants of their report, those at the same pH in this

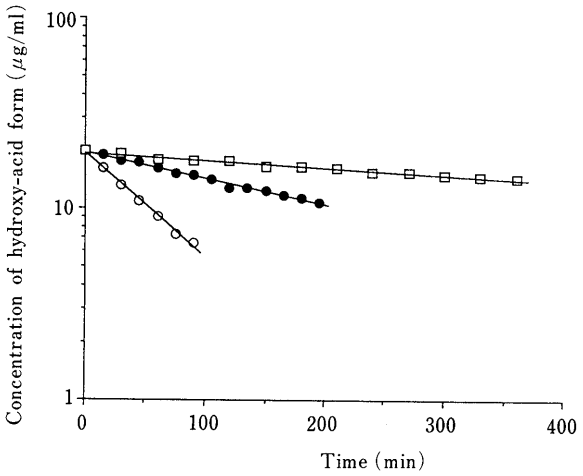

Fig. 5. Time–Courses of the Hydroxy-Acid Form of CPT-11 in Buffer Solutions with Varying pH at 37 °C

○, pH 4.0; ●, pH 5.0; □, pH 6.0.

TABLE II. Kinetic Parameters of Lactonization of CPT-11 in Aqueous Solution

pH	Rate constant (h^{-1})				E_a ($kcal \cdot mol^{-1}$)	A (h^{-1})
	27 °C	32 °C	37 °C	42 °C		
4.0	0.297	0.515	0.822	1.25	17.9	3.77×10^{12}
5.0	0.0653	0.119	0.212	0.359	21.4	2.51×10^{14}
6.0	0.0174	0.0410	0.111	0.195	31.0	7.03×10^{20}

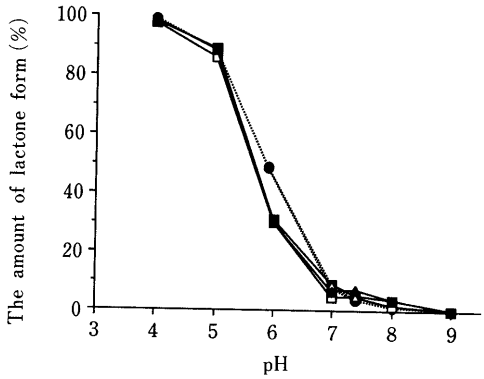

Fig. 6. pH–Amount Profile for Lactone Form of CPT-11

0.2 μg/ml: ○, 32 °C. 2.0 μg/ml: ●, 32 °C. 20.0 μg/ml: □, 27 °C; ■, 32 °C; △, 37 °C; ▲, 42 °C.

TABLE III. Kinetic Parameters of Hydrolysis and Lactonization of SN-38 and Camptothecin

Compound	Reaction	pH	Rate constant (h^{-1})				E_a ($kcal \cdot mol^{-1}$)	A (h^{-1})
			27 °C	32 °C	37 °C	42 °C		
SN-38	Hydrolysis	6.0	0.0297	0.0402	0.0514	0.159	19.7	5.98×10^{12}
		7.0	n.t.	n.t.	0.429	n.t.		
		7.4	n.t.	n.t.	1.27	n.t.		
		8.0	2.79	3.92	4.52	5.32	7.83	1.49×10^{6}
	Lactonization	4.0	0.361	0.555	0.935	1.30	16.4	3.28×10^{11}
		5.0	n.t.	n.t.	0.209	n.t.		
		6.0	0.0295	0.0592	0.0970	0.120	17.7	2.70×10^{11}
Camptothecin	Hydrolysis	6.0	0.0333	0.0565	0.0709	0.0941	12.6	5.33×10^{7}
		7.0	n.t.	n.t.	0.628	n.t.		
		7.4	n.t.	n.t.	2.19	n.t.		
		8.0	3.57	5.26	5.19	9.51	7.83	1.49×10^{6}
	Lactonization	4.0	0.419	0.675	0.996	1.53	16.1	2.14×10^{11}
		5.0	n.t.	n.t.	0.208	n.t.		
		6.0	0.0272	0.0598	0.103	0.0904	17.7	2.70×10^{11}

n.t.: not tested.

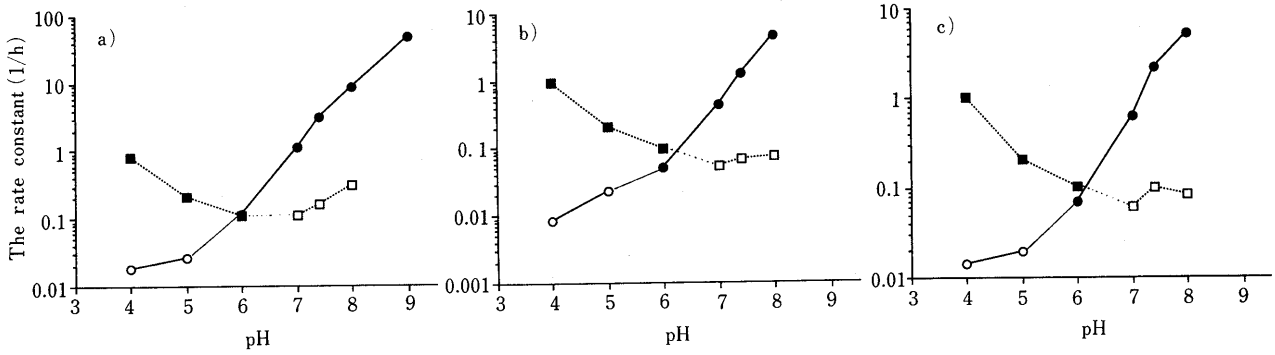

Fig. 7. pH-Rate Profile for the Hydrolysis and Lactonization of (a) CPT-11, (b) SN-38 and (c) Camptothecin at 37 °C
●○, hydrolysis; ■□, lactonization. Closed symbols: observed values. Open symbols: estimated values.

TABLE IV. The Equilibrium Constants for the Conversion of Lactone to the Hydroxy-Acid Form of CPT-11, SN-38 and Camptothecin at 37 °C

Compound	pH					
	4.0	5.0	6.0	7.0	7.4	8.0
CPT-11	0.0225	0.126	2.21	10.3	18.6	27.6
SN-38	0.00908	0.112	1.22	8.01	19.0	61.5
Camptothecin	0.0142	0.0941	1.44	10.5	22.3	61.5

work were large. The reason for this deviation could be due to the difference in buffer systems. The buffers used for both studies seem to produce an effect on the lactone/hydroxy-acid equilibrium. We confirmed that the constants of equilibrium at the same pH of CPT-11 in acetate and Britton–Robinson's buffer solutions were not identical.[7]

As you can see in Fig. 7, the hydrolysis rate increased commensurately as pH increased, but that of lactonization had minimum values at neutral pH. The logarithmic rate constants of hydrolysis were proportional to pH, so the mechanism of hydrolysis was a second-order reaction, dependent on hydroxy-acid anion and lactone concentrations. But that of lactonization was considered to be a combination of more than two elementary processes.

The compounds in this study showed almost identical hydrolysis and lactonization rate constants in buffer solution at each pH and at temperatures ranging 27 to 42 °C. These results led to the conclusion that chemical modification of the quinoline ring of camptothecin had little effect on the reactivity of the lactone ring. This study also clarified the effect of the buffer system on the lactone/hydroxy-acid equilibrium.

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