Synthesis and Properties of the Monoesters of 5-Fluorouridine with 4-Carboxybutyric Acid and Their Conjugates with Chitosan

Hiraku ONISHI,* Yoshiharu MACHIDA, and Tsuneji NAGAI

Faculty of Pharmaceutical Sciences, Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo 142, Japan.
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The mixture of 2′-*O*-(4-carboxybutyryl)-5-fluorouridine (2′-glu-FUR) and 3′-*O*-(4-carboxybutyryl)-5-fluorouridine (3′-glu-FUR), named (glu-FUR(I)), and 5′-*O*-(4-carboxybutyryl)-5-fluorouridine (5′-glu-FUR), named glu-FUR(II), were easily obtained from the reaction of 5-fluorouridine (FUR) with glutaric anhydride. In addition, the chitosan-glu-FUR(I) conjugate and the chitosan-glu-FUR(II) conjugate were prepared. The obtained compounds were investigated regarding their *in vitro* characteristics. Equilibrium between 2′-glu-FUR and 3′-glu-FUR was suggested to be attained quickly in a 1/15 M phosphate buffer of pH 7.4. Glu-FUR(II) was found to be introduced into chitosan more easily than glu-FUR(I). For every compound, chemical hydrolysis was accelerated at weakly basic pH and a gradual regeneration of FUR was observed at physiological pH.

Key words 5-fluorouridine; (4-carboxybutyryl)-5-fluorouridine; conjugate; chemical hydrolysis; chitosan; equilibrium

5-Fluorouridine (FUR) is one of the antitumor fluorinated pyrimidines, which are considered to act as antimetabolic agents. FUR is known to show a marked antitumor effect at a lower dose than 5-fluorouracil (FU) or 2′-deoxy-5-fluorouridine (FUdR).[1-3] As an effective macromolecular prodrug of FUR, Tsukagoshi stated that Divema-FUR exhibited gradual drug release and good antitumor effect at a single low dose.[3] Therefore, the macromolecular prodrug of FUR is proposed to be useful for improving or modifying the efficacy of FUR. In this study, 4-carboxybutyrylated FUR (glu-FUR) was synthesized in order to introduce FUR into various macromolecules with amino groups. Further, chitosan was used as a macromolecular carrier because it was biocompatible[4,5] and biodegradable.[6-10] The synthesis of glu-FUR and chitosan-glu-FUR conjugate was carried out and their physicochemical characteristics, mainly their stabilities in buffered solutions of various pHs, were investigated.

Experimental

Materials FUR was purchased from Sigma Chemical Co. Glutaric anhydride was obtained from Wako Pure Chemical Industries, Ltd. Chitosan with a molecular weight of 8×10^5 (Marine Dew PC-100) was supplied by Ajinomoto Co., Inc. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) was obtained from Nacalai Tesque, Inc. All other chemicals were of reagent grade.

General Procedure Ultraviolet (UV) absorption spectra were measured using a Ubest-30 UV/VIS spectrophotometer (Japan Spectroscopic Co., Ltd.). Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken on a JNM-GX 400 spectrometer (JEOL). A Mini Chemi Pump (Nihon Seimitsu Kagaku) with an octadecyl silica (ODS) column, NEO PACK $5C_{18}$ (20 × 250 mm) (Nishio), and an SPD-2A detector (Shimadzu) (272 nm) was used as the high performance liquid chromatography (HPLC) system for preparation (HPLC(P)). For an HPLC system for analysis (HPLC(A)), an LC-3A apparatus (Shimadzu) with an ODS column, NEO PACK $5C_{18}$ (4.6 × 150 mm) (Nishio), and an SPD-6A detector (Shimazdu) (272 nm) was used. Mixtures of 0.2% aqueous acetic acid–methanol of 5 : 1 (v/v) and 3 : 1 (v/v) were used as mobile phases for HPLC(P) and HPLC(A), respectively.

Synthesis of the Monoesters of FUR with 4-Carboxybutyric Acid FUR (1.573 g, 6.00 mmol) and glutaric anhydride (0.760 g, 6.67 mmol) were added to 15 ml of anhydrous pyridine, and the mixture was stirred at room temperature for 5 d. After evaporation of the solvent, the residue was dissolved in a mixture of 0.2% aqueous acetic acid–methanol (5 : 1, v/v) and separated by HPLC(P). Three main peaks eluted, except for a FUR peak, were collected separately. Their solvent was evaporated to dryness at 40 °C. A quick acyl migration[11] was observed between the first peak compound and the second one. The first and second peaks in HPLC(A) were identified from NMR and HPLC as follows: The residue obtained by evaporation of the mixture of the first and second peaks was dissolved in D_2O. For analysis of the sample in HPLC(A), the ratio of the first peak area to the second one was approximately 1 : 2, both before and after NMR measurement. The NMR spectrum of the sample showed that it was a mixture of 2′-*O*-(4-carboxybutyryl)-FUR (2′-glu-FUR) and 3′-*O*-(4-carboxybutyryl)-FUR (3′-glu-FUR) with the molar ratio of approximately 1 : 2. Namely, ^{1}H-NMR (D_2O) δ: 1.89—1.99 (2H, m, $COCH_2C\underline{H}_2CH_2CO$), 2.43—2.60 (4H, m, $COC\underline{H}_2CH_2C\underline{H}_2CO$), 3.81—3.97 (2H, m, $C_{5'}H_2$), 4.14—4.15 (0.3H, m, $C_{4'}H$), 4.30—4.32 (0.7H, m, $C_{4'}H$), 4.44—4.47 (0.3H, m, $C_{3'}H$), 4.53—4.56 (0.7H, m, $C_{2'}H$), 5.19—5.22 (0.7H, m, $C_{3'}H$), 5.33—5.35 (0.3H, m, $C_{2'}H$), 5.99 (0.7H, d, J = 5.9 Hz, $C_{1'}H$), 6.03 (0.3H, d, J = 4.2 Hz, $C_{1'}H$), 8.07 (1H, d, J = 6.4 Hz, C_5H). From the result of these HPLC and NMR measurements, the first and second peaks were identified as 2′-glu-FUR and 3′-glu-FUR, respectively. The first product, named glu-FUR(I), was obtained as a mixture of 2′-glu-FUR and 3′-glu-FUR. The third peak compound did not show an acyl migration in the preparation and was identified as 5′-*O*-(4-carboxybutyryl)-FUR (5′-glu-FUR) based on the NMR measurement. ^{1}H-NMR (CD_3OD) δ: 1.87—1.97 (2H, m, $COCH_2C\underline{H}_2CH_2CO$), 2.33—2.49 (4H, m, $COC\underline{H}_2CH_2C\underline{H}_2CO$), 4.09—4.11 (1H, m, $C_{3'}H$), 4.14-4.18 (2H, m, $C_{2'}H + C_{4'}H$), 4.31—4.43 (2H, m, $C_{5'}H_2$), 5.80 (1H, d, J = 3.6 Hz, $C_{1'}H$), 7.89 (1H, d, J = 6.8 Hz, C_5H). 5′-Glu-FUR was named glu-FUR(II). The obtained amounts of glu-FUR(I) and glu-FUR(II) were 0.26 and 0.19 g, respectively.

An acyl migration rate from 2′-glu-FUR to 3′-glu-FUR (or from 3′-glu-FUR to 2′-glu-FUR) was checked in some solution systems as follows: The eluted fraction of 2′-glu-FUR (or 3′-glu-FUR) in HPLC(A) and the mobile phase (HPLC(A)) were mixed at the ratio of 1 : 2 (v/v). In addition, the eluted fraction of 2′-glu-FUR (or 3′-glu-FUR), water and a 1/5 M phosphate buffer of pH 5 or 7.4 (μ (= ionic strength) = 0.9, adjusted by addition of NaCl) were mixed at the ratio of 1 : 1 : 1 (v/v/v). These mixtures were incubated at 37 °C and aliquot samples were withdrawn immediately after mixing and after 10, 20, 30 (and 45) min incubations. The samples were analyzed on FUR, 2′-glu-FUR and 3′-glu-FUR by HPLC(A).

Regeneration of FUR from Glu-FUR(I) and Glu-FUR(II) The stability of glu-FUR(I) and glu-FUR(II) and the regeneration of FUR from each were investigated by incubating them in physiological pH (pH = 7.4) and neighboring pHs (pH = 5, 6, 7 and 9) at 37 °C, with 1/15 M phosphate buffers of pH 5, 6, 7, 7.4 and 9 (μ = 0.3, adjusted by addition of NaCl) being used as incubation media. The amounts of glu-FUR(I) and glu-FUR(II) used were determined spectrophotometrically from the UV absorption at 272 nm of the solution in 1/15 M phosphate buffer of pH 6 (μ = 0.3, adjusted by addition of NaCl). Aliquot samples were withdrawn immediately after the start of incubation and after incubation for 3, 7, 24 and 48 h. The regenerated FUR and remaining glu-FUR(I)

* To whom correspondence should be addressed.

and glu-FUR(II) in the aliquots were analyzed by HPLC(A).

Preparation of the Conjugates Chitosan (70 mg) was added to 17.5 ml of water and dissolved by lowering the mixture pH to 3—4 using 1 N aqueous HCl. Next, after the solution pH was adjusted to 6 by the addition of 1 N aqueous NaOH, glu-FUR(I) (70 mg) and EDC (3.5 g) were added to the solution. After the mixture was stirred for 48 h, it was filtered using an ultrafilter unit, USY-5 (Advantec). The residue was washed thoroughly with 1 M aqueous NaCl and subsequently with water. Then, the residue was solidified by the addition of acetone, and subsequently washed with a mixture of acetone and water (4 : 1, v/v). Finally, by drying the residue *in vacuo*, the chitosan-glu-FUR(I) conjugate (chi-glu-FUR(I)) was obtained. For the chitosan-glu-FUR(II) conjugate (chi-glu-FUR(II)), chitosan (100 mg) was dissolved in 25 ml of water in the same way as above, then the solution pH was adjusted to 6 by addition of 1 N aqueous HCl and 1 N aqueous NaOH, and subsequently glu-FUR(II) (100 mg) and EDC (5.0 g) were added. The following separation and purification were carried out in the same way as for chi-glu-FUR(I). The drug content of each conjugate was investigated by alkaline hydrolysis, which was executed by incubating the conjugates in 1 N aqueous NaOH at 40 °C. Aliquot samples were withdrawn after 10, 20 and 30 min incubations. Each aliquot (0.3 ml) was neutralized by the addition of 1 N aqueous HCl (0.3 ml) and the mixture was mixed with 1/10 M phosphate buffer of pH 5 (3 ml) and filtered using USY-5. The filtrate was analyzed spectrophotometrically at 272 nm and injected on HPLC(A). FUR was almost stable during the incubation in 1 N aqueous NaOH at 40 °C for 30 min. The plateau of the released drug was used for the drug content.

Regeneration of FUR from Chi-glu-FUR(I) and Chi-glu-FUR(II) The regeneration of FUR and glu-FUR(I) from chi-glu-FUR(I) and that of FUR and glu-FUR(II) from chi-glu-FUR(II) were investigated by incubating the conjugate in physiological pH (pH = 7.4) and weakly acidic and basic pHs (pH = 5 and 9) at 37 °C, when 1/15 M phosphate buffers of pH 5, 7.4 and 9 (μ = 0.3, adjusted by the addition of NaCl) were used as incubation media. Aliquot samples were withdrawn immediately after the start of incubation and after 3, 7, 24 and 48 h incubations. FUR and glu-FUR(I) or glu-FUR(II) in the aliquots were analyzed by HPLC(A).

Results and Discussion

Glu-FUR(I) and Glu-FUR(II) The chemical structures of FUR and the monoesters of FUR with 4-carboxybutyric acid are shown in Fig. 1. Glu-FUR(I) and glu-FUR(II) were obtained in yields of 12 and 8%, respectively. FUR, 2′-glu-FUR, 3′-glu-FUR and 5′-glu-FUR were eluted in that order in HPLC(A).

Figure 2 shows acyl migration in some solution systems.

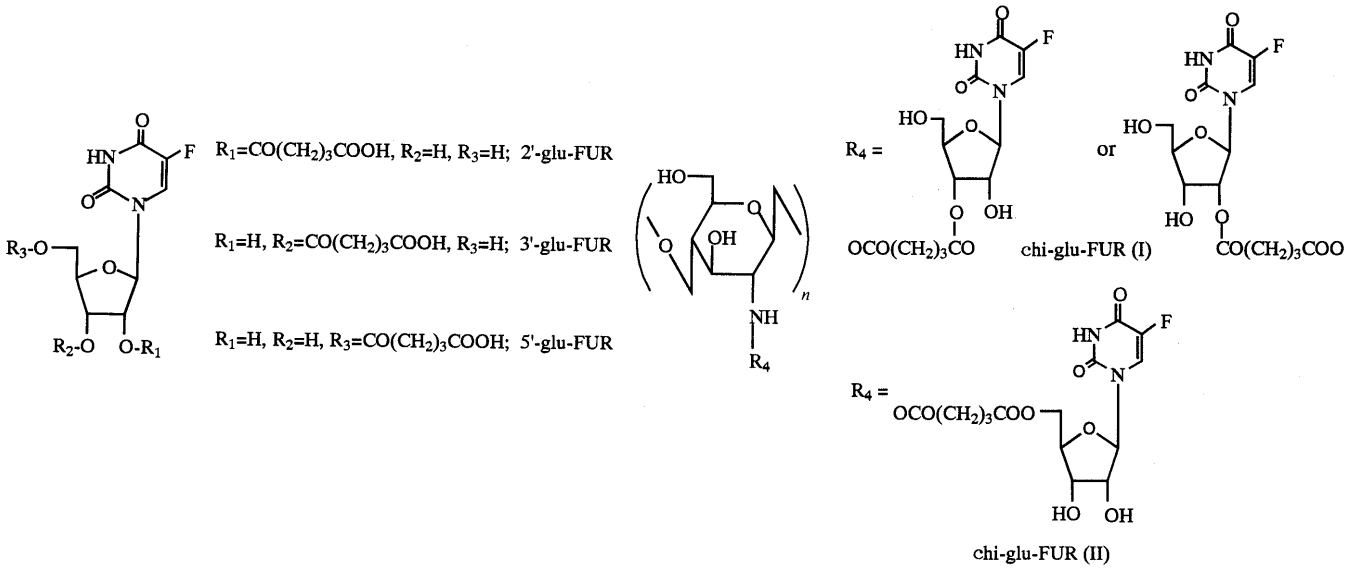

Fig. 1. Chemical Structures of FUR, 2′-Glu-FUR, 3′-Glu-FUR, 5′-Glu-FUR, Chi-glu-FUR(I) and Chi-glu-FUR(II)

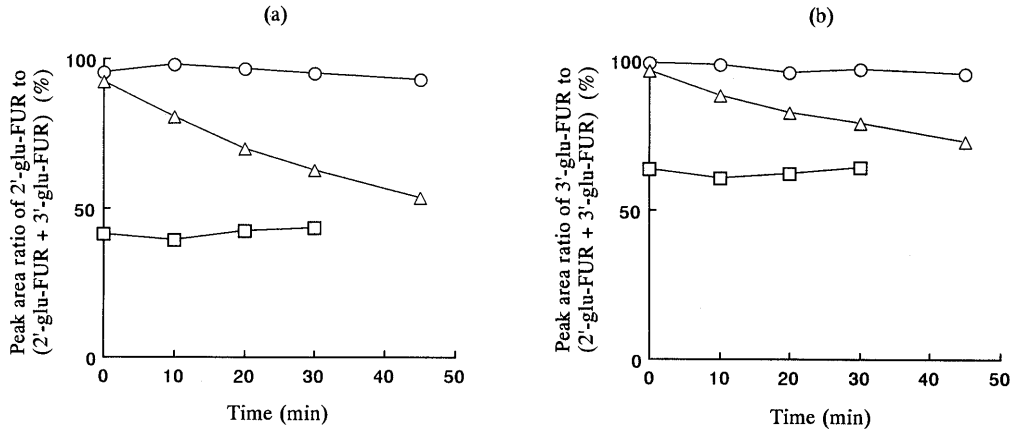

Fig. 2. Acyl Migration between 2′-Glu-FUR and 3′-Glu-FUR in the Incubation of the 2′-Glu-FUR Fraction (a) and the 3′-Glu-FUR Fraction (b) in Several Media at 37 °C

○, in a mobile phase of 0.2% aqueous acetic acid and methanol (3 : 1, v/v); △, in the mixture of 1/5 M phosphate buffer of pH 5 (μ = 0.9), water and mobile phase (1 : 1 : 1, v/v/v); □, in the mixture of 1/5 M phosphate buffer of pH 7.4 (μ = 0.9), water and mobile phase (1 : 1 : 1, v/v/v).

This indicated that the acyl migration rate from 2′-glu-FUR to 3′-glu-FUR (or from 3′-glu-FUR to 2′-glu-FUR) was considerably slow in the HPLC(A) mobile phase (pH≅4.0) and that the migration occurred more quickly at a higher pH. Especially, equilibrium was observed to be achieved immediately after the mix of the peak fraction, water and the 1/5 M phosphate buffer of pH 7.4 ($\mu=0.9$) (1 : 1 : 1, v/v/v). Regeneration of FUR was hardly observed in each incubation. Further, since the summation of the peak areas of 2′-glu-FUR and 3′-glu-FUR were almost constant throughout the incubation of the mixture of 2′-glu-FUR fraction (or 3′-glu-FUR fraction), water and 1/5 M phosphate buffer of pH 5 ($\mu=0.9$), the peak area ratio of 2′-glu-FUR to 3′-glu-FUR was considered to be approximate to the molar ratio of 2′-glu-FUR to 3′-glu-FUR. The acyl migration between the 2′-*O* and 3′-*O* positions in some ribonucleosides has been reported before.[12,13] The acyl migration between 2′-glu-FUR and 3′-glu-FUR was considered to occur because of the easy formation of a cyclic ortho acid ester[11,13] between the 2′-*O* and 3′-*O* positions. However, the acyl migration from 5′-glu-FUR was not observed, which would be due to the difficulty in forming such an ortho ester. The acyl migration rate between 2′-glu-FUR and 3′-glu-FUR was severely affected by the solution pH, and the equilibrium between the two was suggested to be attained quickly in a 1/15 M phosphate buffer of pH 7.4.

Regeneration of FUR from Glu-FUR(I) and Glu-FUR(II) Figure 3(a) shows the regeneration of FUR from glu-FUR(I) at each pH. As pH increased, the regeneration rate was heightened. At each sampling time, from pH 5—7.4, the ratio of 2′-glu-FUR to 3′-glu-FUR was observed to be 1 : 2; that is, the equilibrium between 2′-glu-FUR and 3′-glu-FUR was maintained in those samples. At pH 9, the ratio of 2′-glu-FUR to 3′-glu-FUR was maintained at 1 : 3 in every sample, which indicated that the ratio of 2′-glu-FUR to 3′-glu-FUR under the equilibrium at pH 9 was not 1 : 2 but 1 : 3. The regeneration of FUR from glu-FUR(I) apparently followed pseudo-first order kinetics. Figure 3(b) shows the regeneration of FUR from glu-FUR(II). As the pH increased, the regeneration of FUR from glu-FUR(II) increased. The regeneration almost followed pseudo-first order kinetics. The regeneration rate was calculated from curve fitting by applying monoexponential liberation to their drug regeneration, and the apparent rate constant is shown in Fig. 5. The regeneration rate was generally larger in glu-FUR(I) than in glu-FUR(II) at each pH, except at pH 5. At pH 5, since the regeneration was very small, the estimation of the rate constant at pH 5 was considered to be affected by a deviation in the observations. As for the rate constant at pH 5, a longer observation time will be required for more exact estimation.

Conjugates Since EDC acts as an amide-forming condensation reagent, the structures of chi-glu-FUR(I) and chi-glu-FUR(II) were proposed as described in Fig. 1. Table I shows the reaction conditions for the preparation of chi-glu-FUR(I) and chi-glu-FUR(II) and their drug contents. The drug content of chi-glu-FUR(I) was very small and the condensation of glu-FUR(I) with chitosan by EDC was found not to proceed efficiently. On the other hand, chi-glu-FUR(II) exhibited a moderate drug content. The above difference in drug content was considered to be partly due to a smaller steric hindrance in glu-FUR(II) than in glu-FUR(I) for EDC condensation.

Regeneration of FUR from Chi-glu-FUR(I) and Chi-glu-FUR(II) Chi-glu-FUR(I) and chi-glu-FUR(II) exhibited a pH-dependent drug release as shown in Figs. 4(a) and (b), respectively. Since glu-FUR(I) and glu-FUR(II) were scarcely detected in each incubation, it was considered that FUR was released directly from each conjugate. The drug regeneration almost followed pseudo-first order kinetics

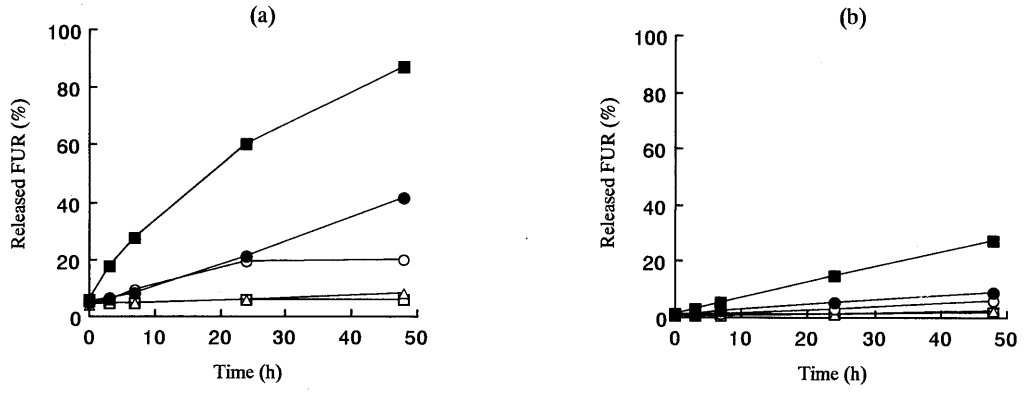

Fig. 3. Release Profiles of FUR from Glu-FUR(I) (a) and Glu-R FUR(II) (b) in 1/15 M Phosphate Buffers ($\mu=0.3$) of pH 5 (□), 6 (△), 7 (○), 7.4 (●) and 9 (■) at 37 °C

TABLE I. Preparation Conditions and Drug Contents of Chi-glu-FUR(I) and Chi-glu-FUR(II)[a]

Chitosan (mg)	Glu-FUR (mg)		EDC (g)	Water (ml)	Reaction time (h)	Drug content (%, w/w)
	I	II				
70	70	—	3.5	17.5	48	0.63
100	—	100	5.0	25.0	48	3.5

a) Each reaction was carried out at pH 6—7 at room temperature.

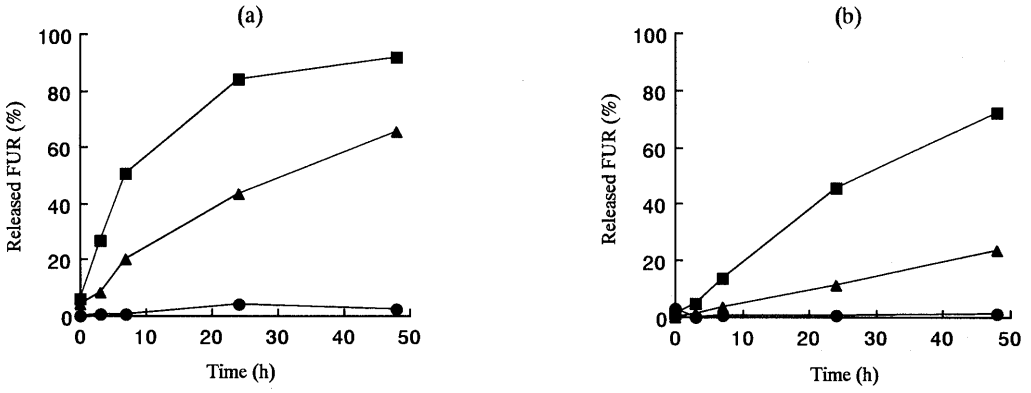

Fig. 4. Release Profiles of FUR from Chi-glu-FUR(I) (a) and Chi-glu-FUR(II) (b) in 1/15 M Phosphate Buffers ($\mu=0.3$) of pH 5 (●), 7.4 (▲) and 9 (■) at 37 °C

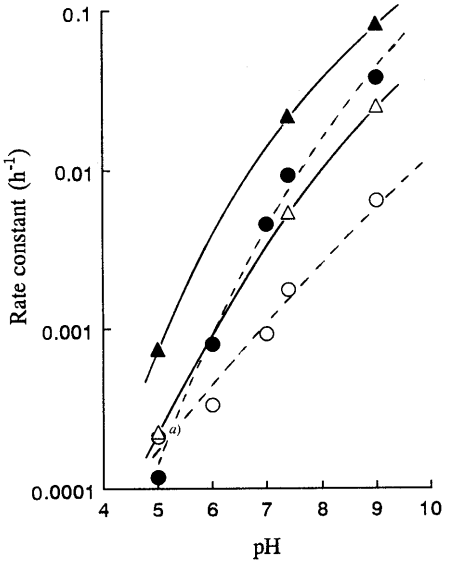

Fig. 5. pH Dependence of the Apparent Rate Constants on the Regeneration of FUR from Glu-FUR(I) (●), Glu-FUR(II) (○), Chi-glu-FUR(I) (▲) and Chi-glu-FUR(II) (△) at 37 °C and $\mu=0.3$

The calculation was carried out based on the results shown in Fig. 3 and 4. *a*) The value of chi-glu-FUR(II) at pH 5 was calculated from observations at 3, 7, 24 and 48 h. In all other values, all the observations, that is, the observations immediately after the start of incubation and at 3, 7, 24 and 48 h, were used for the calculation.

for each conjugate. Drug release was accelerated at weakly basic pH for each conjugate. The release rate of FUR at each pH was higher in the conjugate than in the corresponding glu-FUR. The difference in moiety at the 4th methylene of the 4-carboxybutyryl group appeared to affect the hydrolysis rate. In addition, for the conjugate, the basic amino groups of chitosan might provide the proper microenvironment to accelerate hydrolysis of the ester bond. The drug release rate constants were calculated from curve fitting by applying the monoexponential liberation to the observed drug release profiles. The results are shown in Fig. 5. Chi-glu-FUR(I) exhibited rate constants 3.3—4 times higher than chi-glu-FUR(II) at each pH. It is suggested that the release of FUR can be modified to some extent in the mixture of chi-glu-FUR(I) and chi-glu-FUR(II) by changing the ratio between them.

From these studies, the monoesters of FUR with 4-carboxybutyric acid and the conjugates of chitosan with their esters were characterized based on their behavior in solutions of various pH. Furthermore, it is suggested that their monoesters, glu-FUR(I) and glu-FUR(II), are useful for the preparation of conjugates with macromolecules having amino groups, and that the conjugate can be utilized as a gradual drug release system because of its hindrance to the enzyme, esterase, of an ester introduced to a side chain of a macromolecule.[14,15]

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