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Chondroitin sulfate-based anti-inflammatory macromolecular prodrugs

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ABSTRACT

Macromolecular prodrugs of three non-steroidal anti-inflammatory drugs (NSAIDs), ibuprofen, ketoprofen, and naproxen, were prepared by the covalent attachment of the drugs onto chondroitin sulfate (ChS) using PEG 1000 as a spacer. Drug–PEG adducts were synthesized using 1,1′-carbonyl diimidazole as a coupling agent in dimethyl sulfoxide, followed by the reaction with ChS in highly dilute aqueous solution at pH 6.8 via *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC) as a conjugation agent. The drug–ChS conjugates were confirmed by FTIR, ^{1}H NMR and ^{13}C NMR and the molar percent of drug substitution onto ChS was characterized by ^{1}H NMR using the peak areas of the three protons of –ΦCHCH_3 on the drugs to those of –NHCOCH_3 on ChS. All drug–ChS conjugates are water-soluble. The release amounts of the free drugs from their corresponding drug–ChS conjugates were evaluated in the presence or absence of either esterase or chondroitinase, and the both enzymes in pH 7.4 Tris-buffer solutions at 37 °C by high performance liquid chromatography (HPLC). Keto–ChS conjugates released ~100% ketoprofen within 12 h in the presence of esterase, but the combination with chondroitinase did not accelerate the release rate. The degradation of Keto–ChS conjugates by chondroitinase was confirmed by gel permeation chromatography (GPC). The Keto–ChS conjugates still retained the enzymatic recognition even at the substitution of ketoprofen as high as 56 mol%. The inhibition percent of carrageenan-induced edema of Keto–ChS-56 was comparable to that of a simple blend of ChS and ketoprofen, suggesting that biologically active ChS and ketoprofen could be liberated from the conjugate.

1. Introduction

Chondroitin sulfate (ChS) is a copolymer of D-glucuronic acid and sulfated *N*-acetyl-D-galactosamine in C4 or C6, and is an important structural component in connective tissues and cartilage. ChS can be degraded by anaerobic bacteria, namely *Bacteroides thetaiotaomicron* and *B. ovatus*, which are resident in the large intestine (Saylers, 1979; Salyers and O'Brien, 1980). In the human colon, ChS is present in sloughed epithelial cells and dietary meat. This characteristic suggests that ChS is a potentially good candidate for use as a colon-targeted drug carrier (Rubinstein et al., 1992). ChS has been reported as a good structure/disease modifying anti-osteoarthritis drug (S/DMOAD). The orally administrated ChS reduces the pain

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in osteoarthritis patients especially over long periods compared to the commonly used diclofenac sodium (Morreale et al., 1996; Bourgeois et al., 1998; Volpi, 2004). Although the mechanism of action to alleviate symptoms of osteoarthritis remains unclear, the acceptable mechanism is that ChS inhibits the activities of cartilage degradation enzymes such as collagenase, elastase, and proteoglycanase, etc., and prevents cartilage damage from nitric oxide (NO) free radical (Park et al., 2000).

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used to treat pain, fever, and inflammation. The pharmacological activity of NSAIDs is related to the inhibition of cyclooxygenases (COX) which are responsible for prostaglandin synthesis, and regulate pain and inflammation (Heyneman et al., 2000). The long-term use of NSAIDs may cause gastrointestinal irritation, bleeding and ulceration with side effects including nausea, vomiting, abdominal pain, dyspepsia and diarrhea (Lombardino, 1985). Therefore, it is preferable to temporarily mask the carboxylic group of the NSAID and hence prohibit its direct effect on the gastric mucosa. The "prodrug approach", whereby a drug is linked to a biocompatible polymer via hydrolysable bond, is a widely used method for the controlled and targeted delivery of drugs, while masking side groups that elicit adverse effects of drugs. This methodology also has advantage in the solubilization of drugs, especially to help hydrophobic drugs traverse compartmental barriers. The NSAIDs-linked prodrugs with short chain PEGs have been well studied for dermal applications (Bonina et al., 2001, 2002). All prodrugs are readily hydrolyzed by human plasma and no significant hydrolysis rate is observed with the increase in the length of the oligoethylene chain. These ester prodrugs show the anti-inflammatory activity similar to that of their respective parent drug, but irritate the gastric mucosa to a significantly lesser extent.

NSAIDs-conjugated polysaccharides specifically for colon targeting have been studied on cyclodextrin (Kamada et al., 2002; Yano et al., 2002), dextran (Harboe et al., 1989; Larsen et al., 1991), and pectin (Cheng et al., 2005). Polysaccharide prodrugs of 5-aminosalicylic acid (5-ASA) based on chitosan, hydroxypropyl cellulose, and cyclodextrins have been prepared to examine the effect of solubility of these polysaccharide prodrugs on the release of 5-ASA in the gastrointestinal contents of rats (Zou et al., 2005). Only cyclodextrins-5-ASA releases significantly high amounts of 5-ASA because of its good water solubility. In clinical trials, Rovetta et al. (2004) traced a 2-year study in erosive osteoarthritis (EOA) of the hands using 500 mg naproxen alone (group 1) or a combination with 800 mg ChS (group 2). This study confirmed the partial efficacy of oral ChS in improving some aspects of EOA. In a light review of the above literature, the conjugation of NSAIDs onto polysaccharides not only maintains their biological therapeutic activities of drugs, but reduces the side effects caused by carboxylic groups of NSAIDs as well.

In the present study, the merit of using chondroitin sulfate as a matrix polysaccharide is because of its analgesic and high water-soluble properties. We hypothesize that one conjugate with two different pharmacological moieties would have synergetic anti-inflammatory activity and water solubility even at a high degree of drug substitution. Thus, we conjugated three model NSAIDs, ibuprofen, ketoprofen, or naproxen, onto ChS with the use of a long chain PEG as a spacer. As mentioned before, the anti-inflammatory activity of ChS is due to the inhibition of the NO synthesis and that of NSAIDs is attributed to the inhibition of the COX enzymes. The combination of ChS and NSAIDs as one conjugate may be a promising design in the treatment of degenerative disease in arthritic joints. The temporary masking of free carboxylic groups of NSAIDs via ester linkage in drug–ChS conjugates may prohibit the topical irritancy in GI.

2. Materials and methods

2.1. Materials

Sodium chondroitin sulfate (ChS, oral grade, lot no. OC-97112) was obtained from Tohoku Miyagi Pharmaceutical Co. Ltd. (Tokyo, Japan). Chondroitinase ABC, 1,1′-carbonyl diimidazole (CDI), esterase, 1-hydroxybenzotriazole (HOBT), and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC) were purchased from Aldrich–Sigma Co. (St. Louis, MO) and used as received. Poly(ethylene glycol) PEG with the molecular weight of 1000, 2000, and 4000 was purchased from Showa (Tokyo, Japan). Ibuprofen, ketoprofen, and naproxen (ICI Chemical Co.) were recrystallized from 1:1 v/v of acetone:hexane. Acetonitrile (CH_3CN), chloroform ($CHCl_3$), dimethyl sulfoxide (DMSO), methanol (CH_3OH), and triethylamine were of HPLC grade and were obtained from Tedia (Fairfield, OH). Trizma base and glycine, sodium hydroxide, sodium sulfate were obtained from Fluka (Buchs, Switzerland) and used as received.

2.2. Synthesis of drug–PEG

Poly(ethylene glycol) esters of ibuprofen, ketoprofen, and naproxen were modified from a procedure reported by Bonina et al. (2002) for synthesizing oligoethylene esters of ketoprofen, naproxen and diclofenac. Briefly, 4 mmol of ibuprofen, ketoprofen, or naproxen was separately dissolved in 50 ml of chloroform and then 4.4 mmol of CDI was added portion-wise into each solution and stirred at 0 °C for 4 h. To each solution, 16 mmol of PEG pre-dried overnight in a vacuum, was added. The reaction mixtures were carried out at room temperature for 1 day and then extracted twice sequentially with 50 ml of double deionized (DD) water, 50 ml of 0.1N HCl, 50 ml of DD water, 50 ml of 0.1N NaOH, and 50 ml of DD water. The extracted organic portions were dried over anhydrous sodium sulfate and filtered. The organic solvent was removed by rotary evaporator (Buchs, Switzerland) to yield white solid products. The drug–PEG adducts were abbreviated as Ibu–PEG, Keto–PEG, and Nap–PEG, respectively, for linking with ibuprofen, ketoprofen, and naproxen in the later section.

2.2.1. Ibu–PEG

IR: 1104 cm^{-1} (C–O–C stretching); 1600 cm^{-1} (benzene ring stretching); 1728 cm^{-1} (C=O stretching); 2873 cm^{-1} (C–H stretching); 3445 cm^{-1} (–OH stretching). ^1H NMR (200 MHz, $CDCl_3$), δ (ppm): 0.86 (d, 6H, $-CH(CH_3)_2$); 1.46 (d, 3H, $-\Phi CHCH_3$); 1.70–1.90 (m, 1H, $-CH(CH_3)_2$); 2.40 (d, 2H, ΦCH_2); 3.4–3.7 (m,

–(OCH_2CH_2)$_n$–); 3.75 (m, 1H, –ΦCHCH_3); 4.18 (m, 2H, CO_2CH_2); 7.0–7.3 (m, 4H, Ar*H*). ^{13}C NMR (200 MHz, $CDCl_3$), δ (ppm): 174 (–OCO–); 76–70 (–OCH_2CH_2O–); 45 (–ΦCHCH$_3$); 30 (ΦCH_2); 22 (–CH(CH_3)$_2$); 18 (–ΦCHCH_3).

2.2.2. *Keto–PEG*

IR: 1116 cm^{-1} (C–O–C stretching); 1600 cm^{-1} (benzene ring stretching); 1731 cm^{-1} (C=O stretching); 2874 cm^{-1} (C–H stretching); 3563 cm^{-1} (–OH stretching). ^{1}H NMR (200 MHz, $CDCl_3$), δ (ppm): 1.52 (d, 3H, –ΦCHCH_3); 3.2–3.8 (m, –(OCH_2CH_2)$_n$–); 3.93 (m, 1H, –ΦCHCH$_3$); 4.16 (m, 2H, CO_2CH_2); 7.40–7.85 (m, 9H, Ar*H*). ^{13}C NMR (200 MHz, $CDCl_3$), δ (ppm): 196 (–Φ*C*=OΦ–); 173 (–OCO–); 76–70 (–OCH_2CH_2O–); 45 (–ΦCHCH$_3$); 18 (–ΦCHCH_3).

2.2.3. *Nap–PEG*

IR: 1103 cm^{-1} (C–O–C stretching); 1600 cm^{-1} (benzene ring stretching); 1728 cm^{-1} (C=O stretching); 2875 cm^{-1} (C–H stretching); 3433 cm^{-1} (–OH stretching). ^{1}H NMR (200 MHz, $CDCl_3$), δ (ppm): 1.53 (d, 3H, –ΦCHCH_3); 3.2–3.9 (m, –(OCH_2CH_2)$_n$–, –OCH_3); 3.97 (m, 1H, –ΦCHCH$_3$); 4.21 (m, 2H, CO_2CH_2); 7.11–7.72 (m, 6H, Ar*H*). ^{13}C NMR (200 MHz, $CDCl_3$), δ (ppm): 174 (–OCO–); 76–70 (–OCH_2CH_2O–); 55 (–OCH_3); 45 (–ΦCHCH$_3$); 18 (–ΦCHCH_3).

2.3. *Synthesis of drug–PEG and chondroitin sulfate conjugates (drug–ChS)*

For each drug–PEG, three molar ratios relative to ChS as 2:1, 1:1, and 0.5:1 were prepared. The concentration of ChS was fixed at 1.09 mmol in 150 ml of DD water and the various molar concentrations of drug–PEG were added. After complete dissolution, 1.09 mmol of EDAC and 1.09 mmol of HOBT in 1 ml of 1/1 v/v of H_2O/DMSO were added drop-wise into the reaction solutions, which were adjusted to pH 6.8 by 0.1N HCl or 0.1N NaOH. The reaction was carried out at room temperature for 2 days. Then, the reaction mixture was precipitated into ethanol to remove any unreacted residues. The precipitated products were dried in a vacuum oven at 60 °C. The drug–PEG and ChS conjugates are abbreviated as Ibu–ChS, Keto–ChS and Nap–ChS for ibuprofen, ketoprofen, and naproxen linked.

2.3.1. *Ibu–ChS*

^{1}H NMR (200 MHz, 1/1 v/v, D_2O/DMSO-d_6), δ (ppm): 0.83 (d, 6H, –CH(CH_3)$_2$); 1.32 (d, 3H, –ΦCHCH_3); 1.60–1.80 (m, 1H, –CH(CH_3)$_2$); 1.96 (s, 3H, NHCOCH_3); 2.38 (d, 2H, ΦCH_2); 7.0–7.3 (m, 4H, Ar*H*). ^{13}C NMR (200 MHz, 1/1 v/v, D_2O/DMSO-d_6), δ (ppm): 175.7 (–Drug–COO–); 174.8 (ChS–COO–); 174.3 (–NHCOCH_3–); 69–67 (–OCH_2CH_2O–); 44.5 (–ΦCHCH$_3$); 29.6 (ΦCH_2); 22.4 (–NHCOCH_3); 21.9 (–CH(CH_3) $_2$); 17.9 (–ΦCHCH_3). Yields: ~44.3%

2.3.2. *Keto–ChS*

^{1}H NMR (200 MHz, 1/1 v/v, D_2O/DMSO-d_6), δ (ppm): 1.36 (d, 3H, –ΦCHCH_3); 1.96 (s, 3H, NHCOCH_3); 7.14–7.55 (m, 9H, Ar*H*). ^{13}C NMR (200 MHz, 1/1 v/v, D_2O/DMSO-d_6), δ (ppm): 197.9 (–ΦC=OΦ–); 175.2 (–Drug–COO–); 174.6 (ChS–COO–); 174.1 (–NHCOCH_3–); 69–67 (–OCH_2CH_2O–); 44.5 (–ΦCHCH$_3$); 22.3 (–NHCOCH_3); 17.9 (–ΦCHCH_3). Yields: ~51.9%.

2.3.3. *Nap–ChS*

^{1}H NMR (200 MHz, 1/1 v/v, D_2O/DMSO-d_6), δ (ppm): 1.37 (d, 3H, –ΦCHCH_3); 1.95 (s, 3H, NHCOCH_3); 7.04–7.45 (m, 9H, Ar*H*). ^{13}C NMR (200 MHz, 1/1 v/v, D_2O/DMSO-d_6), δ (ppm): 175.8 (–Drug–COO–); 174.6 (ChS–COO–); 174.1 (–NHCOCH_3–); 69–67 (–OCH_2CH_2O–); 54.9 (–OCH_3); 44.7 (–ΦCHCH$_3$); 22.3 (–NHCOCH_3); 17.4 (–ΦCHCH_3). Yields: ~47.0%.

2.4. *Drug–ChS hydrolysis*

Ten milligrams of each sample was dissolved in 1 ml of Tris-buffer at pH 7.4 at 37 °C. At certain intervals, the solvent was removed by freeze-drying in a vacuum. The free drug was extracted from the polymer residue with 5 ml of acetone. The supernatant acetone solution was then withdrawn and evaporated using a rotary evaporator to produce a white solid. The resultant residue was dissolved in 1 ml of ethanol and analyzed by HPLC on a Hewlett-Packard 1100 system containing a quaternary pump, an online-degassed auto sampler, a HP 1100 photodiode array detector, and a C18 column (HP Spherisob ODS-2 column). Samples were filtered with 0.45 μm millipore filters and eluted with the volume ratio of phosphoric acid solution at pH 3.0:acetonitrile as 65%:35% at 1 ml/min. The eluent was monitored at 254 nm for ketoprofen, 271 nm for naproxen, and 263 nm for ibuprofen, respectively. The column oven was set at 50 °C. Quantities of the hydrolysates in the solution were determined by comparing the HPLC peak area with the calibration intensities obtained from the pure drugs. Each data was averaged with three measurements.

2.5. *Drug–ChS hydrolysis with enzymes*

The enzymatic hydrolysis of drug–ChS was conducted using a similar procedure as stated above except for adding enzymes in Tris-buffer at pH 7.4. The amounts of enzymes are five units for esterase and 0.5 units for chondroitinase ABC. The enzymatic hydrolysis was tested using esterase or chondroitinase ABC individually or in combination.

2.6. *Degradability of Keto–ChS by chondroitinase ABC*

The degradation of Keto–ChS was conducted by dissolving a 10 mg sample in 1 ml Tris-buffer at pH 7.4 with 0.5 units of chondroitinase ABC at 37 °C. At certain intervals, 10 μl of solution was injected into gel permeation chromatography (GPC). The molecular weight was measured using a Shodex sugar KS-G, a KS-804 column equipped with a Water Model 501 pump and a HP 1047 refractive index detector. An aqueous solution of 0.05 M NaCl was used as a mobile phase at a flow rate of 1 ml/min at 50 °C. The column setting was calibrated with four monodisperse dextran standards.

2.7. *Animal experiments*

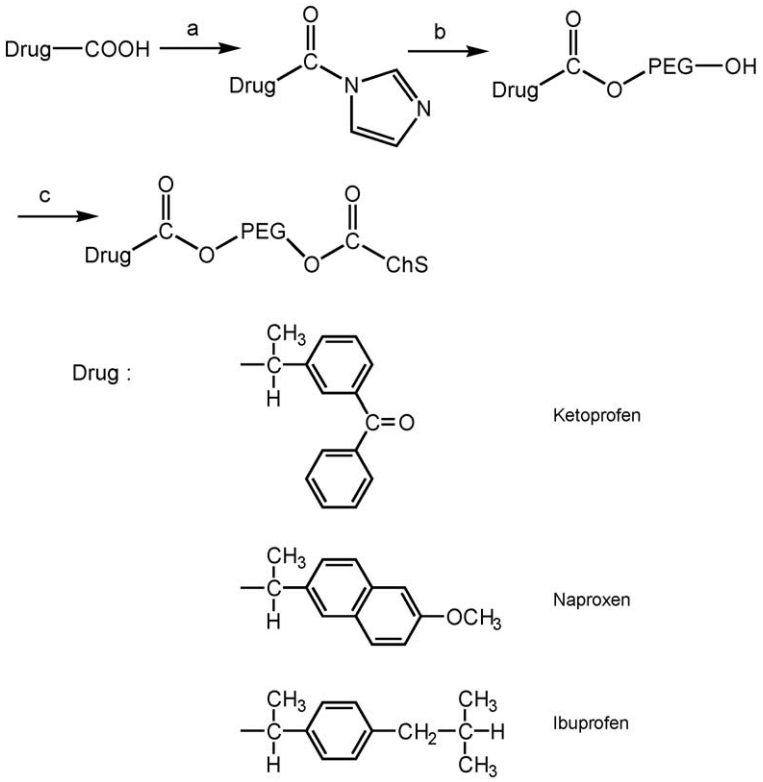
The anti-inflammatory activity was evaluated using a carrageenan-induced edema test in rat paws according to the technique reported by Winter et al. (1962). Male Wistar rats (250–300 g) were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). Rats were fasted for 24 h with free access to water and divided into III

groups. Group I served as a control group without being fed. Groups II and III were fed with ChS (7.14 mg) and ketoprofen (1.00 mg), and with Keto–ChS-56 (8.14 mg), respectively. The individual weight of ChS and ketoprofen used for the blend was calculated based on 56 mol% substitution of ketoprofen onto chondroitin sulfate. Each group had seven rats. Disc pellets were orally administered. Ten minutes after administration, the rats were anaesthetized with an i.p. injection of urethane (3 ml/kg). After 30 min each rat received in its right hind paw a subplantar injection of a 0.1 wt.% carrageenan in normal saline (λ-carrageenan, type IV, Sigma, 0.1 ml/rat). The measurement of the hind paw volume was carried out using an Ugo Basile Plethysmometer model 7150, before any treatment (V_0) and in any intervals (V_t) after the administration. All results were expressed as means ± S.E.M. Statistical evaluations were performed using analysis of variance (ANOVA) followed by the Newman–Keul's test for subgroup comparison.

3. Results and discussion

3.1. *Drug molar percent in drug–ChS*

Scheme 1 illustrates the three-step reaction of drugs onto ChS. Three NSAIDs, ibuprofen, ketoprofen, and naproxen linked to PEG were confirmed by NMR and FTIR. As seen in Fig. 1(a), the FTIR spectrum of Keto–PEG shows a broad stretching absorption around 3500 cm^{-1} attributed to the hydroxyl groups of PEG and a ester absorption at 1731 cm^{-1}, which are absent in pure ketoprofen. The ethylene oxide segments in drug–PEG adducts appear as multiple absorption peaks around 3.2–3.8 ppm in ^1H NMR and 70–76 ppm in ^{13}C NMR besides the characteristic absorption peaks of the drugs. The newly appeared peaks of ester carbon were obtained at 173 or 174 ppm after the conjugation reaction between PEG and the drugs. These confirmed drug–PEG adducts were further used to conjugate with ChS. The FTIR spectra of drug–ChS were combined with the spectra of drug–PEG and pure ChS. Fig. 1(b) illustrates the FTIR spectrum of Keto–ChS. The further supporting data supplied by ^1H and ^{13}C NMR have been indicated in Section 2. The peak areas at 1.32–1.37 ppm due to the three protons of –ΦCHCH_3 on the drugs, and those at 1.95–1.96 ppm due to the three protons of –NHCOCH_3 on ChS were used to calculate the drug molar percent in drug–ChS conjugates. For example, the ^1H NMR spectrum of Keto–ChS in Fig. 2 is the 56 mol% substitution of ketoprofen when the molar feed ratio between Keto–PEG and ChS is 2:1. The ^{13}C NMR spectrum of this conjugate was illustrated in Fig. 3 to show that the four carbonyl signals were observed at 197.9, 175.2, 174.6 and 174.1 ppm, corresponding to –ΦC=OΦ–, –Drug–COO–, ChS–COO–, and –NHCOCH_3, respectively. The drug molar percents were organized in Table 1 using three different feed ratios between drug–PEG and ChS. The numeral after the code indicates the drug molar percent in a conjugate. The drug molar percent increases with the increase in the feed ratio of drug–PEG/ChS and maintains a similar value in the conjugates with the same feed ratio of 1/2 or 1/1 among three model drugs. When the molar



Scheme 1 – The conjugation reactions of drugs and chondroitin sulfate using PEG as a spacer. (a) CDI, $CHCl_3$, 0 °C, 4 h; (b) PEG, RT, 1 d; (c) ChS, EDAC/HOBT, DDW, rt, 2 d.

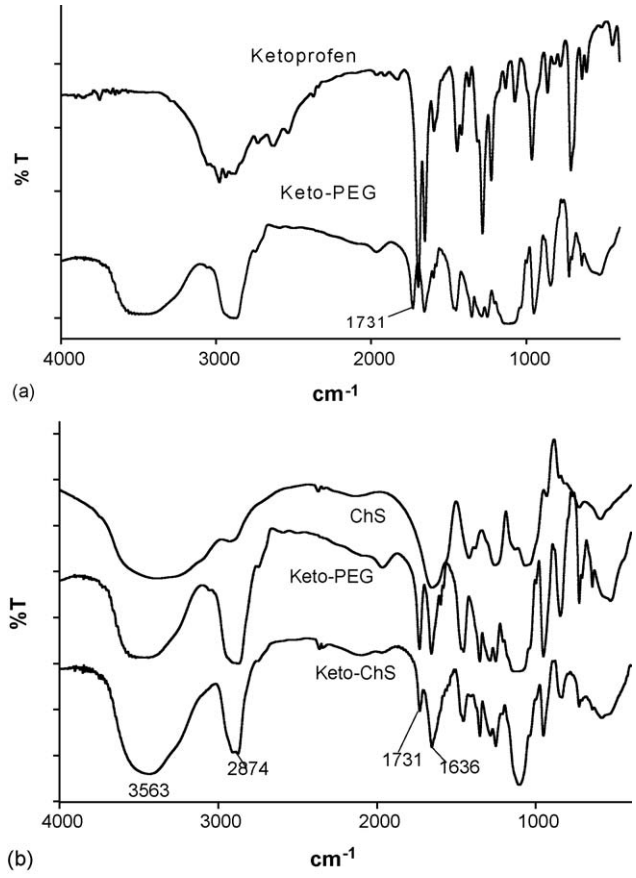


Fig. 1 – (a) FTIR spectra of ketoprofen and Keto–PEG; (b) chondroitin sulfate, Keto–PEG, and Keto–ChS.

Table 1 – Molar percent of drug in drug–ChS with various molar feed ratios of drug–PEG and ChS

	Molar ratio in feed ChS:drug–PEG	Molar percents of drug[a]	Code name
Ibuprofen	2:1	10.7	Ibu–ChS-10
	1:1	28.3	Ibu–ChS-28
	0.5:1	65.2	Ibu–ChS-65
Ketoprofen	2:1	9.2	Keto–ChS-9
	1:1	24.4	Keto–ChS-24
	0.5:1	56.1	Keto–ChS-56
Naproxen	2:1	10.4	Nap–ChS-10
	1:1	26.6	Nap–ChS-26
	0.5:1	66.8	Nap–ChS-66

[a] Determined by NMR.

ratio of drug–PEG/ChS is 2/1, the difference of the 10 mol% was observed between Keto–ChS-56 and Nap–ChS-66. Since the minimum energies of trimers of these three drugs linked with 2-hydroxyethyl methacrylate (HEMA) have been calculated from the molecular modeling (Wang et al., 2002), the results show minimum energy increases in the sequence of Ibu–HEMA, Nap–HEMA, and Keto–HEMA. The largest minimization energy value indicates the largest steric hindrance to free rotation along the polymer chain. Thus, the larger steric hindrance may result in the lower degree of substitution.

3.2. *Hydrolysis of drug–ChS*

Two hydrolysable ester groups are present in the drug–ChS conjugates. In principle, the free drugs can be released using two routes as illustrated in Scheme 2. The drug–ChS conjugates were hydrolyzed through a competing mechanism: to

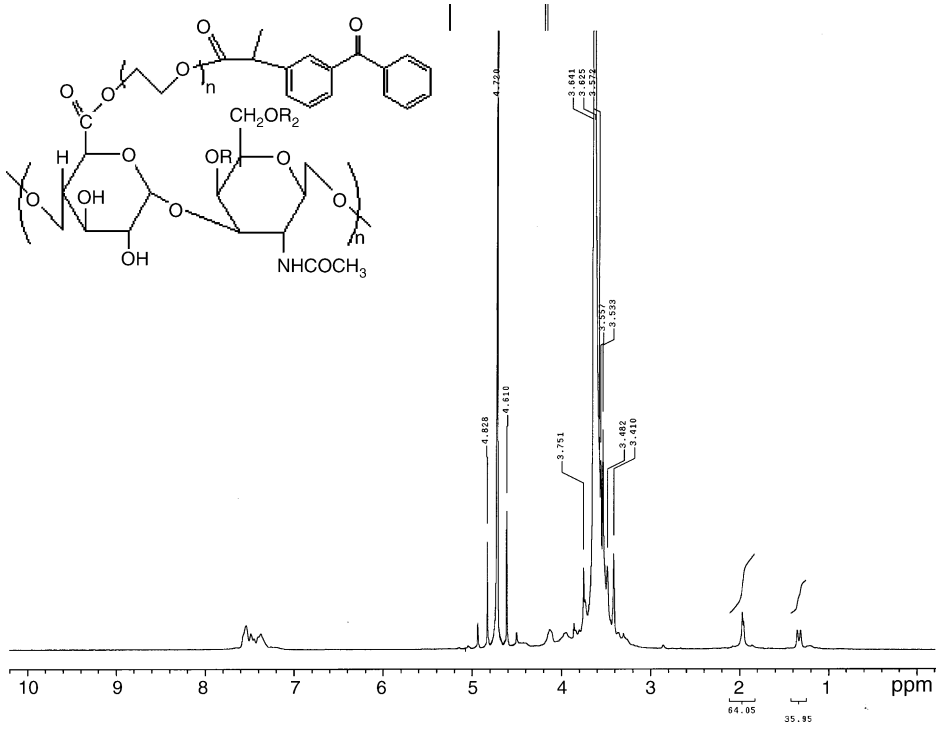


Fig. 2 – ^{1}H NMR spectrum of Keto–ChS-56.

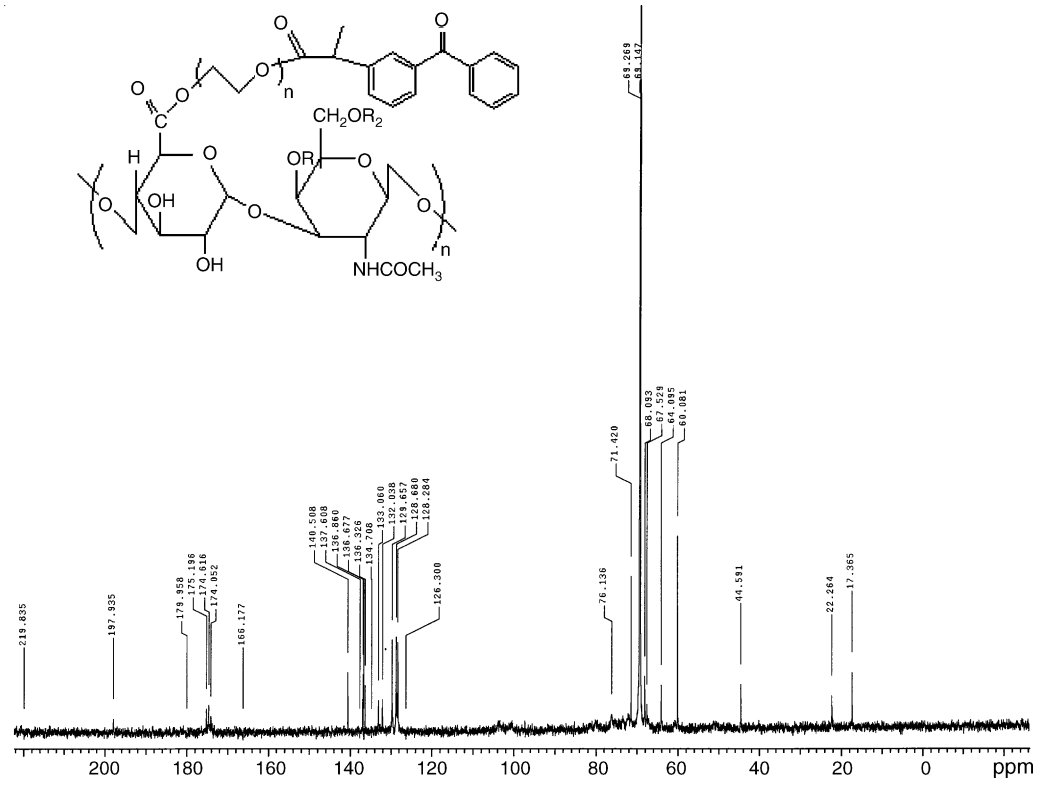
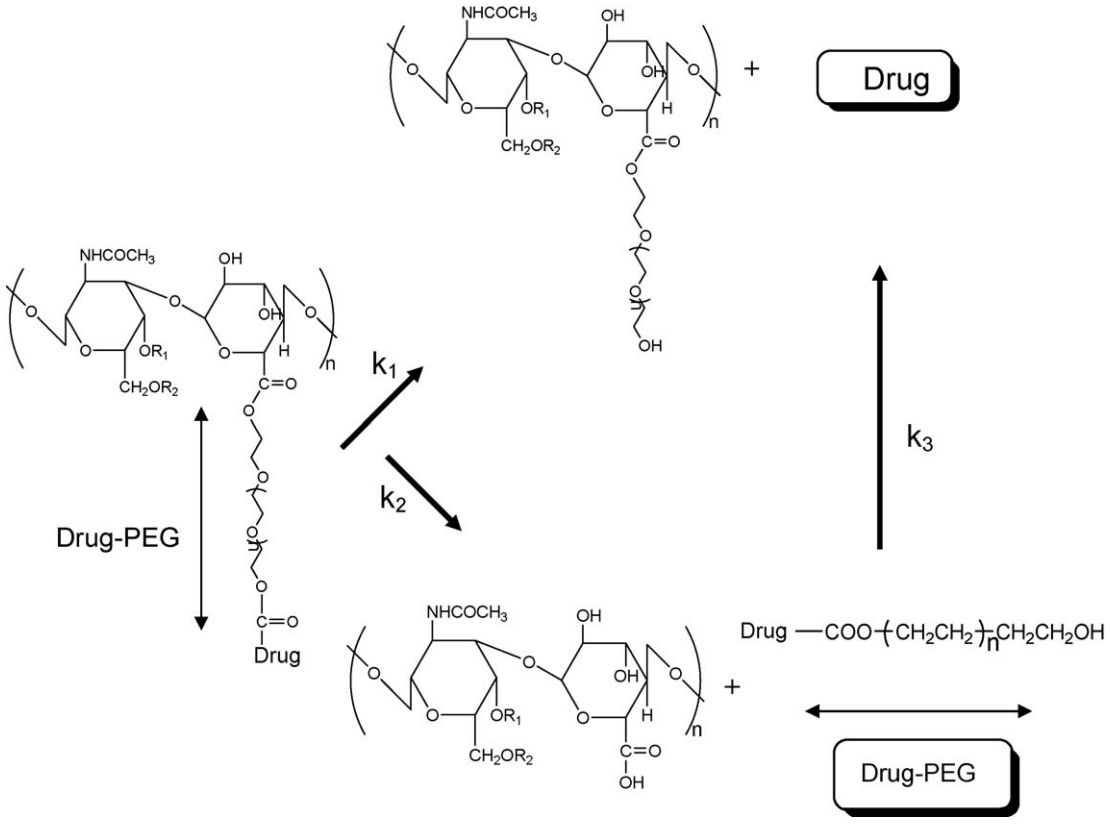


Fig. 3 – ^{13}C NMR spectrum of Keto–ChS-56.



Scheme 2 – The hydrolysis routes of free drugs from drug–ChS conjugates.

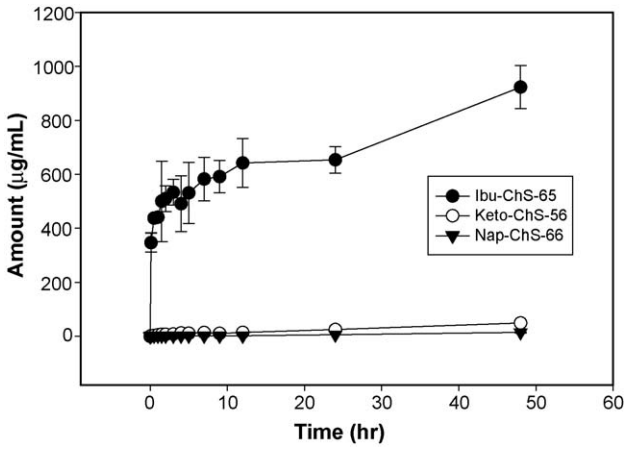


Fig. 4 – Hydrolysis profiles of drug–ChS conjugates at pH 7.4 Tris-buffer.

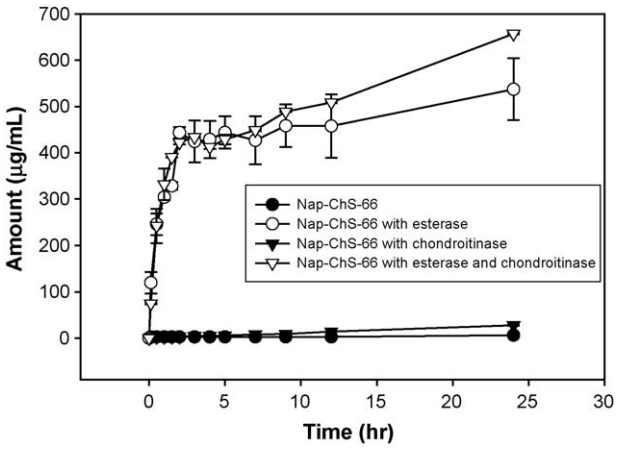


Fig. 6 – Hydrolysis profiles of Nap–ChS-66 in the different enzyme conditions at pH 7.4 Tris-buffer.

release free drugs directly (k_1) or drug–PEG adducts (k_2), which could be further hydrolyzed into free drugs and PEG (k_3). However, due to the inability to separate the large molecules such as drug–ChS and drug–PEG in the HPLC study, only the total amounts of the free drugs were quantitatively measured in the drug–ChS conjugates regardless of the hydrolysis routes. Fig. 4 displays the accumulated amounts of free drugs in Tris-buffer at pH 7.4 using the highest drug contents in the drug–ChS conjugates. As shown, the release amount of ibuprofen is ~600 g/ml from Ibu–ChS-65 within 24 h but is unnoticeable from Keto–ChS-56 and Nap–ChS-66. The hydrolysis studies were further carried out in the presence of esterase or chondroitinase alone or in combination. The release amount of ibuprofen is slightly higher in the presence of esterase but no synergetic effect was observed using esterase and chondroitinase together. The same experiments were carried out in Keto–ChS-56 and Nap–ChS-66 and the results are presented in Figs. 5 and 6. Apparently, the amounts of the free drugs released from Keto–ChS-56 or Nap–ChS-66 are much higher in the presence of esterase. A combination of chondroitinase and esterase does not increase the release amounts of ketoprofen or naproxen. This insensitivity of the molecular weight to the

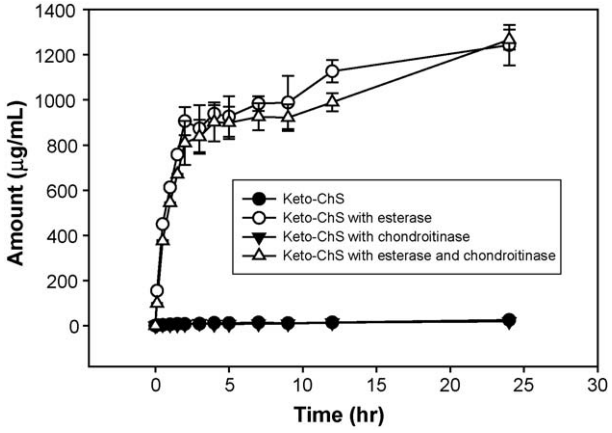


Fig. 5 – Hydrolysis profiles of Keto–ChS-56 in the different enzyme conditions at pH 7.4 Tris-buffer.

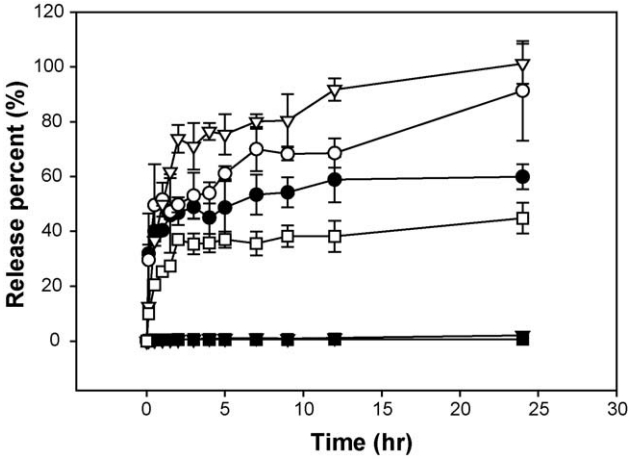


Fig. 7 – Drug release percentage based on the theoretical drug concentrations determined by NMR. (●, ○) Ibu–ChS-65; (▼, ▽) Keto–ChS-56; (■, □) Nap–ChS-66. The filled legends represent the hydrolysis at pH 7.4 Tris-buffer and the open ones are using esterase.

drug release from drug–ChS, is in agreement with the findings in dextran–naproxen ester prodrugs (Harboe et al., 1989). All exhibit 100% bioavailability to parent naproxen independent of the molecular weights of dextran (molecular weight was used from 10,000 to 500,000 g/mol). An alternative plot was adopted to easily compare the catalytic efficiency of enzymes to substrates among the three drug–ChS conjugates. The drug release percentage (relative to the drug content determined by NMR) was plotted in Fig. 7. The drugs released from the drug–ChS conjugates appears massively at 2–3 h and slightly increases thereafter. It is obvious that Keto–ChS-56 shows the highest efficiency in the hydrolysis of ester bonds. Almost 100% of ketoprofen is released within 12 h under esterase catalysis.

The release percentage of Ibu–ChS-65 in the presence and absence of esterase is similar at the first 5 h, but subsequently increases in the presence of esterase. The catalytic efficiency by esterase in Ibu–ChS-65 is not as promising as in Keto–ChS-56 or Nap–ChS-66. Similar results have been found

in the copolymers of methacrylic acid and 2-hydroxyethyl methacrylate linked with the three drugs (Wang et al., 2002). The enzymatic hydrolysis of ester bonds is highly sensitive to the hydrophilicity of the substitute itself. The solubility values are 0.205, 0.0264 and 0.0111 g/l, respectively to ketoprofen, naproxen and ibuprofen in water at 37 °C (Kyuki, 1982). Although the three drug–ChS conjugates are still highly water-soluble, we believe that the characteristics of a pure drug itself impact the hydrolysis behavior significantly.

Because ChS has been modified by the drug moieties at the high degree of substitution (DS), it may alter the enzyme-specific recognition. Thus, the molecular weight of Keto–ChS was checked after enzymatic degradation. Fig. 8 shows the GPC profiles of pure ChS and the Keto–ChS conjugates. The retention peak that appeared around 7.8 min does not shift with the introduction of the ketoprofen in Keto–ChS-9 and Keto–ChS-24, but a slight shoulder peak around 6.2 min appears in Keto–ChS-56. The number average molecular weight calculated based on four mono-disperse dextran standards are 159, 145, 143 and 127×10^3 g/mol corresponding to Keto–ChS-56, Keto–ChS-24, Keto–ChS-9, and ChS, respectively. The molecular weight increases with the molar percent substitution of ketoprofen onto the Keto–ChS conjugate. The degradation profiles of Keto–ChS-56 in the presence of chondroitinase (Fig. 9) are similar to those of pure ChS (Tsai et al., 2005). That the retention peak appeared at 7.8 min shifts into the longer time implies the recognition between chondroitinase and the Keto–ChS substrate even at the 56 mol% of ketoprofen substitution. To our knowledge, no polysaccharides have been reported to be degradable at this high DS. The flexible PEG 1000 spacer definitely plays an important role because in the drug–HEMA systems, the conjugates become insoluble when the drug molar percents are higher than 30 mol% (Wang et al., 2002). The improvement of therapeutic efficacy of compounds has been reported in using long chain PEGs as the spacer (Conforti et al., 1991; Greenwald et al., 2003) but in NSAIDs-linked prodrugs with short chain PEGs (Bonina et al., 2001, 2002), no significant hydrolysis rate was reported with the increase in the length of the oligoethylene chain, therefore the effect of the PEG length on the hydrolysis rate of drug–ChS conjugates is most interesting. Fig. 10 shows the hydrolysis

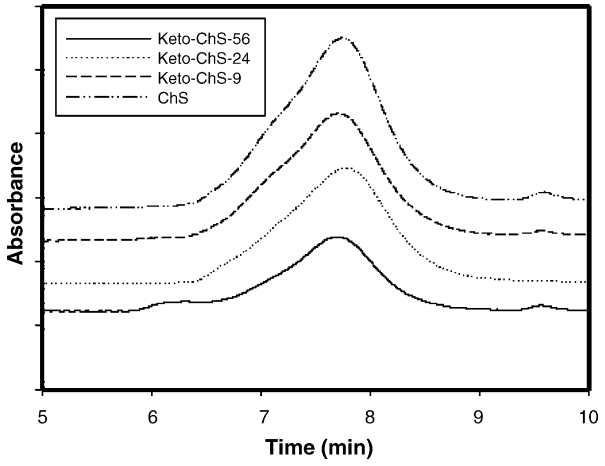


Fig. 8 – GPC diagrams of Keto–ChS with the various drug contents.

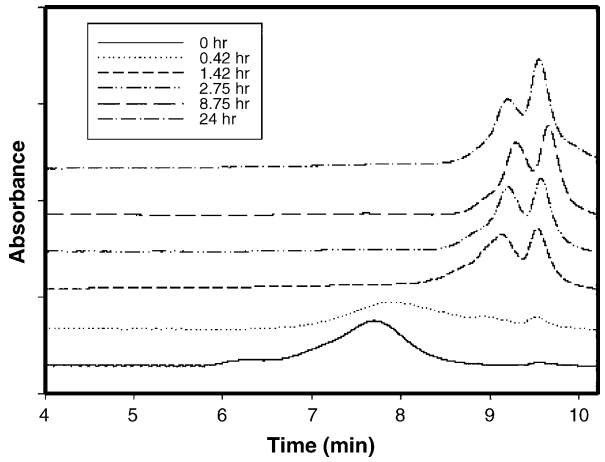


Fig. 9 – Degradation profiles of Keto–ChS-56 vs. time in the presence of chondroitinase by GPC.

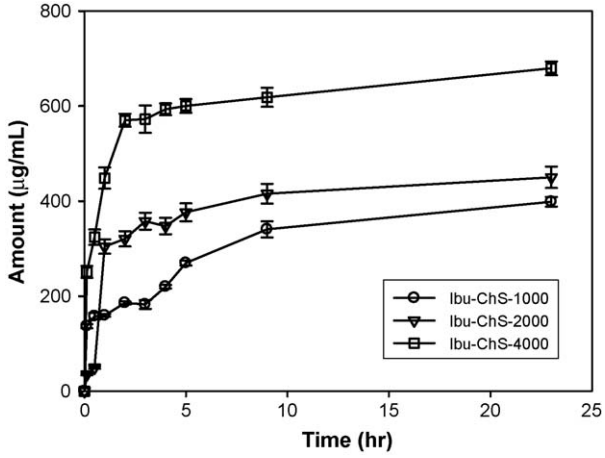


Fig. 10 – Hydrolysis profiles of Ibu–ChS conjugates with the various PEG molecular weight at pH 7.4 Tris-buffer.

profiles for three Ibu–ChS conjugates with the PEG molecular weights of 1000, 2000, and 4000 when the feed ratio of Ibu–PEG/ChS was fixed at 1/2. The hydrolysis rate increases with the PEG molecular weight in the Tris-buffer solutions at pH 7.4. Using the long chain PEG as the spacer indeed accelerates the release rate in this study.

3.3. *Anti-inflammatory test*

The anti-inflammatory effect is represented by the percentage of swelling and inhibition calculated using Eqs. (1) and (2):

$$\text{Swelling (\%)} = \frac{V_t - V_0}{V_0} \times 100 \quad (1)$$

$$\text{Inhibition (\%)} = \frac{(V_t - V_0)_{\text{control}} - (V_t - V_0)_{\text{treat}}}{(V_t - V_0)_{\text{control}}} \times 100 \quad (2)$$

where V_0 is the average volume in the hind paws of rats ($n = 7$) before any treatment and V_t the average volume after the inflammatory agent injection. Pretreatment with

Table 2 – Percentage of swelling (inhibition) carried out on carrageenan-induced edema test in rats ($n = 7$)

Time (h)	2	5	7	8	9	11	13	15
Control	22.40 ± 9.28	45.93 ± 7.21	49.93 ± 7.78	42.57 ± 9.04	60.43 ± 6.46	56.17 ± 7.68	51.38 ± 9.20	40.34 ± 10.45
ChS + Keto	14.89 ± 12.30 (33.53)	11.60 ± 6.28* (74.74)	15.14 ± 11.98* (69.68)	13.08 ± 9.90* (69.27)	12.76 ± 11.89* (78.88)	12.86 ± 8.97* (77.10)	18.26 ± 13.00* (64.46)	16.40 ± 14.67* (59.34)
Keto–ChS-56	18.95 ± 8.99 (15.40)	22.67 ± 7.15* (50.64)	20.34 ± 5.08* (59.26)	18.56 ± 8.51* (56.40)	15.67 ± 6.27* (74.07)	13.37 ± 6.81* (76.20)	8.56 ± 6.87*,# (83.33)	10.87 ± 6.36*,# (73.05)

Data are presented as means ± S.D., $n = 7$, of swelling (%) calculated from Eq. (1), with inhibition (%) calculated from Eq. (2) given in parentheses.

* $P < 0.05$, compared with control group.

\# $P < 0.05$, compared with Keto + ChS.

ChS + Ketoprofen and Keto–ChS-56 started to reduce swelling percentages magnificently at 5 h but the high swelling percentage was maintained in the control group for all time points (Table 2). The inhibition level of Keto–ChS-56 increased up to 13 h. The simple blend of ChS and ketoprofen reduced the swelling promptly but the Keto–ChS conjugate resulted in the reduction of edema swelling over a prolonged period of time. The inhibition percent of free ketoprofen reached the maximum value of 67.6% at 2 h and decayed after 6 h (Wang et al., 2002) but that of Keto–ChS-56 increased up to 83.3% within 13 h. The synergetic anti-inflammatory activity with the use of NSAIDs and ChS has been found to be maintained over long periods compared with use of an NSAID only (Morreale et al., 1996; Tsai et al., 2005).

4. Conclusions

Three NSAIDs (ibuprofen, ketoprofen, or naproxen)-linked ChS prodrugs were successfully synthesized with the use of PEG as a spacer. This design not only solved the low water-solubility of NSAIDs but also temporarily masked the free carboxylic groups of NSAIDs with ester linkage to prohibit the topical irritancy in GI. Moreover, one conjugate of the analgesic ChS and NSAIDs could bring about a synergetic anti-inflammatory effect and the delayed release of the drugs. The hydrolysis of labile ester linkages between the drugs and ChS were accelerated with the use of esterase. However, no synergetic catalysis was observed in the presence of esterase and chondroitinase in the three conjugates. The large amount of ketoprofen was released from Keto–ChS-56 probably due to its high water solubility. The degradation of ChS sugar main chains did not accelerate the enzymatic hydrolysis of the ester bonds between the drugs and ChS, and the enzymatic recognition was still retained in Keto–ChS-56, that is the highest degree of substitution found in literature. The anti-inflammatory activity of Keto–ChS-56 was better than pure ketoprofen in the carrageenan-induced edema test. In principle, any drugs containing carboxylic functional groups can be linked onto ChS with PEG as a spacer using the same conjugation chemistry in this study.

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