Stability and Degradation Kinetics of 5′-Deoxy-5-fluoro-N^4-(3,4,5-trimethoxybenzoyl)cytidine in Aqueous Solution

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The stability of 5′-deoxy-5-fluoro-N^4-(3,4,5-trimethoxybenzoyl)cytidine (Ro 09-1390) in buffered solutions at 37 °C was studied. Seven degradation products were identified by high-performance liquid chromatography (HPLC) and spectroscopy. The degradation products were different depending on the pH of the solution.

Ro 09-1390 was unstable at pH 2.1 and stable at around pH 5.6 in spite of forming several decomposition products; and its degradation rate did not change in the pH range from 8 to 12. The degradation of Ro 09-1390 followed apparent first order kinetics and the decomposition pathway at alkaline conditions was different from the one at acidic conditions. On the other hand, at neutral pH the other decomposition products were formed.

Keywords 5′-deoxy-5-fluoro-N^4-(3,4,5-trimethoxybenzoyl)cytidine (Ro 09-1390); 5′-deoxy-5-fluorouridine (DFUR); 5′-deoxy-5-fluorocytidine (DFCR); stability; kinetics; pH-profile; aqueous solution; decomposition product; pseudo first order reaction

5′-Deoxy-5-fluoro-N^4-(3,4,5-trimethoxybenzoyl)cytidine, Ro 09-1390, is a prodrug of 5′-deoxy-5-fluorouridine (DFUR; doxifluridine), an antitumor agent. This prodrug is under development as a successor to doxifluridine because its intestinal toxicity (diarrhea) is lower.[1)]

Ro 09-1390 rapidly decomposes to DFUR in a solution of pH 2.1; however, it is very stable in an unsolubilized state.

For the preformulation study of Ro 09-1390, the following investigation was initiated to determine whether degradation products are formed at 37 °C in buffer solutions ranging from pH 2 to 12. This work covers the decomposition kinetics and identification of the degradation products.

Experimental

Materials 5′-Deoxy-fluorocytidine (DFCR), DFUR, 5-fluorocytosine (FC), 5-fluorourasil (FU) and Ro 09-1390 were synthesized by F. Hoffmann-La Roche Ltd., Basel, Switzerland. All reagents were of analytical grade and used without further purification. The water used was distilled and deionized by Advantec Aquarius GS-50.

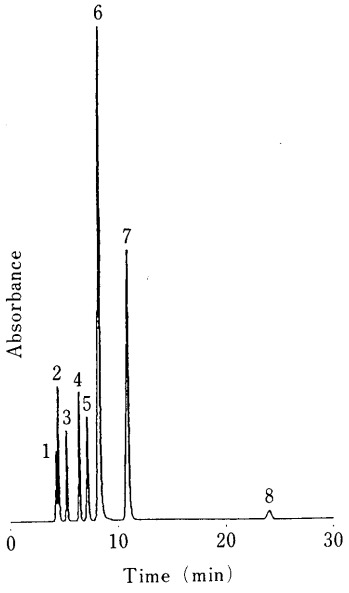

Fig. 1. A Typical HPLC Chromatogram for the Separation of Ro 09-1390 and Other Authentic Compounds

1=DFCR; 2=FC; 3=TMB amide; 4=FU; 5=DFUR; 6=Ro 09-1390; 7=IS (methyl *p*-hydroxybenzoate); 8=TMB acid. Injection: 0.84 μg of Ro 09-1390 in 20 μl of 8% acetonitrile solution.

HPLC System for Analysis The high-performance liquid chromatography (HPLC) system for analyzing the aqueous solutions consists of a Jasco Trirotar-V pump, a Jasco Uvidec-100-V detector set at 260 nm, a Rheodyne 7125 injector, a System Instruments 8000A integrator and a Tosoh TSK-gel DEAE-2SW (5 μm, 250×4.6 mm i.d.) anion-exchange column with diethylaminoethyl (DEAE) groups bonded to hydrophilic silica gel. The eluent is a mixture of 5 mM phosphate buffer (pH 7.0) and acetonitrile (92:8) at a flow rate of 0.8 ml/min. A typical chromatogram for the separation of Ro 09-1390 and standard compounds is shown in Fig. 1.

Spectral Instruments The proton nuclear magnetic resonance (^{1}H-NMR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra were taken on a JEOL GSX-400 spectrometer in deuterated solvent with tetramethylsilane as an internal standard. The infrared (IR) spectra were measured in KBr disks on a Jasco IR-810 spectrometer. The fast atom bombardment-mass (FAB-MS) spectra of samples in a mixture of glycerol and dimethylsulfoxide (1:1) were recorded with a JEOL JMX-DX-300 machine. The ultraviolet (UV) spectra were obtained with a Waters photodiode array detector 991J connected to the HPLC system.

Preparation of the Reaction Solution A stock solution of Ro 09-1390 in acetonitrile (10.5 mg/ml) was prepared in a volumetric flask. Aliquots were taken from the stock solution and diluted with buffers in the range of about pH 2 to 12 to get final concentrations of 0.21% (w/v), and the ionic strength was adjusted to $\mu=0.1$ with NaCl. All sample solutions were stored at 37 °C for the prescribed period.

Isolation of Unknown Decomposition Products A solution of about 2 g of Ro 09-1390 in 100 ml of 20% (v/v) acetonitrile solution, adjusted to pH 7.7, was kept at 50 °C for 39 h. This was neutralized to pH 7.0 with 200 ml of 0.1 M phosphate buffer and then concentrated under reduced pressure at 37 °C until the acetonitrile was removed. In the next step, the concentrated solution was extracted with two 165-ml portions of chloroform. The combined chloroform layer was evaporated to dryness under reduced pressure below 37 °C and the residue was dissolved in 25 ml of chloroform again. Then, 400 μl of the chloroform solution was put on a silica gel column (220×10 mm i.d., Wako) for isolation and eluted with a mixture of chloroform and methanol (40:1) at a flow rate of about 0.3 ml/min and fractionated (1 ml/tube). The fractions were run on a silica-gel, thin-layer chromatography (TLC) plate (0.25 mm, E. Merck) with a mixture of chloroform, ethanol and water (40:20:1) as the mobile phase and checked with UV detection at 254 nm. By repeating the chromatographic procedure, we extracted the desired fractions from the gel and about 320 mg of a yellow material was obtained after evaporation.

This material behaved as if it was a single compound: the TLC gave a single spot and the HPLC gave a single sharp peak. However, the NMR spectrum revealed that it was not a single compound, because peaks originated from 5′-methyl group on ^{1}H- and ^{13}C-NMR and similar peaks from 1′—4′ protons on ^{1}H-NMR were detected in the ratio of *ca.* 1:2, respectively. Therefore, it was subjected to further separation. Namely, it was dissolved in 19.2 ml of a mixture of ether, *n*-hexane and acetonitrile (25:10:3), and a 500 μl aliquot of the solution was applied to the HPLC

system consisting of the same instrumentation, except for a normal-phase column (Inertsil PREP-SIL, 10 μm, 250 × 10 mm i.d., GL Sciences) and eluted with a mixture of ether, *n*-hexane and acetonitrile (10:4:1) at a flow rate of 7.0 ml/min.

The material was separated into two peaks and each was fractionated.

Each fraction was evaporated to dryness under reduced pressure; then, 230 mg from the major peak and 90 mg from the minor one were obtained as yellow materials.

Results and Discussion

Degradation Kinetics of Ro 09-1390 Using the above-mentioned HPLC system for analysis in the stability test solutions at various pH values, we determined the contents of Ro 09-1390 by the internal standard method and the ratio of Ro 09-1390 and resultant decomposition products by area percent. Figure 2 shows that the decrease in the concentration of Ro 09-1390 with time followed apparent first order kinetics at pHs ranging from 2 to 12.

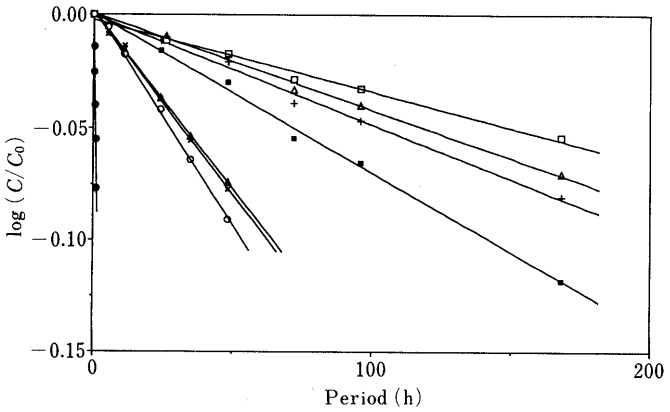

Fig. 2. Apparent First Order Plots for Ro 09-1390 Degradation at Various pH Values, at 37 °C, and μ=0.1

●, pH 2.1; ■, pH 4.4; △, pH 5.0; □, pH 5.6; +, pH 6.6; ▲, pH 8.1; ×, pH 10.4; ○, pH 12.1.

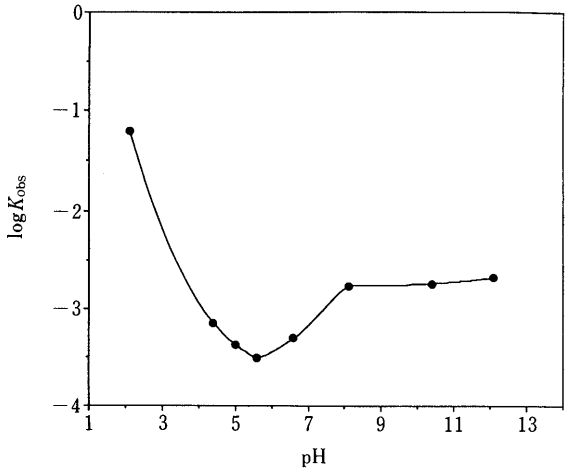

Fig. 3. log K_{obs}–pH Profile for Ro 09-1390 Degradation at 37°C, and μ=0.1

Figure 3 shows a log*K*–pH profile which we constructed by the use of decomposition rate constants obtained from the slopes of the lines shown in Fig. 2. These results show that Ro 09-1390 is unstable at pH 2.1 and is stable at around pH 5.6 even though it forms several decomposition products and its degradation rate does not change in the pH range from 8.1 to 12.1.

The inflection near pH 8 in Fig. 3 should indicate that the dissociation equilibrium of the amide group in Ro 09-1390 (pK_a 8.0) influences the hydrolysis rate.[2]

From these facts, it seemed that the deprotonated type of Ro 09-1390 decomposed exclusively *via* the water-catalysis in the pH range 8.1 to 12.1.[3]

Degradation Pathway The presence of decomposition products, DFCR, DFUR, 3,4,5-trimethoxybenzoic acid

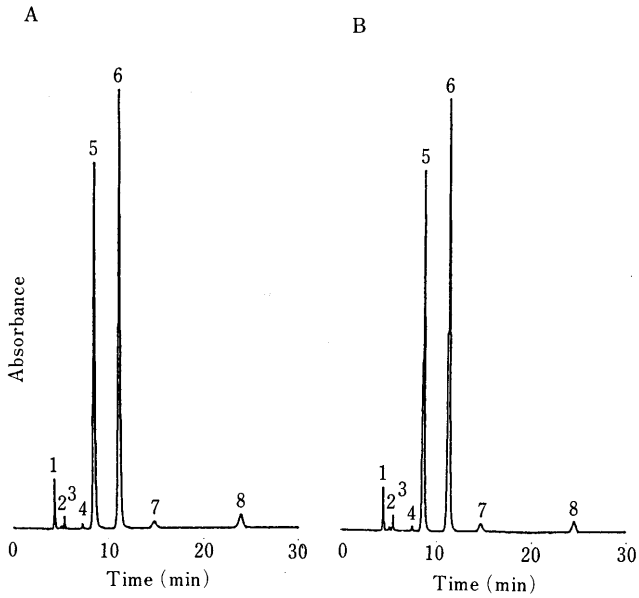

Fig. 4. Chromatograms of the pH 6.6 Reaction Mixture after 168 h (A) and the pH 8.1 Reaction Mixture after 48 h (B) at 37 °C, Respectively (Taken in the Condition for Analysis)

1=DFCR; 2=unknown X; 3=TMB amide; 4=DFUR; 5=Ro 09-1390; 6=I.S. (methyl *p*-hydroxybenzoate); 7=unknown Y; 8=TMB acid.

TABLE I. Ratio of Ro 09-1390 and Its Decomposition Products at Various pHs (μ=0.1, 37 °C)

pH of solution	Reaction time (h)	Compounds (%)[a]						
		Ro 09-1390	DFCR	DFUR	TMB amide	TMB acid	Unknown X	Unknown Y
2.1	1.25	83.4	0	7.0	9.4	0.2	0	0
4.4	168	77.2	1.6	8.0	10.7	2.5	0	0
5.0	168	87.5	2.1	2.7	4.2	3.6	0	0
5.6	168	90.7	2.3	1.0	1.8	3.8	0	0.5
6.6	168	85.7	3.6	0.7	1.5	4.5	0.5	3.5
8.1	48	85.4	3.3	0.8	1.6	3.8	0.7	4.4
10.4	48	86.2	4.2	1.3	2.1	6.2	0	0
12.1	48	84.2	4.7	1.5	2.5	7.2	0	0

a) Listed values are average of four replicate assay values by peak area percentage method by HPLC which are corrected with molar extinction coefficient except for unknown X and Y.

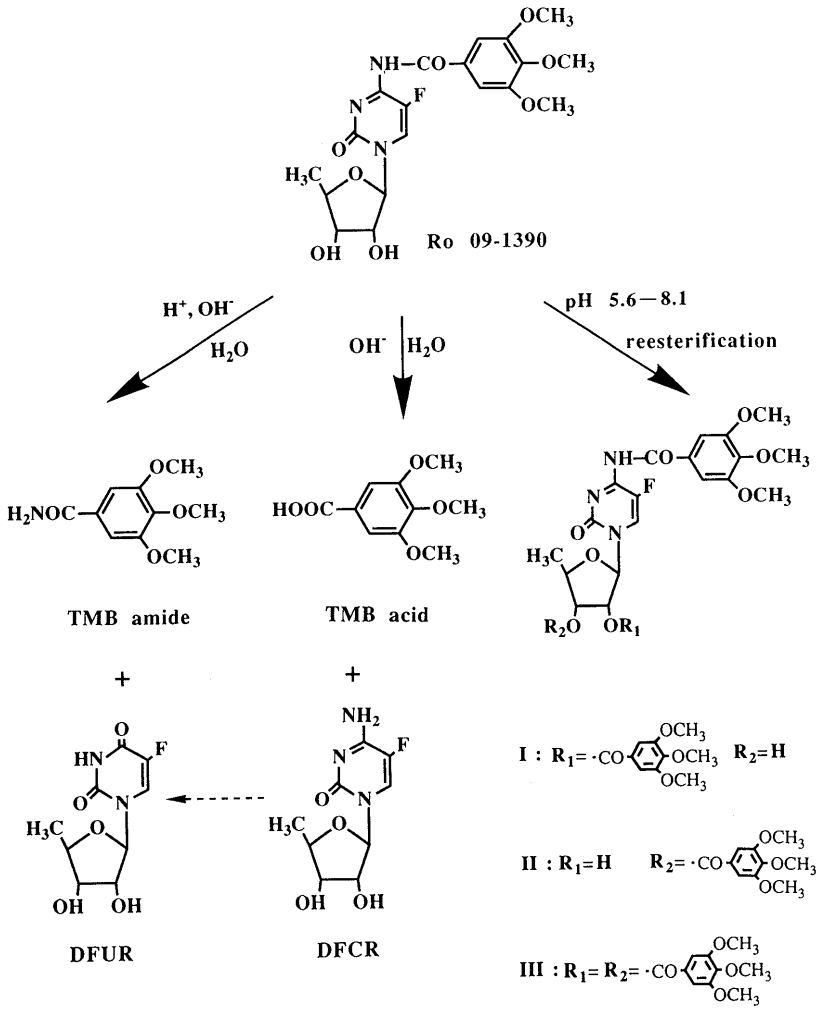

Fig. 5. Pathways of Degradation of Ro 09-1390

(TMB acid) and 3,4,5-trimethoxybenzamide (TMB amide) were confirmed by HPLC, two TLC systems and IR, UV, NMR and MS spectra in comparison with their authentic substances.

Even though only DFUR and TMB amide were observed at pH 2.1, an equal amount of DFCR and DFUR was observed at pH 5.0; and when the pH rose from 5.0 to 12.1 the formation ratio of DFCR increased (Table I). Above pH 4.4, DFUR, DFCR, TMB acid, and TMB amide were observed. At pHs 6.6 and 8.1, new degradation compounds were formed (Fig. 4). No appreciable effect on the degradation of Ro 09-1390 at these pH values was observed for any buffer species used in this study.

Identification of Unknown Decomposition Products The molecular weights of unknown compounds were confirmed to be both *m/z* 634 by the FAB-MS spectra, and from the NMR spectra they were assigned to be compound I, 5′-deoxy-5-fluoro-N^4,$O^{2'}$-bis(3,4,5-trimethoxybenzoyl)citidine for the minor component and compound II, 5′-deoxy-5-fluoro-N^4,$O^{3'}$-bis(3,4,5-trimethoxybenzoyl)cytidine for the major component (Fig. 5).

We found that peak 7 in Fig. 4 contained these two compounds in the ratio of *ca.* 1 : 2.3.

In addition, another substance was eluted from the silica gel column used for isolation before the mixture of compounds I and II were eluted. From this band, 8 mg was obtained, which was different from peak 2 in Fig. 4 because this compound was not eluted out by the HPLC for analysis. Its molecular weight was shown to be *m/z* 827 by the FAB-MS spectrum and was identified to be compound III, 5′-deoxy-5-fluoro-N^4,$O^{2'}$,$O^{3'}$-tris(3,4,5-trimethoxybenzoyl)cytidine by NMR (Fig. 5) but its existence ratio is very small.

It is generally well-known that H^+ catalyzes esterification between alcohol and carboxylic acid. Therefore, the reaction hardly occurs in a relatively strong alkaline region where OH^- is predominant, while a minimal amount of H^+ to catalyze is enough in the reaction.

On the other hand, since esterification is an equilibrium reaction, it does not proceed without sufficient TMB acid as the reactant even at the region where necessarily sufficient H^+ exist. Accordingly, the reesterification was thought to occur only in the pH range from 5.6 to 8.1.

Conclusion

In the stability test of Ro 09-1390 in buffered solutions at 37 °C, it was found that Ro 09-1390 was stable at about pH 5.6 in spite of forming several decomposition products and its degradation followed apparent first order kinetics.

Moreover, it was found that at the pHs of 2.1 and 4.4, Ro 09-1390 degrade mainly to DFUR and TMB amide, and at the pHs of 5.0, 10.4, 12.1 it degraded to the above

two compounds as well as DFCR and TMB acid, but in the neutral region, at least three compounds (I, II and III) were produced in addition to the four main products as shown in Fig. 5. These compounds resulted from the reesterification between the 2′- and/or 3′-hydroxy group of Ro 09-1390 and TMB acid (product of Ro 09-1390 degradation).

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References and Notes

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