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Chitosan–Drug Conjugate Microspheres: Preparation and Drug Release Properties of Microspheres Composed of the Conjugate of 2′- or 3′-(4-Carboxy-butyryl)-5-Fluorouridine with Chitosan

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ABSTRACT

The conjugate microspheres (Chi-glu-FUR-m) were prepared by the dry-in-oil method using chitosan–5-fluorouridine conjugate. Chi-glu-FUR-m were characterized by drug content, particle shape and size, swelling property, and drug release. Their characteristics were compared with those of the simple microspheres (Chi/FUR-m), which were prepared under similar conditions using a mixture of chitosan and 5-fluorouridine. Both microspheres prepared showed a high retention of the drug after preparation and similar particle size and shape. Swelling ratios after incubation in aqueous buffers of pH 7.4 for 6 hr were similar for both microspheres. Chi-glu-FUR-m swelled quickly in aqueous buffers of pH 7.4 and the disintegration was observed to occur gradually from 24 hr after the incubation. Chi-glu-FUR-m showed a gradual drug release (50% release time = 61 hr), while Chi/FUR-m released the drug very rapidly. Such characteristics of Chi-glu-FUR-m as swelling, slow disintegration, and gradual drug release propose its usefulness for localization or chemoembolization therapy.

INTRODUCTION

Chitosan is produced to a large amount from a natural product, chitin, and is obtained easily and inexpensively. Further, it exhibits high biocompatibility (1,2) and biodegradability (3–7). These properties of chitosan have often provided usefulness as a drug carrier. Chitosan was found useful as an additive for tablets or granules for oral administration (8–10). Further, since it is soluble in an acidic aqueous solution, it was used as a drug carrier for microspheres (11,12) and macromolecule-drug conjugates (13–17).

Generally, microspheres are well known to be useful as a target drug delivery system because their distribution in the body can be controlled by their particle size (18–20), or they can be localized to the target site (21–23). Then, the particle size, the stability as particles, and the drug release properties affect the utility of the microspheres. However, the chitosan microspheres previously reported did not show satisfactory results for drug release properties (11,12). Namely, the drug release from the chitosan microspheres was dependent on the drug species, and the drug release profiles were often complicated. On the other hand, some of macromolecule–drug conjugates previously reported showed a good prolonged release profile. Therefore, it is proposed that chitosan microspheres showing a suitable drug release could be obtained by utilization of conjugates in the preparation of chitosan microspheres. Since chitosan is hardly soluble at physiological pH, the microspheres formed from the chitosan–drug conjugate are considered to be able to act as microspheres under physiological conditions.

MATERIALS AND METHODS

Materials

5-Fluorouridine (FUR) was purchased from Sigma Chemical Co. Daichitosan VL, the viscosity of which was 7 cps at the concentration of 5% (w/w) in 1% (w/w) acetic acid aqueous solution at 20°C, was supplied by Dainichi Seika Color and Chemicals Mfg. Co. Ltd. and was used as chitosan (Chi). Soybean oil and glutaric anhydride were obtained from Wako Pure Chemical Industries, Ltd. Sorbitan sesquioleate (SO-15) was purchased from Nikko Chemicals, Japan. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was obtained from Nacalai Tesque, Inc. All other chemicals were of reagent grade.

Preparation of Microspheres

Preparation of Simple Microspheres (Chi/FUR-m)

Chitosan (100 mg) was dissolved in aqueous acetic acid and then FUR (3 mg) was added into the chitosan solution. The volume and pH of the mixture were finally adjusted to 3 ml and pH 4–4.5, respectively, with the addition of aqueous acetic acid. The mixture was dropped continuously into 150 ml of soybean oil containing 1% (v/v) SO-15 which was stirred at 200 rpm and 40°C. After that, while stirring the obtained oily mixture under the same conditions, the pressure of the system was reduced for 3 hr by an aspirator and subsequently for 1.5 hr by a vacuum pump. The oily mixture was filtered and the residue was washed sufficiently with ether, ethanol, and ether in that order. Thus, the microspheres were separated and dried in vacuo overnight. The obtained microspheres were used as the simple microspheres (Chi/FUR-m).

Preparation of Conjugate Microspheres (Chi-glu-FUR-m)

The conjugate of FUR with chitosan was prepared following the previous method (17). Briefly, 2′- or 3′-(4-carboxybutyryl)-FUR (glu-FUR) was synthesized by the reaction of FUR and glutaric anhydride at the molar ratio of 1:1.2, and separated with an ODS reversed-phase column using a mixture of 0.2% acetic acid aqueous solution and methanol (3:1, v/v) as an elution solvent. The conjugate (Chi-glu-FUR) was prepared by the reaction of glu-FUR with chitosan using EDC as follows. Chitosan was dissolved in water of pH 3–4 adjusted by 1 N HCl aqueous solution and the solution pH was increased to 6.5 using 1 N NaOH aqueous solution. To the chitosan solution, glu-FUR and EDC were added and stirred for 2 days at room temperature. The conjugate was precipitated with the addition of acetone of 3 times volume. The precipitate was washed with a mixture of acetone and water (3:1, v/v). After drying, the Chi-glu-FUR conjugate was obtained. The conjugate (100 mg) was dissolved in aqueous acetic acid. The volume and pH of the mixture were adjusted to 3 ml and pH 4–4.5, respectively, with the addition of aqueous acetic acid. The mixture was dropped continuously into 150 ml of soybean oil containing 1% (v/v) SO-15. The procedures and conditions of the preparation, washing, and drying of the conjugate microspheres were the same as those of Chi/FUR-m. The obtained microspheres were used as the conjugate microspheres (Chi-glu-FUR-m).

The FUR content of both kinds of the microspheres was estimated spectrophotometrically at 272 nm after they were dissolved in 0.1 M acetate buffer of pH 4.0. Further, the conjugate microspheres were treated by 1 N NaOH aqueous solution at 40°C for 60 min. During the treatment, the aliquot samples were withdrawn at appropriate times. After the aliquots were neutralized by 1 N HCl aqueous solution, the concentrations of FUR in the samples were determined by high-performance liquid chromatography (HPLC). The content of FUR was also estimated from the amount of the regenerated FUR which attained a plateau level.

Characteristics of Size and Shape

The obtained microspheres were investigated for size distribution and mean diameter. The particle diameters of more than 200 of the microspheres were measured at random by microscopic observation for Chi/FUR-m and Chi-glu-FUR-m after drying and after incubation in phosphate-buffered saline (PBS) of pH 7.4 and 1/15 M phosphate buffer of pH 7.4 at 37°C for 6 hr.

Similarly, Chi-glu-FUR-m was investigated for swelling and change of shape by microscopic observation during the incubation in PBS of pH 7.4 and 1/15 M phosphate buffer of pH 7.4 at 37°C. The aliquot samples were taken at appropriate times and the microspheres were observed for size and shape using a microscope equipped with a camera.

In Vitro Release Experiment

Fifteen milligrams of Chi/FUR-m was dispersed in 20 ml of PBS of pH 7.4 and in 20 ml of 1/15 M phosphate buffer of pH 7.4. Two milligrams of Chi-glu-FUR-m was dispersed in 2 ml of 1/15 M phosphate buffer of pH 7.4. The mixtures were incubated in the water bath at 37°C by shaking at 60 rpm using an EYELA Uni Thermo Shaker NTS-1300. The aliquot samples were withdrawn at appropriate times. The amount of FUR released from Chi/FUR-m was determined by ultraviolet (UV) spectroscopy at 272 nm. The amount of released FUR from Chi-glu-FUR-m was determined by HPLC.

HPLC Analysis

HPLC was executed using a Shimadzu LC-6A apparatus equipped with a NEO-PACK 120A $5C_{18}$ column (4.5 × 150 mm) and an SPD-6A detector set at 272 nm. The mixture of 0.05 M acetate buffer of pH 4 and methanol (3:1, v/v) was used as a mobile phase. The samples obtained in the experiments using the buffered solution were directly injected on the HPLC. The sample from an alkaline medium in the drug content study was neutralized by 1 N HCl aqueous solution and then injected on the HPLC. The concentration of FUR was calculated using a standard curve.

RESULTS AND DISCUSSION

Drug Content, Shape, and Size of Microspheres

After formation of microspheres, Chi/FUR-m and Chi-glu-FUR-m were satisfactorily recovered for the amounts of the Chi/FUR mixture and conjugate used. The preparation conditions and drug content are shown in Table 1. The FUR content of Chi/FUR-m was estimated to 1.9% (w/w). This content was somewhat smaller than the value of 3.2% (w/w) expected from the added amount. The FUR content of Chi-glu-FUR-m was 3.3% (w/w) from UV spectroscopy but 2.5% (w/w) from HPLC. Since the overlap of the UV absorption, which was considered to be derived from glutarylated chitosan, was observed in the UV profile of the conjugate, the result in HPLC was adopted as the drug content of Chi-glu-FUR-m. Although the FUR content of Chi-glu-FUR conjugate was checked only by UV spectroscopy before being processed into the microspheres, it was slightly higher than that of Chi-glu-FUR-

Table 1

Preparation Conditions and Drug Contents of Microspheres

Microsphere	Chitosan (mg)	FUR or glu-FUR (mg)	EDC (mg)	Water (ml)	Reaction Time (hr)	Drug Content (%, w/w)
Chi/FUR-m	99	3.3	—	—	—	1.9
Chi-glu-FUR(1)-m	100	76	1000	12	48	2.5

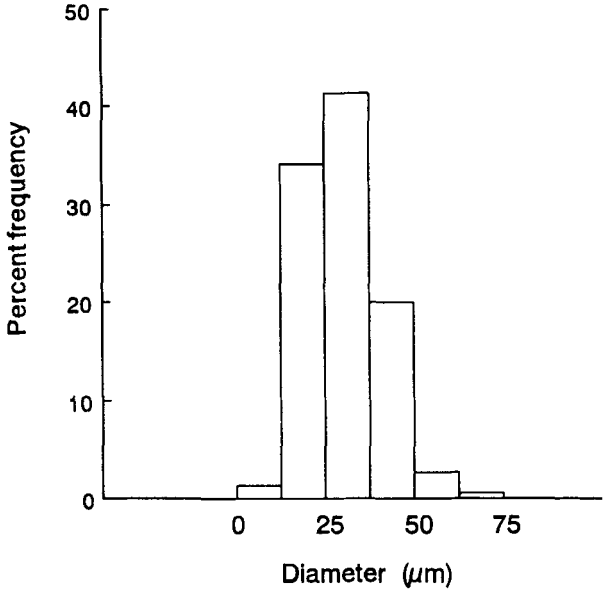

Figure 1. Particle size distribution of the diameters of dried Chi/FUR-m.

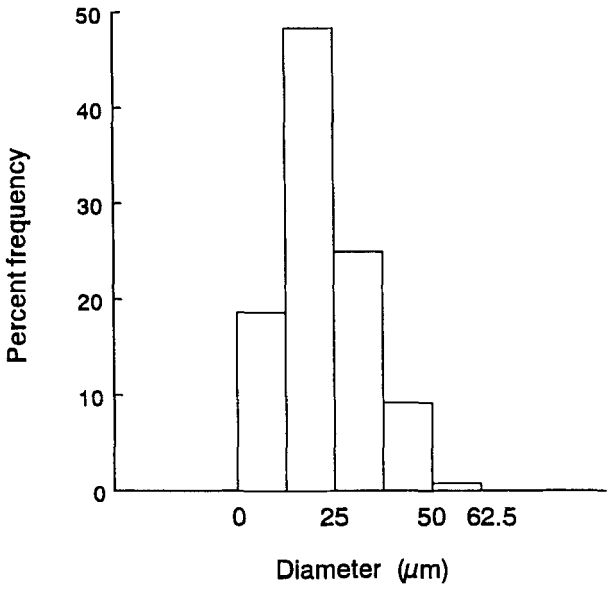

Figure 2. Particle size distribution of the diameters of dried Chi-glu-FUR-m.

m. The drug content of Chi-glu-FUR was considered to be slightly lowered by the conversion to microspheres.

Figures 1 and 2 show the size distributions of the diameters of dried Chi/FUR-m and dried Chi-glu-FUR-m, respectively. Both showed a spherical shape. Chi/FUR-m showed a mean diameter of 30 μm, and the sizes varied from 6.25 to 68.75 μm. Chi-glu-FUR-m showed an average diameter of 22 μm, and the sizes distributed from 6.25 to 56.25 μm.

Shape and Swelling of the Conjugate Microspheres at Physiological pH

Table 2 shows the mean diameters of Chi/FUR-m and Chi-glu-FUR-m before—that is, dried—and after incubation for 6 hr in PBS of pH 7.4 and 1/15 M phosphate buffer of pH 7.4. Both microspheres showed a spherical shape before and after incubation. After incubation of 6 hr, both microspheres swelled and each mean diameter increased to approximately twice that of the dried microspheres. The shapes and mean diameters of the microspheres after swelling were similar in both PBS of pH 7.4 and 1/15 M phosphate buffer of pH 7.4.

The photomicrographs in Fig. 3 show Chi-glu-FUR-m before—that is, dried—and after incubation in PBS of pH 7.4; and Fig. 4 the Chi-glu-FUR-m before—that is, dried—and after incubation in 1/15 M phosphate buffer of pH 7.4, respectively. The swelling was recognized to occur rapidly and the microsphere sizes and shapes hardly changed at 2–24 hr. However, disintegration of the microspheres was observed to occur gradually after incubation for 24 hr. This might be because the polymer chains were gradually disentangled due to the swelling, and their binding force weakened. This natural dis-

Table 2

Mean Diameters of Microspheres: Dried and After Incubation in PBS of pH 7.4 and 1/15 M Phosphate Buffers of pH 7.4 for 6 hr at 37°C

Microsphere	Size, Dried (μm)	Size (μm) After Incubation for 6 hr in:	
		PBS	1/15 M Phosphate Buffer
Chi/FUR-m	30	50	52
Chi-glu-FUR-m	22	41	42

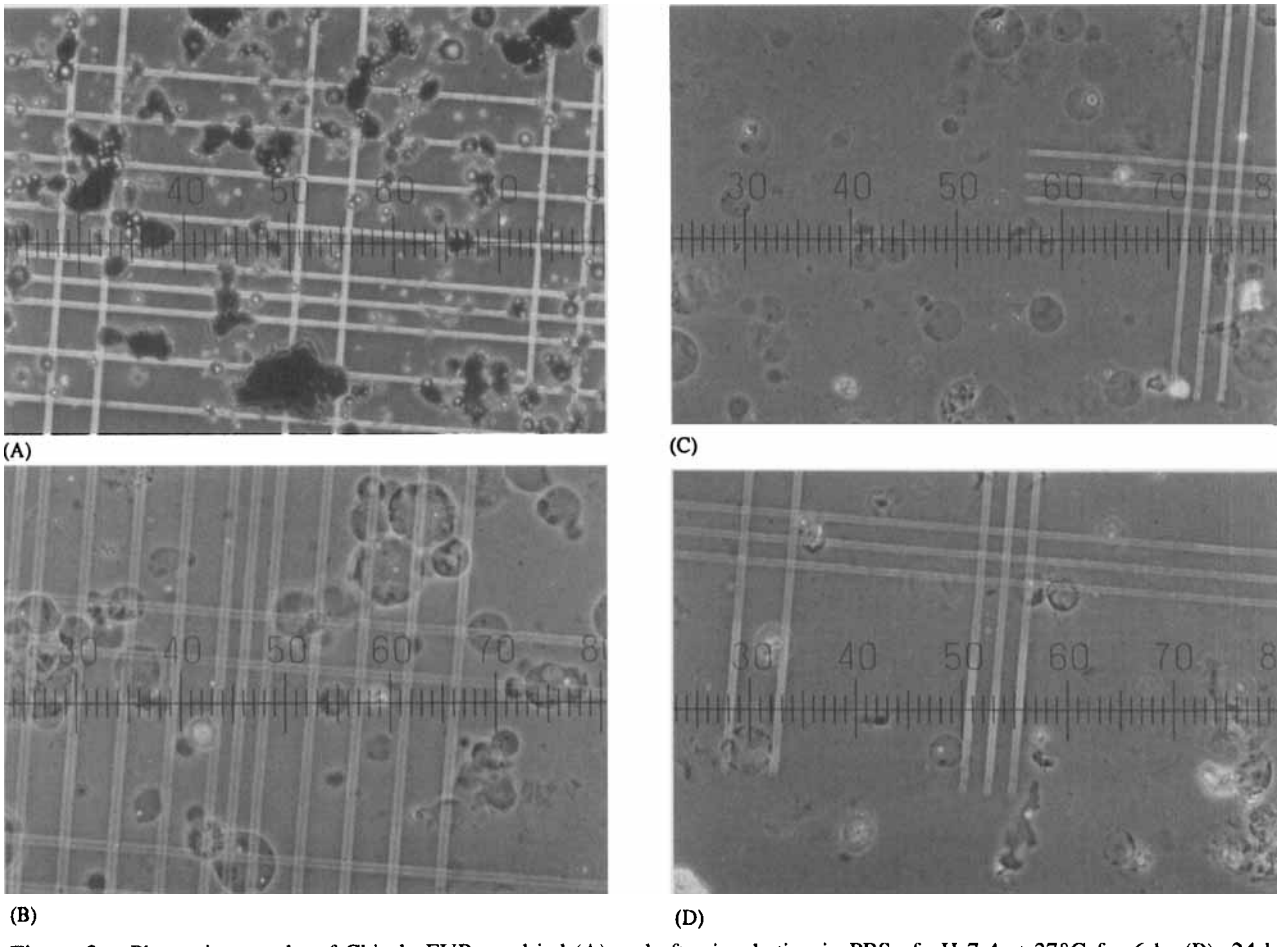

Figure 3. Photomicrographs of Chi-glu-FUR-m: dried (A) and after incubation in PBS of pH 7.4 at 37°C for 6 hr (B), 24 hr (C), and 48 hr (D). Scale bars = 100 μm.

integration property of Chi-glu-FUR-m might be adequate for degradation in tissues or organs.

Drug Release from Microspheres

Drug release profiles from Chi/FUR-m and Chi-glu-FUR-m are shown in Fig. 5. Chi/FUR-m released FUR very quickly in PBS of pH 7.4 and 1/15 M phosphate buffer of pH 7.4. This suggested that both buffers should permeate into the microspheres very rapidly. Since Chi/FUR-m swelled quickly and since FUR is very water soluble and a small molecule, FUR dispersed in the microspheres was considered to be released rapidly out of the microspheres. On the other hand, Chi-glu-FUR-m released FUR slowly in 1/15 M phosphate buffer of pH 7.4. The release profile of Chi-glu-FUR-m was similar to that of the conjugate, Chi-glu-FUR, itself (17). When the FUR release from Chi-glu-FUR-m was approximated to the pseudo-first-order kinetics, its rate constant was calculated to be 0.011 h^{-1}. It was smaller to some extent compared to that from the conjugate itself (17). As Chi-glu-FUR-m swelled quickly, the regeneration of FUR was considered not to be changed so much between Chi-glu-FUR-m and the conjugate itself. Also, the regenerated FUR from Chi-glu-FUR would be released rapidly out of the spheres. As a result, Chi-glu-FUR-m might show a drug release profile similar to that of the conjugate itself. Since the hindrance to the enzyme, esterase, of the ester introduced to a side chain of a macromolecule was previously reported (16,24), Chi-glu-FUR-m is suggested to act as a microsphere showing a gradual drug release in a body fluid.

Chi-glu-FUR-m is proposed to be useful as a microparticulated gradual drug release system. The property of slow disintegration after swelling will be useful for

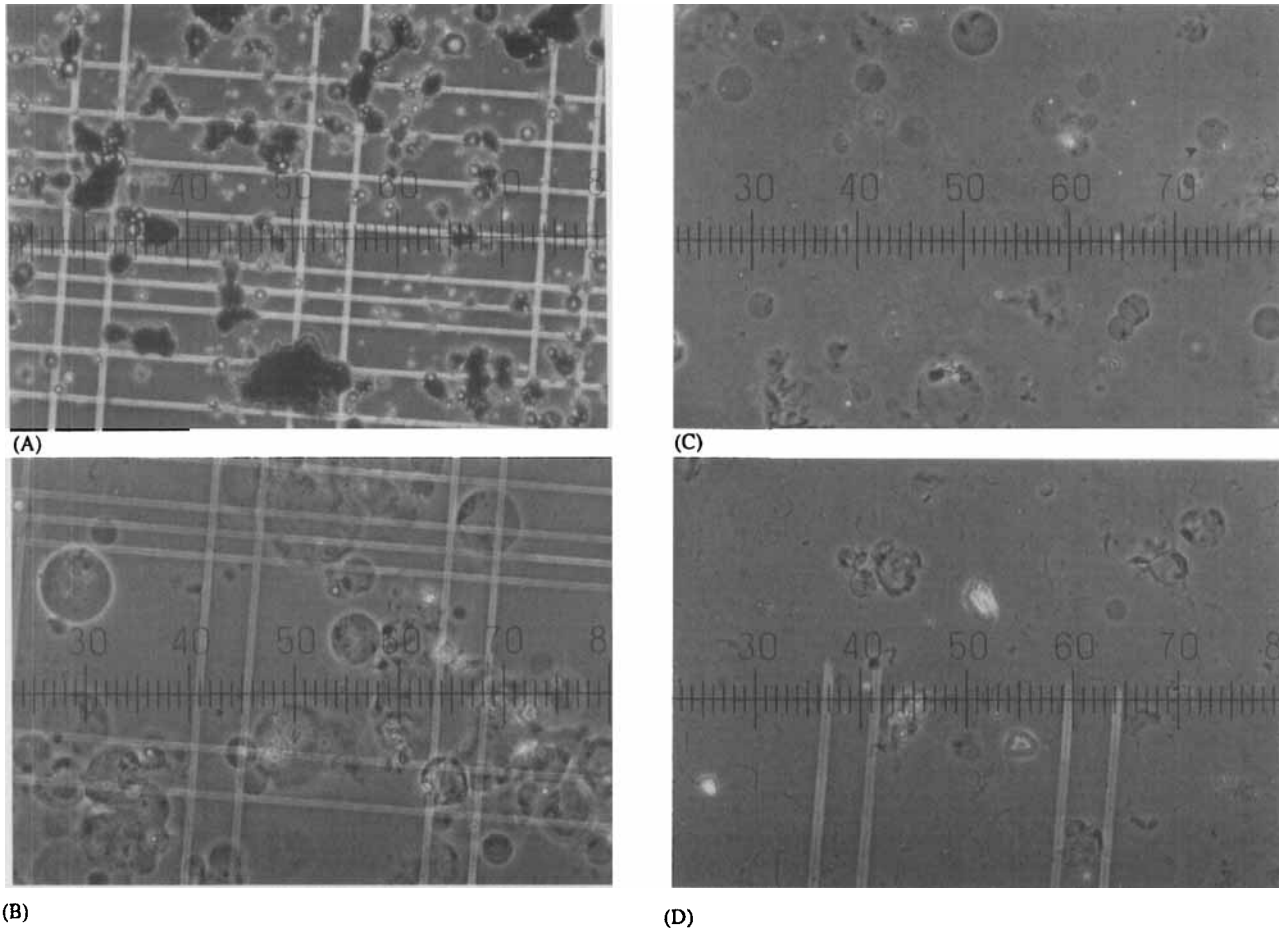

Figure 4. Photomicrographs of Chi-glu-FUR-m: dried (A) and after incubation in 1/15 M phosphate buffer of pH 7.4 at 37°C for 6 hr (B), 24 hr (C), and 48 hr (D). Scale bars = 100 μm.

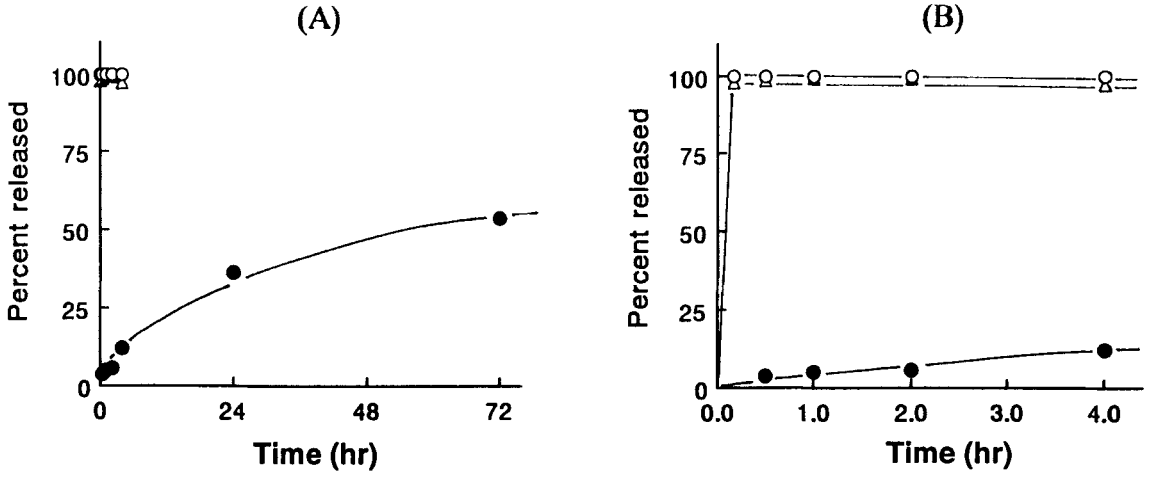

Figure 5. Drug release profiles from Chi/FUR-m during incubation in PBS of pH 7.4 (Δ) and 1/15 M phosphate buffer of pH 7.4 (○) at 37°C; and from Chi-glu-FUR-m during incubation in 1/15 M phosphate buffer of pH 7.4 (●) at 37°C. A, Release during incubation for 0–72 hr. B, Release during incubation for 0–4 hr.

chemoembolization therapy because the particles can be eliminated naturally from the embolized part after the drug release. Chi-glu-FUR-m will be able to be better localized than the conjugate itself because of the nature of the solid particle. Further, it will be possible to dispose Chi-glu-FUR-m to specific tissues or organs, dependent on the particle size characteristics. Thus, Chi-glu-FUR-m should be more controllable as to localization and disposition of the drug than the conjugate itself. The drug release rate and profile, the size of the dried and swollen particles, and the disintegration properties of the microspheres will have to be considered together on an evaluation of the utility of the chitosan-drug conjugate microspheres.

REFERENCES

1. T. Sannan, in *Abstract of Papers*, 13th Conference on Pharmaceutical Technology (The Academy of Pharmaceutical Sciences and Technology, Jpn.), Shirakabako, July 1988, p. 104.
2. J. F. Prudden, P. Miegel, P. Hanson, L. Friedrich, and L. Balassa, Am. J. Surg., 119, 560 (1970).
3. R. C. Davies, A. Neuberger, and B. M. Wilson, Biochim. Biophys. Acta, 178, 294 (1969).
4. K. Amano and E. Ito, Eur. J. Biochem., 85, 97 (1978).
5. S. H. Pungburn, P. V. Trescony, and J. Heller, Biomaterials, 3, 105 (1982).
6. C. Yomota, T. Komuro, and T. Kimura, Yakugaku Zasshi, 110, 442 (1990).
7. F. Nakamura, H. Onishi, Y. Machida, and T. Nagai, Yakuzaigaku, 52, 59 (1992).
8. K. Inouye, Y. Machida, and T. Nagai, Drug Design Deliv., 1, 297 (1987).
9. K. Inouye, Y. Machida, T. Sannan, and T. Nagai, Drug Design Deliv., 2, 165 (1988).
10. K. Inouye, Y. Machida, T. Sannan, and T. Nagai, Drug Design Deliv., 2, 55 (1989).
11. Y. P. Li, Y. Machida, T. Sannan, and T. Nagai, STP Pharma Sci., 1, 363 (1991).
12. T. Yoshino, Y. Machida, H. Onishi, and T. Nagai, STP Pharma Sci., in press.
13. T. Ouchi, T. Banba, T. Matsumoto, S. Suzuki, and M. Suzuki, J. Bioact. Comp. Polym., 4, 362 (1989).
14. Y. D. Sanzgiri, C. D. Blanton, Jr., and J. M. Gallo, Pharm. Res., 7, 418 (1990).
15. H. Ichikawa, H. Onishi, T. Takahata, Y. Machida, and T. Nagai, Drug Design Discovery, 10, 343, (1993).
16. H. Ichikawa, H. Onishi, M. Sato, Y. Machida, and T. Nagai, Yakuzaigaku, 55, 59 (1995).
17. H. Onishi, Y. Machida, and T. Nagai, Chem. Pharm. Bull., 43, 340 (1995).
18. K. Sugibayashi, Y. Morimoto, T. Nadai, Y. Kato, A. Hasegawa, and T. Arita, Chem. Pharm. Bull., 27, 204 (1979).
19. M. Kanke, G. H. Simmons, D. L. Weiss, B. A. Bivins, and P. P. Deluca, J. Pharm. Sci., 69, 755 (1980).
20. T. Yoshioka, M. Hashida, S. Muranishi, and H. Sezaki, Int. J. Pharm., 8, 131 (1981).
21. T. Kato, K. Unno, and A. Goto, in *Methods in Enzymology* (K. J. Widder and R. Green, eds.), Academic Press, Tokyo, 1985, p. 139.
22. T. Kato, R. Nemoto, H. Mori, M. Takahashi, Y. Tamakawa, and M. Harada, J. Am. Med. Assoc., 245, 1123 (1981).
23. M. Minabe, M. Iida, K. Takeuchi, T. Hori, S.-H. Hyon, and Y. Ikada, Drug Delivery System, 6, 201 (1991).
24. H. Onishi, T. Kawaguchi, and T. Nagai, Chem. Pharm. Bull., 35, 3370 (1987).