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In Vitro and *in Vivo* Evaluation of Sustained Release Chitosan-Coated Ketoprofen Microparticles

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Simple ketoprofen microspheres (MS) were prepared by the dry-in-oil method using ethylcellulose (EC) as a matrix polymer. Further, the microspheres modified by addition of polyethylene glycol (PEG) and hydroxypropyl cellulose (HPC), called MS-P and MS-H, respectively, were prepared. The *in vitro* release from MS, MS-P and MS-H was examined in the JP XIII second fluid, pH 6.8, at 37°C and 60 rpm. Chitosan-coated ketoprofen microparticles (Chi-MP) were prepared by the precipitation of droplets of chitosan solution containing MS, and their adhesion to the rat small intestinal mucosa was tested. The plasma concentrations after duodenal administration were investigated for ketoprofen powder suspension, MS and Chi-MP. The particle size was raised with the increase in amount of ketoprofen added. The drug content and addition of PEG or HPC affected the drug release rate. The microspheres with moderate drug content, prepared by addition of modest amount of PEG, exhibited better gradual drug release. Chi-MP showed a good mucoadhesion. The maximum plasma concentration of ketoprofen for Chi-MP was less than one-third of that for ketoprofen powder suspension. Chi-MP tended to show the higher and steadier plasma levels than MS.

Key words——chitosan-coated microparticle; simple microsphere; ;ketoprofen; plasma concentration; mucoadhesion

INTRODUCTION

Ketoprofen is a widely used nonsteroidal anti-inflammatory drug.[1] Although this drug exhibits strong anti-inflammatory actions, the improvement of some properties have been attempted. Since ketoprofen is low water-soluble, various techniques such as dry elixir,[2] the solid dispersion,[3] water-soluble prodrug[4] or complexation[5] have been applied for enhancement of the solubility. Further, frequent dosing of ketoprofen is required for therapeutic maintenance because of its fairly fast elimination from the body.[6,7]. Exposure of the stomach to high levels of ketoprofen can cause gastric damage such as ulceration or bleeding.[8] To improve this disadvantage, sustained release or enteric-coating dosage forms have been developed, resulting in less frequent dosing and less gastrointestinal disturbance.[9] Thus, in the present study, the sustained release microspheres of ketoprofen have been developed using ethylcellulose (EC), which is often utilized as a matrix for preparation of prolonged release dosage forms.[10] Since localization and retention of the drug to the absorption site are known to influence the absorption,[11,12] chitosan coating, which was reported to enhance the accessibility and localization to the absorptive membrane via bioadhesion,[13,14] has been further applied. The drug absorption after duodenal administration has been compared among ketoprofen suspension, simple microspheres and chitosan-coated microparticles.

MATERIALS AND METHODS

1. Materials Ketoprofen was purchased from Sigma Chemical Co. (St. Louis, MO). Ethylcellulose (EC; 49% ethoxy, 100 cp grade) and polyethylene glycol (PEG; MW 20,000) were obtained from Wako Pure Chemical Industries, Ltd. Daichitosan H (MW 650,000, deacetylation degree 82%) was supplied from Dainichiseika Color & Chemicals Mfg. Co., Ltd., and used as chitosan. Hydroxypropylcellulose (HPC; type H) was purchased from Nippon Soda Co., Ltd. Sorbitan tristearate (SS-30) and sorbitan sesquioleate (SO-15) were purchased from Nikko Chemicals Co., Ltd. All other chemicals were of reagent grade.

Fluorescein isothiocyanate (FITC)-labeled chitosan, i.e. fluorescein thiocarbamyl (FTC)-chitosan (FTC-Chi) was prepared as follows: One g of chitosan was dissolved in 1 liter of water with the pH adjusted to 3 with 1 M HCl, and the pH of the solution was then adjusted to 6.5. Then, 30 mg of FITC was dissolved in 60 ml of water, and the solution was added to the chitosan solution. The mixture was stirred for 24 h at room temperature in the dark. The crude product was precipitated by increasing the pH

of the solution to 9 using 1 M NaOH, and collected by centrifugation at 3000 rpm for 5 min. It was washed by dissolution in 1 M HCl and subsequent precipitation by increasing the pH to 9 using 1 M NaOH. Finally, the precipitate was washed with water, and the product, FTC-Chi, was obtained by lyophilization of the aqueous suspension. The content of FTC in FTC-Chi was determined to be 0.6% (w/w) from spectrophotometric (490 nm) measurement after dissolving FTC-Chi in 0.1 M acetate buffer, pH 5.

2. Preparation of Ketoprofen Microspheres and Chitosan-Coated Ketoprofen Microparticles Simple ketoprofen microspheres (MS) were prepared using EC and ketoprofen by the dry-in-oil method as follows: EC (1.5 g) and ketoprofen (0.9, 1.5 or 2.25 g) were dissolved in 25 ml of acetone. The solution was added drop-wise to 250 ml of liquid paraffin containing SS-30 at 2% (w/v) and stirred at 600 rpm and 20°C. The emulsion was stirred at room temperature for 1 h, then at 35°C for 5 h and finally at 57°C for 1 h. One hundred ml of n-hexane warmed at 55°C was added to the mixture, and filtered using a membrane with a pore diameter of 0.45 μm. The residue was washed with n-hexane warmed at 55°C to yield MS.

Ketoprofen microspheres with PEG (MS-P) were prepared as follows: PEG (0.075, 0.15 or 0.3 g) was dissolved in 25 ml of acetone warmed at 35°C. After cooling the solution at 20°C, 1.5 g of ketoprofen and 1.5 g of EC were added. The subsequent procedures were the same as those described for MS.

Ketoprofen microspheres with HPC (MS-H) were produced as follows: HPC (0.075, 0.15 or 0.3 g) was added to 25 ml of acetone at 20°C, and stirred vigorously at 14000 rpm for 1 min. Then, 1.5 g of ketoprofen and 1.5 g of EC were added to the suspension, and dissolved. The subsequent procedures were the same as those described for MS.

Chitosan-coated ketoprofen microparticles (Chi-MP) were prepared by coating MS with FTC-Chi as shown in Fig. 1A. MS prepared from the solution of EC (1.5 g) and ketoprofen (1.5 g) in 25 ml of acetone was used. FTC-Chi (100 mg) was dissolved in 5 ml of 2% (v/v) acetic acid aqueous solution. MS (100 mg) was suspended in the solution, and added drop-wise to 20 ml of liquid paraffin containing SO-15 at 1% (w/v) and stirred at 600 rpm. The suspension was added drop-wise to 500 ml of double solvent layers of n-hexane/1 M NaOH (2 : 3, v/v) and stirred at 300 rpm. One min later after end of dropping, the particles precipitated in 1 M NaOH aqueous layer were collected by filtration using mesh (opening 150 μm), washed with 500 ml of water, and dried in a desiccator in vacuo at room temperature to produce Chi-MP.

4. Particle Characteristics MS, MS-P, MS-H and Chi-MP were prepared once; therefore, their characterization was performed using each single lot. The Green diameter was checked for randomly selected 200 particles. After 10 mg of MS, MS-P or MS-H were dissolved in chloroform, the drug content was determined from UV absorption of the solution at 256 nm.

The contents of ketoprofen and FTC-Chi in Chi-MP were investigated as follows: Twenty mg of Chi-MP were added to 0.1 M acetate buffer, pH 5, to dissolve the FTC-Chi coating layer. After centrifugation of the mixture at 3000 rpm for 5 min, the UV absorp-

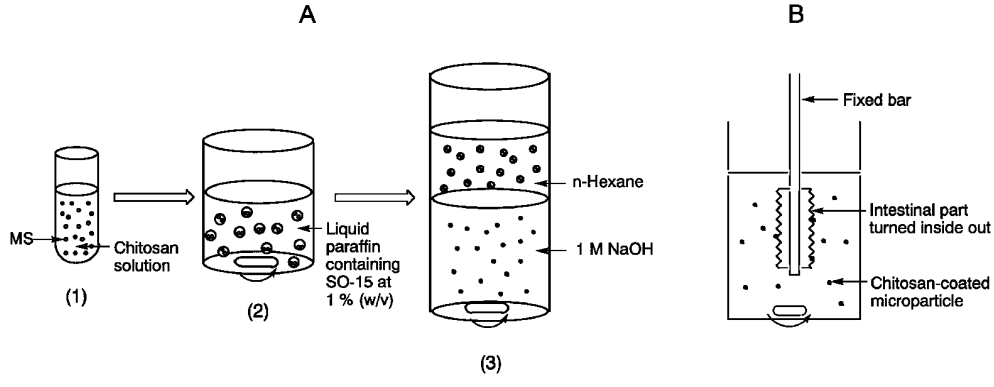

Fig. 1. Schematic Representation of Chitosan-Coating of MS (A) and Bioadhesive Test of Microparticles (B)

A: (1) MS (formation 2 in Table 1) were suspended in chitosan solution. (2) MS suspension in chitosan solution was emulsified in liquid paraffin containing SO-15 at 1% (w/w). (3) Liquid paraffin was washed off in the upper layer (n-hexane) and chitosan was precipitated around MS in the bottom layer (1 M NaOH).

tion of the supernatant at 490 nm was measured to determine the amount of FTC-Chi. The amount of ketoprofen in the supernatant was determined by subtracting UV absorption at 256 nm of the contained Chi-FTC from that of the supernatant. The precipitate was dried in vacuo and dissolved in chloroform. The solution was measured spectrophotometrically at 256 nm to determine the amount of ketoprofen. The content of ketoprofen in Chi-MP was determined by summation of the amount obtained in 0.1 M acetate buffer, pH 5, and that recovered in chloroform.

5. Drug Release Tests The release test was performed at 37°C according to the JP XIII paddle method. MS, MS-P or MS-H (30—50 mg) were added to 300 ml of the JP XIII second fluid, pH 6.8, which was stirred at a moderate rate, 60 rpm, to reflect the in vivo situation; this rate was similar to that in other release studies.[2,7,10] Aliquots (2 ml) were withdrawn at appropriate times. Immediately after each sampling, the aliquot was filtered with a membrane filter (0.45 μm in pore diameter) and the same volume of fresh JP XIII second fluid at 37°C was supplemented to the test medium. The amount of released ketoprofen was determined by measuring UV absorption of the filtrate at 256 nm.

6. *In Vitro* Bioadhesion of Chi-MP Male Wistar rats weighing 200 g were fasted for 24 h, and the intestinal part (5 cm in length) located 5—10 cm below the pylorus was surgically excised under anesthesia with ether. Immediately after that, the animals were sacrificed by excessive anesthesia with ether. The excised part was turned inside out, and 2 cm of the tube in length was taken by cut and used for the experiment. A cylindrical glass bar (4 mm in diameter) was passed through the tube. This tube was immersed and fixed such that its top was 5 mm below the solvent surface of the JP XIII second fluid (8 ml) containing 10 mg of Chi-MP, which was set in the cylindrical bottle (1.8 cm I.D.$\times$4 cm height) and stirred at 200 rpm. The intestine tube was taken out of the medium after immersion for 20, 50 or 120 min, immediately put at the same position into the similar cylindrical bottle containing fresh JP XIII second fluid (8 ml) with no sample, and the fluid was stirred at 200 rpm for 5 min. Then, the tube was taken out, and the particles on the intestinal mucosa were collected by scraping the tube with a spatula and dissolved in 5 ml of 0.1 M acetate buffer, pH 4.5. The FTC-Chi concentration was determined fluorometrically (Ex. 490 nm; Em. 520 nm), and the amount of adhering Chi-MP was calculated from the FTC-Chi amount.

7. Plasma Concentration after Duodenal Administration Male Wistar rats weighing 200 g were fasted for 24 h, then anesthetized by intraperitoneal injection of urethane solution in saline at 0.9 g/4 ml/kg. As to MS in this animal experiment, MS prepared from the solution of EC (1.5 g) and ketoprofen (1.5 g) in 25 ml of acetone was used. The abdominal skin and membrane were cut open, and the small rift was made by the cutter at the duodenal part 5 cm below the pylorus. The suspension (1 ml) of ketoprofen powder, MS or Chi-MP in saline was administered downward from the rift at a dose of 6.25 mg ketoprofen/kg using the thin glass tube. The area just below the rift was ligated to prevent the leakage of the drug. After that, the incisions in the abdominal membrane and skin were sutured. The rats were placed on their back on a warm plate set at 40°C throughout the experiment (n=5). At appropriate times after administration, the blood samples (300 μl) were withdrawn from the jugular vein. Plasma was obtained by centrifugation of the blood at 3000 rpm for 5 min. One hundred μl of 1 M HCl aqueous solution was added to 100 μl of plasma, and shaken vigorously for 1 min. Then, 2.5 ml of diethyl ether was added to the mixture, and shaken vigorously for 1 min. After centrifugation of the mixture at 2000 rpm for 5 min, the whole organic layer was filtered through a membrane (0.45 μm in pore diameter), and the solvent in the filtrate was evaporated under nitrogen gas at 40°C. The residue was dissolved in 200 μl of the mixture of 0.02 M phosphate buffer, pH 3.0, and acetonitrile (65 : 35, v/v) (mobile phase in high performance liquid chromatography (HPLC)) and analyzed by HPLC.

8. HPLC Assay The HPLC analysis was performed at room temperature as follows: Twenty μl of the sample was injected into a Lichrosorb RP-18 column (4.6 mm in inner diameter$\times$150 mm in length; Sumika Chemical Analysis Service) attached to a Shimadzu LC-6AD apparatus with a Shimadzu SPD-10AV UV detector set at 256 nm. A mixture of 0.02 M phosphate buffer, pH 3.0, and acetonitrile (65 : 35, v/v) was used as a mobile phase.

RESULTS AND DISCUSSION

1. Particle Characteristics of MS, MS-P and MS-H The mean particle size and drug content of MS, MS-P and MS-H were described in Table 1. For all the microspheres, the particle size was distributed from several μm to several dozen μm. The particle size tended to be larger overall with increase in ketoprofen amount added, resulting in the increase in mean particle size. This was considered to be due to increase in amount of ketoprofen in the emulsion formed in the dry-in-oil method. The drug content was also raised with increase in amount of ketoprofen added. The particle size and drug content were little affected by addition of PEG and HPC probably because the amounts of PEG and HPC added were small. Taking the yield of the microspheres and the drug content into account, most of EC added was recognized to be recovered in the microspheres (data not shown). When the ideal drug content was calculated as a ratio of added ketoprofen amount to total amount of drug and additives, the ratio of the observed drug content to the ideal one, named incorporation efficiency, was calculated (Table 1). The incorporation efficiency was relatively low, suggesting that ketoprofen should leak to the outer phase to a fair extent. This was supposed to be based on the solubility and partition coefficient of the drug in the W/O system. The incorporation efficiency tended to be larger under the condition of higher amount of ketoprofen added, which was possibly because the amount of the drug leaking to the outer phase was relatively smaller. The incorporation efficiency ranged from 25.4 to 37.6 % (w/w) in the formations with combination of EC and ketoprofen (1 : 1, w/w).

2. Drug Release Characteristics The drug release from MS, MS-P and MS-H was shown in Fig. 2. For MS, the percentage released was raised with increase in the drug content. Formation 2 seemed to present a gradual release fairly well. Addition of PEG increased the percentage release for 24 h; especially, the initial release was enhanced by PEG. Since PEG is water-soluble, ketoprofen incorporated near PEG was considered to be released fast following hydration or dissolution of PEG. The addition of HPC tended to raise an overall release, but its influence was not marked. This observation was probably because hydration and subsequent dissolution of HPC took more time than those of PEG.

3. Particle Characteristics and Bioadhesion of Chi-MP For Chi-MP, the drug content was 11% (w/w), the content of FTC-Chi was 30% (w/w). Therefore, ketoprofen was hardly lost in the coating process. The particle size was approximately 200 μm, and ten or more MS were contained in one particle of Chi-MP. The adhesive profile of Chi-MP to the intestinal mucosa is shown in Fig. 3. Chi-MP exhibited quick adhesion to the mucosa. The decrease in the ratio of adhering Chi-MP at 50 min after the start of the test was considered due to dissolution and exfoliation of part of the mucosa. This suggested that Chi-MP should have a fairly good adhesion to the intestinal mucosa.

4. Plasma Concentration after Duodenal Administration Figure 4 shows the plasma concentration-time profiles after duodenal administration at the dose of 6.25 mg ketoprofen/kg. Ketoprofen suspension exhibited maximum concentration (14.2 μg/ml) at 1 h after administration, and then the plasma level decreased to nearly 1/10 of the maximum value at 24 h after administration. MS exhibited the maximum concentration (2.4 μg/ml) at 1 h after adminis-

Table 1. Particle Characteristics of MS, MS-P and MS-H Prepared by Various Formulations

Particle type	EC (g)	Ketoprofen (g)	PEG (g)	HPG (g)	Mean particle diameter (μm)	Drug content (%, w/w)	Incorporatoin efficiency (%, w/w)	Formation No.
MS	1.5	0.9	—	—	12.4	7.9	21.1	1
	1.5	1.5	—	—	17.4	14.2	28.4	2
	1.5	2.25	—	—	18.3	22.1	36.8	3
MS-P	1.5	1.5	0.075	—	18.6	12.4	25.4	4
	1.5	1.5	0.15	—	18.9	14.7	30.9	5
	1.5	1.5	0.3	—	19.1	13.6	29.9	6
MS-H	1.5	1.5	—	0.075	18.9	15.2	31.2	7
	1.5	1.5	—	0.15	20.9	16.8	35.3	8
	1.5	1.5	—	0.3	20.4	17.1	37.6	9

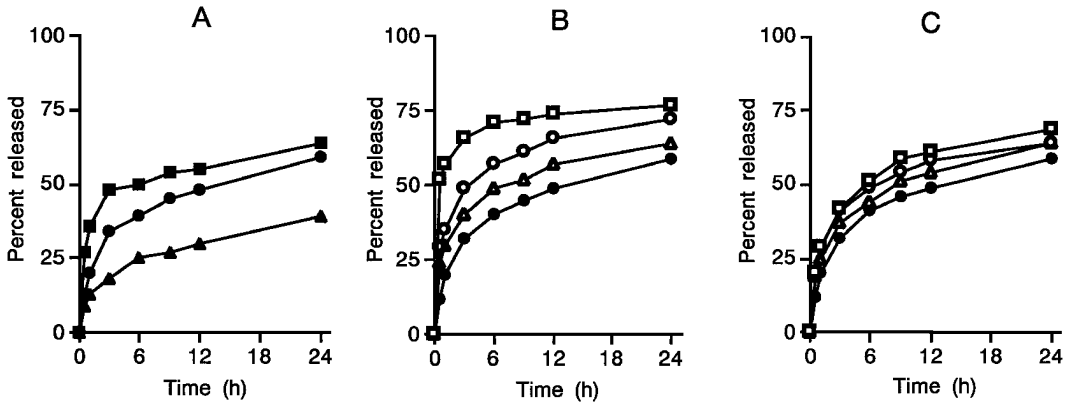

Fig. 2. Release Profiles of Ketoprofen from MS (A), MS-P (B) and MS-H (C) Prepared at Different Formulations in the JP XIII Second Fluid at 37°C

The results are for the formations described in Table 1. A: Formations 1 (▲), 2 (●) and 3 (■) in Table 1. B: Formations 2 (●), 4 (△), 5 (○) and 6 (□) in Table 1. C: Formations 2 (●), 7 (△), 8 (○) and 9 (□) in Table 1.

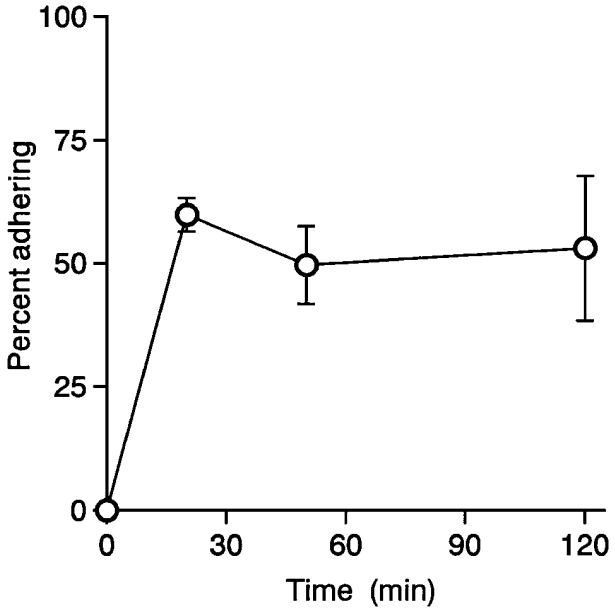

Fig. 3. *In Vitro* Bioadhesion of Chi-MP to the Rat Intestinal Mucosa

The time means the stirring time after the start of the test. The results are expressed as the mean ± S.D. ($n=3$).

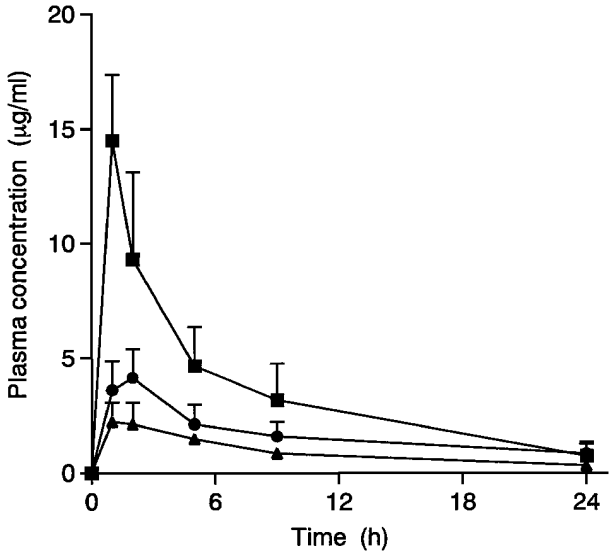

Fig. 4. Plasma Concentration-Time Profiles of Ketoprofen after Duodenal Administration of Ketoprofen Suspension, MS and Chi-MP at the Dose of 6.25 mg Ketoprofen/kg in Rats

■: Ketoprofen suspension, ▲: MS (formation 2 in Table 1), ●: Chi-MP. Each point represents the mean ± S.D. ($n=5$).

tration, and the plasma level was less than 0.5 μg/ml at 24 h after administration. For Chi-MP, the plasma level reached its maximum (4.1 μg/ml) at approximately 2 h after administration, and at 24 h post-administration the plasma concentration was near that of ketoprofen suspension.

The pharmacokinetic parameters are shown in Table 2, where the moment values, $AUC_{0-24\,h}$, $MTR_{0-24\,h}$ and $VTR_{0-24\,h}$, were calculated using the trapezoidal method. From the simple comparison of the $AUC_{0-24\,h}$ values, the relative bioavailabilities of Chi-MP and MS to ketoprofen suspension were calculated to be 49 and 29%, respectively. One of the reasons for the lower relative bioavailabilities of Chi-MP and MS was possibly due to the incomplete drug release from MS for 24 h, which was shown in Fig. 2. Although the absolute bioavailabilities were unknown, the plasma concentration profile given by ketoprofen suspension was fairly parallel to that in oral administration at 5 mg/kg reported by Kitagawa

Table 2 Pharmacokinetic Parameters for Ketoprofen Suspension, MS and Chi-MP after Duodenal Adminstration at a Dose of 6.25 mg Ketoprofen/kg in Rats

Dosage form	C_{max} (μg/ml)	T_{max} (h)	$AUC_{0-24\,h}$ (μg·h/ml)	$MRT_{0-24\,h}$ (h)	$VRT_{0-24\,h}$ (h^2)
Ketoprofen suspension	14.2±2.8	1.0±0.0	84.2±27.0	6.2±1.1	31.0±10.6
MS	2.4±0.9	1.2±0.5	24.4±14.2	7.4±1.3	35.0±15.1
Chi-MP	4.1±1.3	1.6±0.6	41.0±17.5	8.2±1.6	48.4±14.3

The results are expressed as the mean±S.D. ($n=5$).

et al.[15] who had reported the drug absorption was caused well and fast at oral administration. Therefore, ketoprofen suspension in the present comparative study was considered to give the proper plasma level of ketoprofen. Chi-MP tended to maintain the plasma level higher and more steadily than MS. The values of T_{max}, $MTR_{0-24\,h}$ and $VRT_{0-24\,h}$ also proposed that Chi-MP should tend to give better systemic retention of ketoprofen.

It has been reported that chitosan coating shows the bioadhesion to the intestinal mucosa, which allows strong and close contact with mucosal membrane and localization of particles at sites showing good absorption, resulting in enhancement of drug absorption.[13,14] These properties may facilitate ketoprofen absorption in Chi-MP; the good bioadhesive property was observed *in vitro* (Fig. 3). However, it will be required for more detailed evaluation to further examine how chitosan coating modifies absorption of the drug.

CONCLUSION

Ketoprofen microspheres were prepared by the dry-in-oil method using ethylcellulose as a matrix polymer and allowed prolonged drug release. The release rate could be modified by addition of polyethylene glycol or hydroxypropylcellulose. Ketoprofen microspheres could be coated easily with chitosan by the precipitation technique using double solvent layers of n-hexane-alkaline aqueous solution. The obtained chitosan-coated ketoprofen microparticles showed good intestinal adhesion *in vitro* and steady maintenance of plasma concentration *in vivo*. Ethylcellulose-induced sustained release and bioadhesion by chitosan might be useful to facilitate sustained action of ketoprofen.

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