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Degradation Kinetics of Fluorouracil-Acetic-Acid-Dextran Conjugate in Aqueous Solution

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ABSTRACT The degradation kinetics of fluorouracil-acetic-acid-dextran conjugate (FUAC-dextran) was investigated in various buffer solutions with different pH value and physiological saline solution at 60°C and 37°C, respectively. The hydrolytic reaction displayed pseudo-first-order degradation kinetics. Hydrolytic rate constant obtained was the function of pH value and independent of species of buffering agents. The smallest rate constant was observed at pH round 3.00. The activation energy of the hydrolytic reaction was estimated from Arrhenius equation as 88.73 ± 6.00 kJ·mol^{-1}. The special base catalytic degradation of the conjugate was observed from acidic to slight alkaline condition and the special base catalytic rate constants were calculated. The conjugate was more stable in physiological saline than that in buffer solution at pH 7.00 or 9.00 at 37°C. The results revealed that the conjugate was stable in acidic condition and will degrade in alkaline condition.

KEYWORDS Fluorouracil, Dextran, Degradation kinetics, Chemical stability

With development of a drug delivery system, macromolecular prodrugs have been gained great attention due to their application on either passive drug targeting or controlled release (Hoste et al., 2004). As a natural macromolecular, dextran has been investigated as the most promising carrier for delivery of drugs (Mehvar, 2000). 5-Fluorouracil (5-FU) is widely used for the treatment of various kinds of cancer including colon cancer. The antitumor mechanism of 5-FU was ascribed to 5-FU inhibits thymidylate synthetase and depletes dTTP and forms nucleotides that can be incorporated into RNA and DNA and induces p53-dependent apoptosis. To achieve good therapeutic effects the concentration of 5-FU should be kept at low level and sustained released (Longley et al., 2003). However as an antimetabolic agent 5-FU has also been found to cause serious side effects, many efforts have been made to decrease its side effects and increase its therapeutic index. Among those efforts, fluorouracil acetic acid was synthesized (Tada, 1975) with a moderate antitumor ability and decreased its side effect, it was widely used in the macromolecular prodrug (polymer prodrug) containing fluorouracil (Chung et al., 1991; Mdró & Pató, 1990; Ouchi et al., 1998; Kang et al., 2002).

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Oral colon-specific drug delivery can be achieved in different ways, including pH or enzymes-dependent approaches utilizing the changes in pH or enzymes along the GI tract. Dextran conjugate has also been used for oral colon-targeted drug delivery systems for many years (Chourasia & Jain, 2004). After oral administration of the dextran conjugates dextran backbone can be specifically degraded by enzymes of the colonic bacteria, and its chemical bond between the parent drug and dextran was broken. Dextran ester prodrug was confirmed as stable in acidic condition and tended to release parent drug in neutral and weak alkaline condition (Larsen, 1989b). So the dextran prodrug can be used for colon-specific drug release in pH and enzymes dependent method. Several dextran ester prodrugs have been prepared and characterized for colon-specific drug release (Lee et al. 2001; Pang et al. 2002; Chourasia & Jain, 2004) after oral administration.

The fluorouracil-acetic-acid–dextran conjugate was synthesized from 5-FU and dextran (M = 70,000) (Hao et al., 2006). The conjugate was confirmed by ultraviolet spectrum (UV), infrared spectrum (IR), and nuclear magnetic resonance spectrum (NMR) with the degree of substitution (DS) (Lee et al., 2001) of fluorouracil acetic acid (FUAC) 15.1% (Hao et al., 2006). In this report, the chemical stability of the conjugate in different buffer solutions and physiological saline solution was investigated. The investigation is beneficial to the evaluation of the conjugate in oral colon-targeting drug delivery of 5-FU or FUAC in pH dependent manner.

MATERIALS AND METHODS

Fluorouracil was purchased from Nantong Jinghua Pharmaceutical Co. Ltd, Jiangsu Province, China. Dextran (T-70) was purchased from Pharmacia-Amersham, Uppsala, Sweden. Fluorouracil acetic acid was synthesized as reported (Tada, 1975). The fluorouracil-acetic-acid-dextran conjugate was synthesized by the esterification of fluorouracil acetic acid with dextran (Hao et al., 2006). All other solvents and chemicals were obtained from commercial sources and used without further purification.

HPLC Analysis of Fluorouracil Acetic Acid (FUAC) and Fluorouracil

The concentration of FU was determined by HPLC (Nassim et al., 2002) and concentration of FUAC was also determined simultaneously. The HPLC system consisted of a pump (LC-10ATvp, Shimadzu, Japan) and a 250 × 4.6 mm, 5 μm kromasil C_{18} column (Elete, Dalian, China) maintained at room temperature, protected by C_{18} guard column filled with the same materials and a UV detector (model SPD-10Avp, Shimadzu, Japan). The mobile phase consisted of 50 mM KH_2PO_4 buffer solution filtered through 0.45 μm membrane filter before use. The mobile phase was eluted at a flow rate of 1.0 mL/min and monitored by measuring the absorbance at 271 nm at a sensitivity of AUFS 0.002. The retention time of FUAC and fluorouracil was about 4.6 min and 8.5 min, respectively. ANASTAR software was employed for the data analysis.

Hydrolytic Degradation of the Conjugate in Buffer Solution

The buffer solution used in the investigation was as following: hydrochloric acid (pH 1.24), citrate (pH 2.96), acetate (pH 4.95), phosphate (pH 6.80), borate (pH 8.75). The concentration of all buffers was maintained 0.1 M and adjusted to an ionic strength of 0.5 M by the addition of NaCI. The stock solution of the conjugate was added to the preheated buffer solution to give a final concentration of 50 μM (based on FUAC). For the conjugate the concentration of fluorouracil acetic acid was calculated as follows:

$$\begin{aligned} 50\mu\mathrm{M} &= 50\times10^{-6}\times \mathrm{M}_{(\mathrm{FUAC})}(\mathrm{mg}_{(FUAC)}/ml) \\ &= \frac{50\times10^{-6}\times M_{(FUAC)}}{DS}(mg_{(conjugate)}/ml) \\ &= \frac{50\times10^{-6}\times188}{0.151}(mg_{(conjugate)}/ml) \\ &= 0.0623 mg_{(conjugate)}/ml \end{aligned} \quad (1)$$

The buffer solution was incubated at 37°C (or 60°C) for 8 h for buffers with pH below 7.00 and for 4.0 h with pH above 7.00. A 0.2 mL portion of the reaction mixture was removed at 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0 h, respectively, for buffers with pH below 7.00 and 0.125, 0.25, 0.375, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0 h respectively, for the buffers with pH below 7.00 and filtered through 0.45 μm membrane filter; 20 μl of filtrate was injected into HPLC for analysis.

Buffer Effect

To eliminate the effect of the species of buffer agents on the degradation of the conjugate, the chemical stability of the conjugate was also investigated in 0.1 M phosphate buffer solution at different pH value. The buffer solutions were selected with the pH value of 1.96, 3.01, 5.66, 6.84, 8.62, respectively. The concentration of all buffer solution was maintained 0.1 M and free of NaCI avoiding the effect of the ionic strength. The method of the stability investigation was the same as above-mentioned.

Temperature Effect

The temperature of 60°C and 37°C was selected to investigate the chemical stability at elevated temperature and physiological temperature. The method of the stability investigation was the same as above-mentioned.

Hydrolytic Degradation in Physiological Saline Solution

The chemical stability of the conjugates in the physiological condition was investigated with the mentioned method, only replacing the buffer solution with physiological saline solution.

RESULT AND DISCUSSION

Chemistry

The FUAC-dextran conjugate was synthesized from the fluorouracil acetic acid and dextran through a ester bond. The ester bond was broken down during the hydrolytic degradation of the conjugates and FUAC was released as shown in Fig. 1.

The observed hydrolytic rate constant (k_{obs}) was defined as following:

$$k_{obs} = k_{ester} + k_{FUAC} \tag{2}$$

where k_{obs} is the overall hydrolytic rate constant of the conjugate, k_{ester} is the hydrolytic rate constant of the conjugate, K_{FUAC} is the hydrolytic rate constant of FUAC. Theoretically the hydrolytic degradation of the conjugate will lead to the formation of both FUAC and FU. But when the conjugate hydrolyzed in buffer solution and physiological saline solution, only FUAC was obtained throughout the investigation and

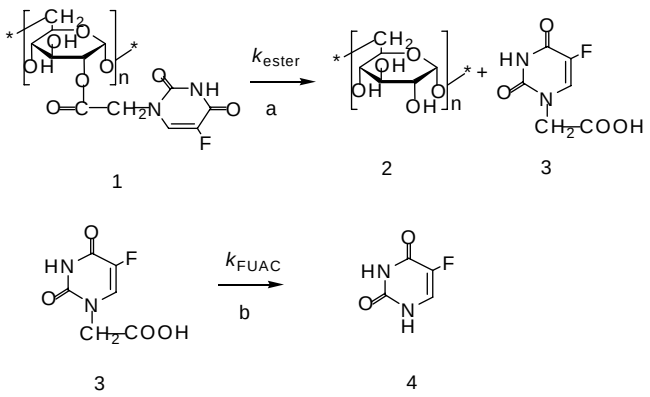

FIGURE 1 **The Hydrolysis of FUAC-Dextran Conjugate. 1: FUAC-dextran conjugate, 2: Dextran, 3: FUAC, 4: Fluorouracil.**

no detectable FU was observed. The reason may be that FUAC was stable in the buffer solution (Chung et al., 1991). Therefore Eq. 2 was simplified as the following Eq. 3:

$$k_{obs} = k_{ester} \tag{3}$$

The hydrolytic degradation of the conjugate displayed the pseudo-first-order kinetics. The pseudo-first-order rate constants were calculated from the concentration of the conjugate at different time by Eq. 4.

$$\ln(c_0 - c) = -k_{obs}t + \ln c_0 \tag{4}$$

where c_0 is the total concentration of conjugate in the solution (based on FUAC), c is the consumed concentration of the conjugate in hydrolytic reaction at time t (based on FUAC), t is the time of the hydrolysis. The consumed amount of conjugated FUAC of the conjugate was equal to the concentration of free FUAC in the reaction mixture. Therefore the parameter c_0 and c were both calculated based on the concentration of FUAC. A straight line was constructed from the value of $ln\ (c_0 - c)$ versus the time of hydrolysis (t) and a typical line was conducted in Fig. 2. The pseudo-first-order rate constant was the negative value of the slope of the $ln\ (c_0 - c)$ – time line.

Because the pseudo-first-order rate constant is a function of temperature, according to the Arrhenius equation, the Arrhenius observed activation energy for the hydrolysis of the conjugate could be calculated

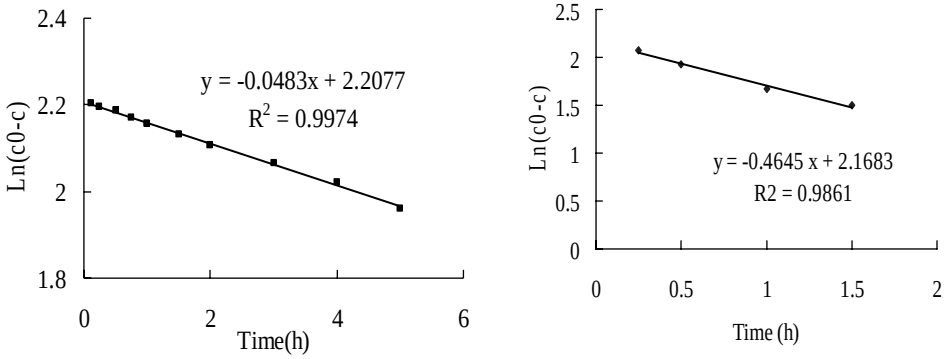

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FIGURE 2 **Semi-Logarithm Plot of the Content of the Residual Conjugated FUAC Versus Time in 0.1M Phosphate Buffer Solution of pH 6.84. A: 37°C, B: 60°C.**

from the rate constant at 37°C and 60°C respectively, according to Eq. 5.

$$\ln \frac{k_{T2}}{k_{T1}} = -\frac{E_{obs}}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \tag{5}$$

Degradation Kinetics

The different buffer solution was selected because the same buffer solutions were used for the investigation of the hydrolytic degradation of other dextran ester (McLeod et al., 1993). The concentration of buffer and the total ionic strength were the same as the previous investigation (McLeod et al., 1993). So the concentration and ion strength of the buffer solution had no significant influence on the hydrolysis rates, the rate constant was only the function of pH value and temperature. The pseudo-first-order rate constant was calculated as the above-mentioned method and was shown in Table 1 and Table 2.

Stability profiles of the conjugate in buffer solution with varied pH at 37°C and 60°C was shown in Fig. 3 and Fig.4. The semi-logarithm plot of the rate constant versus pH value was constructed in Figs. 3A and 4A. From Figs. 3A and 4A, the lowest log (k_{obs}) was observed at pH of 2.96 and 3.01, which indicated that the conjugate was more stable in buffer solution with pH around 3.0. The results also revealed that hydrolysis of conjugate accelerate gradually with the pH value increasing from 2.96 to 8.75. This result was consistent with that of hydrolytic degradation of the glucocorticoid-dextran esters (McLeod et al., 1993). The reason may be the formation of the intramolecular hydrogen bond in acid condition between the ester bond and the adjacent hydroxyl group which conflict the hydrolytic degradation (Larsen, 1989a). With the pH increasing the hydrogen bond was destroyed and the hydrolytic rate constant increased.

Buffer Effect

To eliminate the effects of the kinds of buffer solution and ionic strength on the hydrolysis, the hydrolytic degradation of the conjugate was also

TABLE 1 **Kinetic Data for Hydrolytic Degradation of Conjugate in Buffer Solution (μ = 0.5M)**

		37°C		60°C	
Buffer solution	pH value	K_{obs}/h^{-1}	$t_{½}$/h	K_{obs}/h^{-1}	$t_{½}$/h
Hydrochloric acid	1.24	1.6×10^{-3}	430	9.30×10^{-3}	74.5
Citrate	2.96	7.0×10^{-4}	990	1.50×10^{-3}	462
Acetate	4.95	9.0×10^{-3}	770	1.52×10^{-2}	45.6
Phosphate	6.80	4.9×10^{-2}	14.1	0.391	1.63
Borate	8.75	0.797	0.87	4.21	0.165

TABLE 2 Kinetic Data for Hydrolytic Degradation of Conjugate in 0.1 M Phosphate Buffer Solution at Different pH Value

	37°C		60°C	
pH value	K_{obs}/h^{-1}	$t_{1/2}/h$	K_{obs}/h^{-1}	$t_{1/2}/h$
1.96	5.0×10^{-4}	1.39×10^{3}	4.7×10^{-3}	147
3.01	5.0×10^{-4}	1.39×10^{3}	6.9×10^{-3}	100
5.66	7.1×10^{-3}	97.6	7.57×10^{-2}	9.16
6.84	4.83×10^{-2}	14.3	0.465	1.49
8.62	0.388	1.78	4.73	0.147

investigated in 0.1 M phosphate buffer solution at different pH value and free of salt. The hydrolysis constant of the conjugate was the function of pH value and temperature. The rate constants were shown in Table 2. The semi-logarithm plot of the rate constant versus pH value was constructed in Fig. 4A. From Fig. 4A the lowest log (k_{obs}) was observed at pH of 3.01. The result indicated that the conjugated was more stable in buffer solution (0.1 M) at pH of 3.01. The result was consistent with that obtained in the above buffer solution (μ = 0.5 M) and that of other dextran ester degradation. From the results in Table 2 and Table 3, there is no obvious difference observed in the degradation kinetics of the conjugate hydrolysis.

pH Value Effect

The hydrolytic rate constant increased with the pH value increasing from about 3.0 to about 9.0. From the results shown in Fig. 3 and Fig.4, the pH-rate profiles exhibited straight-line proportion at pH range from

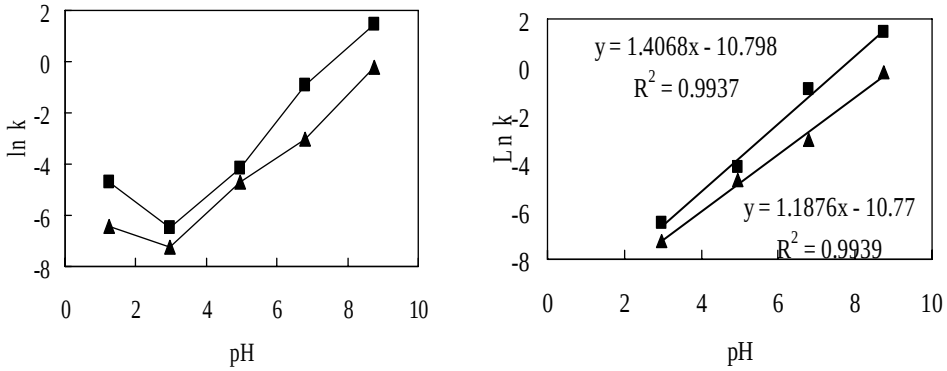

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FIGURE 3 Plots of Rate Constant Versus pH Value in Different Kinds of Buffer Solution (μ = 0.5 M). ■, at 60°C. ▲, at 37°C. Buffer Solution(pH Value): Hydrochloric Acid (pH 1.24), Citrate (pH 2.96), Acetate (pH 4.95), Phosphate (pH 6.80), Borate (pH 8.75). A: ln k ~ pH Profiles, B: Linearization of the Data in A.

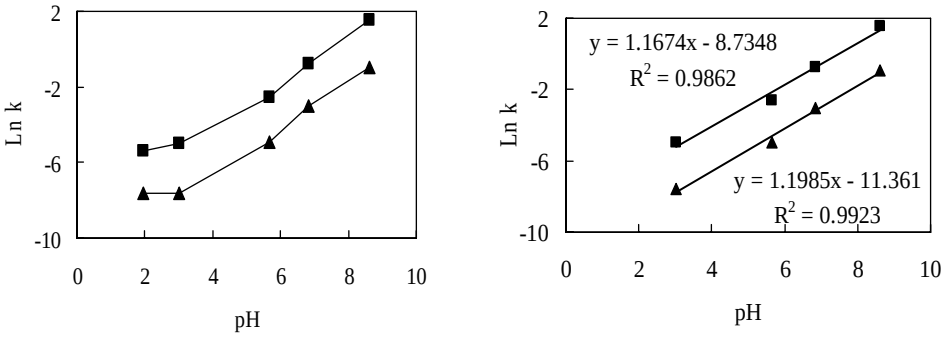

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FIGURE 4 Plots of Rate Constant Versus pH Value in 0.1M Phosphate Buffer Solution. ■, at 60°C. ▲, at 37°C. pH Value of 1.96, 3.01, 5.66, 6.84, 8.62. A: ln k ~ pH Profiles, B: Linearization of the Data in A.

TABLE 3 Special Base-Catalytic Rate Constants

Solution	37°C K_{OH} ($L \cdot mol^{-1} \cdot h^{-1}$)	60°C K_{OH} ($L \cdot mol^{-1} \cdot h^{-1}$)
0.1 M phosphate	3.71×10^4	9.86×10^4
Buffer (μ = 0.5 M)	5.79×10^4	7.55×10^4

2.96 to 8.75 in Fig. 3B and from 3.01 to 8.62 in Fig. 4B with a slope close to unity.

This phenomena denoted that there was the special base-catalytic hydrolysis within pH range investigated. Thus the hydrolytic rate of the conjugate was proportional to the hydroxide ion activity, α_{OH}, in accordance with the following rate expression:

$$k_{obs} = k_o + k_{OH}\alpha_{OH} \tag{6}$$

where k_0 is water catalysis rate constant, k_{OH} is the second-order rate constant for special base-catalyzed hydrolysis, α_{OH} was calculated according to Harned and Hamer (1933).

$$\log \alpha_{OH} = pH - 13.62 \ (37^\circ C) \tag{7}$$

$$\log \alpha_{OH} = pH - 13.02 \ (60^\circ C) \tag{8}$$

The values of k_{OH} calculated were listed in Table 3. The special base catalysis on the hydrolysis was usually observed in the neutral and weak alkaline solution in the previous studies. But in this report, the special base-catalyzed hydrolysis of the conjugate was observed at pH range of from acidic to slight alkaline solution. Compared with the hydrolytic rate constant of the conjugate in buffer solution at pH 8.75 ($\mu = 0.5$) ($k_{obs} = 0.797\ h^{-1}$) with that of the naproxen dextran ester in buffer solution at pH 8.78 ($\mu = 0.5$) ($k_{obs} = 0.072\ h^{-1}$) (Larsen, 1989b), it can be found that the hydrolytic rate constant of the FUAC-dextran conjugate was ten times larger than that of naproxen-dextran ester. The reason may be that naproxen was more lipophilic than FUAC. The conjugated FUAC was more likely to be attacked by hydroxide ion.

TABLE 4 The Kinetic Data of Hydrolytic Degradation of the Conjugate in Physiological Saline Solution (n = 3)

Solution	Physiological saline
k (h^{-1}) 37°C	0.0391
k (h^{-1}) 60°C	0.0674

From the hydrolytic rate constants in 0.1 M phosphate buffer solution at 37°C and 60°C listed in Table 2, the observed activation energy (E_{obs}) were calculated. The E_{obs} was calculated according to Eq. 5. The obtained E_{obs} was 88.73 ± 6.00 $kJ \cdot mol^{-1}$ (n = 5). This was indicative for the breakage of a sigma bond, proving once again that FUAC was covalently bonded to the polymeric backbone.

Temperature Effect

The hydrolytic rate constant increased with the temperature increasing from 37°C and 60°C as shown in Table 1, Table 2, Table 3, and Table 4. It was observed that, as other ester hydrolysis reaction, the hydrolysis of the conjugate accelerate with increase of reaction temperature.

Chemical Stability in Physiological Saline Solution

The hydrolytic degradation in physiological saline solution was also investigated and the kinetic data were shown in Table 4. From the results we can come to the conclusion that the conjugate was more stable in physiological saline solution (0.154 M NaCI) than that in 0.1 M phosphate buffer solution at pH 6.84. The reason may be that there is less buffer capacity in physiological saline solution than that in phosphate buffer solution and there was no catalysis of the phosphate ion in physiological saline solution. Therefore the pseudo-first-order rate constant in physiological saline solution was smaller than that in 0.1 M phosphate buffer solution at same pH value.

CONCLUSION

The degradation kinetics for the dextran conjugate in buffer solution revealed that the conjugate was more stable at pH 3.00 around. The hydrolysis rate constant of the conjugate was the function of pH value and independent of kinds of buffer solution. The special base-catalytic hydrolysis of the conjugate in buffer

solution was observed at pH from acidic to slight alkaline. The stability investigation also revealed that the conjugate was more stable in physiological saline solution than that in 0.1 M phosphate buffer at 37°C.

From the investigation we can anticipate that after orally administration of the conjugate, fluorouracil acetic acid would keep its chemical bond to dextran backbone through the upper gastrointestinal tract and would be slowly released down along the gastrointestinal tract because of the pH value change along the gastrointestinal tract. So the dextran conjugate would be utilized to achieve colon-specific drug delivery for the treatment of local carcinoma of the large intestine in pH sensitive and enzymes dependent method (Chourasia & Jain, 2004, Larsen et al., 1989). The ability of the conjugate colon-specific drug delivery was further evaluated in vivo.

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