Development of conjugate of prednisolone with chondroitin sulfate and its potentiality for the treatment of arthritis

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CONTENTS

LIST OF PUBLICATIONS

Conjugate between chondroitin sulfate and prednisolone with a glycine linker: preparation and *in vitro* conversion analysis.: Onishi H., Matsuyama M., Chem. Pharm. Bull. (Tokyo) 61(9):902-912 (2013)

In Vivo evaluation of chondroitin sulfate-glycyl-prednisolone for anti-arthritic effectiveness and pharmacokinetic characteristics.: Onishi H., Isoda Y., Matsuyama M., Int. J. Pharm. 45, 113-20 (2013)

Chondroitin Sulfate-glycyl-prednisolone conjugate as arthritis targeting system: localization and drug release in inflammatory joints.: Onishi H., Yoshida R., and Matsuyama M., Biol. Pharm. Bull. (accepted)

ABBREVIATIONS

AUC	Area under the plasma concentration–time curve
CDI	*N,N*-Carbonyldiimidazole
CS	Chondroitin sulfate
CS-GP	Conjugate of chondroitin sulfate-glycyl-prednisolone
DDS	Drug Delivery System
DMAP	4-Dimethylaminopyridine
DMARDs	Disease-modifying anti-rheumatic drugs
EI-MS	Electron ionization-mass spectrometry
EPR	Enhanced permeability and retention
GP	Glycine ester of PD
HPLC	High performance liquid chromatography
MALDI-TOF-MS	Matrix assisted laser desorption/ionization-time-of-flight mass spectrometry
MW	Molecular weight
NHS	*N*-Hydroxysuccinimide
NSAIDs	Non-steroidal anti-inflammatory drugs
PBS	Phosphate-buffered saline
PD	Prednisolone
PEG	Polyethylene glycol
RA	Rheumatoid arthritis
TLC	Thin layer chromatography
TMS	Tetramethylsilane
Tr-GP	*N*-Trityl-glycine ester
WSC	1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride

GENERAL INTRODUCTION

There are many effective drugs in clinical usage, but some are restricted for dosage or dosing period due to several adverse effects. The reasons of these adverse effects are thought to be caused by the non-specific body distribution and subsequent unwanted biological function. In order to maximize efficacy and minimize adverse effect of drugs, it is necessary to control the pharmacokinetics and body distribution of drugs and achieve the optimum conditions for an appropriate time period. Such an approach is so called Drug Delivery System (DDS). The technique to deliver and release a drug to a target region in the body is called drug targeting, and lot of researches have been attempted because it could lead to enlarge the beneficial potential of existing and new drugs.

The drug targeting therapy has been examined in cancer and inflammatory diseases. This therapy utilized the phenomenon called enhanced permeability and retention (EPR) effect. The neovascular vessels surrounding cancer and inflammatory lesions have poor formation of endothelial cells and a tendency to extravasate the high molecular plasma contents, which is not observed in normal blood vessels. In addition, although the extravasation is resorbed via the lymphatics in normal tissues, such a resorption system is not fully developed, resulting in the accumulation of the extravasation.[1-3)]

High molecule weight polymer-linked drugs, and liposomes or nanoparticles containing drugs have been developed actively, and many have been examined as a targeted therapy. They have achieved certain success to deliver the drugs to target region effectively.[4-9)] However, some problems such as prolonged remaining of the residual material in target tissue after the release of pharmaceuticals and immunological responsiveness to the systems still remain, leading to the requirement of further improved systems.[8, 9)]

Steroids are one of the most potent drugs for treatment of inflammatory

diseases including rheumatoid arthritis.[10-12)]

On the other hand, the drugs are used carefully in clinical practice, because of several risk of adverse effects, for example increased susceptibility to infection, osteoporosis, peptic ulcer, and hyperlipidemia.[13-16)]

In this thesis, novel high molecular weight polymer-drug conjugate was synthesized, in which prednisolone (PD) is linked to chondroitin sulfate (CS), and examined its pharmacological effect using rats with adjuvant-induced arthritis, which is one of the arthritis models widely used for evaluation of anti-rheumatic drugs.[10, 17)] Finally, in order to elucidate the targeting mechanism of the conjugate, its pharmacokinetic characteristics and drug distribution to the arthritic tissues were investigated using the same model.

As for the linkage between PD and CS in the conjugate, an ester bond which is susceptible to hydrolysis under physiological conditions was chosen;[18-22)] that is an amino acid, glycine, was used as a linker for the combination between CS and PD. Although an ester bond is easily cleaved by enzymes in physiological conditions, the hydrolysis of the ester bond is expected to be suppressed in the conjugated form, because CS might form a steric barrier and protect the ester from enzymes.[23, 24)] In addition, CS is one of glycosaminoglycans, and used as an injectable drug or health care supplement; namely, it has high safety and thought to be reasonable material as drug carrier.[25-33)]

In chapter 1, a novel conjugate between PD and CS with glycine as a linker was prepared and examined the property and feasibility as a prodrug of PD *in vitro*. First, PD was converted to the *N*-trityl-glycine ester (Tr-GP), and the glycine ester of PD (GP) was obtained by detritylation of Tr-GP. Then, GP and CS were condensed with water-soluble carbodiimide to yield the conjugate (CS-GP). The characteristics as a prodrug of PD were estimated by measuring the release of PD in buffered solutions of different pH values. The release tests

were performed under the conditions with rat plasma. Furthermore, as a preliminary study, the efficacy of CS-GP was estimated using rats with adjuvant-induced arthritis.[34)]

In chapter 2, CS-GP with different PD contents was synthesized and estimated their conversion characteristics at different pH conditions. Next, anti-inflammatory effects of CS-GP, PD, CS and CS/PD mixture were examined using rat adjuvant-induced arthritis, and their efficacies were compared one another. Also, the pharmacokinetic properties of CS-GP and organ distributions were investigated in normal rats.[35)]

In chapter 3, in order to analyze the pharmacological mechanism of CS-GP, pharmacokinetic features and drug localization and retention were examined for CS-GP and PD. CS-GP and PD were administered to rats with adjuvant-induced arthritis, and plasma concentrations of the free PD released from CS-GP and the total PD (conjugated PD and free PD) were monitored. Further, the drug distribution in the inflammatory joints was investigated at 1, 7 and 24 h after the administration to rats with adjuvant-induced arthritis.[36)]

Chapter 1

Conjugate between Chondroitin Sulfate and Prednisolone with a Glycine Linker: Preparation and *in Vitro* Conversion Analysis

1. Introduction

Rheumatoid arthritis (RA) is a chronic disease that causes inflammation of the synovial membrane located inside the articular capsule. It is characterized by progressive destruction of the joints, involving synovial hyperplasia, synovial cell activation and articular inflammation.[37-39] Currently, the disease is a major problem worldwide, with many people suffering.[40] The symptoms of the disease, including persistent pain, stiffness and joint swelling, lead to deterioration of the quality of life (QOL). Both genetic and environmental factors are considered to be involved in the etiology.[4, 41, 42] The treatment of RA includes surgery to improve joint function, physiotherapy to recover motor function, and pharmacotherapy to ameliorate inflammation and joint destruction. As pharmacotherapy, non-steroidal anti-inflammatory drugs (NSAIDs),[10, 11, 43] disease-modifying anti-rheumatic drugs (DMARDs)[44] and glucocorticoids have been mainly used to manage various disease states.[10-12, 45, 46] In addition, recently, many antibody drugs have been developed actively, and their use is spreading progressively[47-50]; however, the aforementioned conventional drugs still play an important role in the treatment of RA. In particular, glucocorticoids are very highly potent and fast-acting agents in the treatment of RA; however, their chronic use often causes severe systemic side effects such as diabetes, osteoporosis and adrenal failure, resulting in their limited use.[13-16]

Therefore, recently, various attempts have been made to improve the use of glucocorticoids in the treatment of RA.[4-6, 23, 51, 52] As their efficacy is associated with the drug concentration at the diseased site, specific delivery of the drug to the target site is considered to be a key to the promotion of efficacy At the same time, such specific delivery to the target site is considered to enable the reduction of systemic side effects by decrease in their transfer to the other parts and/or reduction of the dose. These approaches are known as targeted delivery.

For example, liposomal formulations, polymer–drug conjugates and nanoparticles have been delivered to inflammatory sites such as swelling joints.[4-7] These systems are based on the enhanced permeability and retention (EPR) effect, which is caused physiologically because neovascular vessels develop at the diseased site of RA.[5-7, 53]

In this study, we focused on chondroitin sulfate (CS) as a carrier macromolecule, because CS is highly safe and can be applied intravenously and intramuscularly.[25-27] To date, no toxic or adverse effects have been reported with the use of CS.

Although CS, injected intravenously, is excreted into urine to a large extent, some of the excreted CS-related molecules exhibit a high molecular weight similar to that of the original polymer, and some of them appear in the degradation form of oligosaccharides or inorganic sulfate ions.[52, 54] From these pharmacokinetic features, CS is considered to behave as a polymer to a certain extent in the systemic circulation. Namely, CS is expected to contribute to systemic retention. In fact, prolonged systemic circulation and elevation of the area under the plasma concentration–time curve (AUC) were reported in the CS–cisplatin conjugate.[55] These safety and pharmacokinetics of CS suggest that CS should be a useful drug carrier for a delivery system of RA treatment.

As far as we know, conjugates between CS and glucocorticoids for the treatment of RA have hardly been reported. Regarding CS–drug conjugates for anti-inflammatory therapy, Peng et al. reported conjugates of CS and NSAIDs, which were tested for their effect on carrageenan-induced edema.[26] These conjugates were found to exhibit a prolonged effect. In the present study, prednisolone (PD), used often for the treatment of RA, was chosen as the glucocorticoid agent. The design of the conjugate of PD with CS was performed by taking into account the following matters. i) In order to enable the hydrolysis at the inflammatory acidic pH, the chemical bond such as hydrazone and cis

aconityl group could be proposed as an adequate linker because they are susceptible to hydrolysis at acidic pH with the fairly high stability at physiological pH.[56, 57] Also, a peptide linker is another choice because they can be hydrolyzed by various peptidases in the tissues or cells after the localization there.[58] However, in the case of glucocorticoid drugs with hydroxy groups, those chemical combinations are not necessarily easy to synthesize. In the case of PD, being one of the glucocorticoid drugs, the ester linkage is the most simple. In fact, various ester prodrugs for glucocorticoid drugs, including macromolecule–glucocorticoid conjugates, have been examined by many researchers.[18-22] ii) If macromolecule–glucocorticoid conjugates are used for the delivery to the arthritic inflammatory site, it is required that they are stable to a fair extent at the systemic physiological condition and that they can release the active drug at an adequate rate at the target site. Although an ester bond is generally more susceptible to hydrolysis at higher pH, such requirements are considered to be achieved to a fair extent with the ester linkage of the macromolecular prodrugs. Namely, the ester bond is generally fairly stable at the systemic pH of 7.4, though it depends on the chemical structure. Furthermore, the ester linkage in the macromolecular prodrugs is generally stable against enzymatic hydrolysis due to the steric hindrance to the esterase.[23,24] These stability characteristics of the ester bond in the macromolecular prodrugs suggest that the pH-dependent drug release might be caused simply at the tissues, which enables *in vivo* release to be more predictable as compared to other designs of prodrugs. iii) In addition, although the pH value tends to be acidic at the arthritic inflammatory site, it is reported to vary from pH 7.4 to 6.0, dependent on the diseased states,[61] suggesting that the ester bond, subjected to hydrolysis widely at weakly basic, neutral and weakly acidic pH, should be adequate as a linker for the activation. iv) In the case of design of the ester prodrug of PD with CS as a carrier, a bi-functional linker

with a carboxy group and an amino group is considered to be useful for the combination with the hydroxyl group of PD and the carboxy group of CS. Therefore, amino acids and peptides are proposed as an available linker; in particular, a simple amino acid, glycine, is suggested as a candidate for the linker. For instance, Conover et al. produced the conjugate of polyethylene glycol and camptothecin using a glycine linker, and investigated the pharmacokinetics and antitumor effect; the conjugate showed the gradual release in the conditions of physiological pH and rat plasma and achieved the fairly fast delivery to the diseased site, resulting in high antitumor effect.[59] Considering these chemical and biological conditions on the arthritis pharmacotherapy, glycine was employed as a linker in the present study. The produced conjugate was examined here for the detailed characteristics for the stability, drug release and pharmacological potential. The preparation of the conjugate was attempted as follows. First, PD was derivatized to a glycine ester of PD, named GP, and then the conjugate between CS and GP, called CS-GP, was prepared by a carbodiimide coupling method. GP and CS-GP were analyzed for *in vitro* conversion behaviors in different pH media and for the effect of plasma on the conversion. Also, a preliminary pharmacological study was conducted using rats with adjuvant-induced arthritis[10, 17] to confirm the effectiveness of CS-GP.

2. Materials and Methods

2.1. Materials

PD, *N,N*-carbonyldiimidazole (CDI), 4-dimethylaminopyridine (DMAP), *N*-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride(WSC) and chondroitin sulfate C (CS) sodium salt (CS-Na) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The CS-Na was derived from shark cartilage and contained 2.6 % N and 6.6 % S. For its composition, the ratio of 6-sulfate to 4-sulfate was 9 : 1, that is, 6-sulfate is

major and the molecular weight (MW) was 40,000–80,000. *N*-Tritylglycine (Tr-G) was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Heat-killed and desiccated *Mycobacterium tuberculosis* M37Ra was obtained from Difco Laboratories (Detroit, MI, U.S.A.) and used as an adjuvant. All other chemicals were of reagent grade.

2.2. Physical Measurements

A UV-VIS. absorption spectra were recorded using a Beckman DU640 spectrophotometer. NMR spectra were measured with a JEOL Lambda-500 (500 MHz) spectrometer, in which tetramethylsilane (TMS) was used as a reference with chemical shift of 0 ppm. MS spectra were obtained with a JEOL JSM600 mass spectrometer for electron ionization-mass spectrometry (EI-MS) and with an AXIMA–CRF PLUS mass spectrometer (Shimadzu Corp., Kyoto, Japan) for matrix assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS).

2.3. Animals

Male Wistar rats (7 weeks old; weighing 200–210 g) were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). Lewis female rats (8 weeks old, 150–160 g) were obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan). They were bred on the breeding diet MF supplied by Oriental Yeast, Co., Ltd. (Tokyo, Japan) with water ad libitum at 23±1 °C and relative humidity of 60±5 %. They were used for the experiments a few days after purchase. The experimental protocol was approved by the Committee on Animal Research of Hoshi University, Japan. The animal experiments were performed in compliance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, Japan.

2.4. Preparation of CS-PD Conjugate with a Glycine Linker

Glycyl-prednisolone (GP) was synthesized by the two step method. First, the Tr-G ester of PD, named Tr-GP, was synthesized as follows. Tr-G (477 mg, 1.5 mmol) and CDI (243 mg, 1.5 mmol) were dissolved in 10 mL tetrahydofuran (THF) at 0 °C, and stirred for 30 min at 0 °C. DMAP (15 mg, 0.12 mmol) and PD (270 mg, 0.75 mmol) were added to the solution, and the mixture was stirred at room temperature for 4.5 h. The solvent was evaporated, and the resultant residue was dissolved in several milliliters of a mixture of chloroform and methanol (90 : 1, v/v). The resultant solution was added to a silica gel column [3 cm (inner diameter)×30 cm (length); silica gel: 230–400 mesh size (Merck KGaA, Darmstadt, Germany)], and eluted using a mixture of chloroform and methanol (90 : 1, v/v) as a solvent at a flow rate of 3 mL/min using a pump. The eluted solution was fractionated, and the eluted compounds were assessed by thin-layer chromatography (TLC), which was performed using pre-coated DC Kieselgel 60 F_{254} plates (No. 1.05715.0001; Merck). The spots on TLC were detected by UV light at 254 nm. The fractions [*Rf*=0.08 with chloroform–methanol (90 : 1, v/v)] were collected, and the solvent was evaporated. The yield of Tr-GP was 400 mg. ^{1}H-NMR (DMSO-d_6), δ (ppm): 7.20–7.42 (m, 16H, Tr-G Tr-H, PD C1-H), 6.15–6.18 (d, 1H, PD C2-H), 5.92 (s, 1H, PD C4-H), 5.02–5.05 (d, 1H, PD C21-H´), 4.72–4.75 (d, 1H, PD C21-H), 4.29 (s, 1H, PD C11-H), 2.97–2.99 (m, 2H, Tr-G C-H_2). EI-MS m/z: 659 (M+·).

GP was obtained by detritylation of Tr-GP using aqueous acetic acid. Namely, 75 % (v/v) aqueous acetic acid (10 mL) was added to Tr-GP (300 mg) and heated at 75 °C to dissolve Tr-GP completely. Immediately after the white powder started to precipitate, the mixture was cooled on the ice and kept for 30 min. After the white precipitate was removed by the filtration, the solvent of the filtrate was evaporated. The residue was dissolved in several milliliters of a mixture of chloroform and methanol (15 : 1, v/v) and underwent column

chromatography using a silica gel column in the same manner as stated above except that the mixture of chloroform and methanol (15 : 1, v/v) was used as the elution solvent. Each fraction was assessed by TLC as above. The fractions in which *Rf* was 0.23 [chloroform–methanol (15 : 1, v/v)] were collected, and the solvent was evaporated. The yield of GP was 60 mg. ^{1}H-NMR (DMSO-d_6), δ (ppm): 7.31–7.33 (d, 1H, PD C1-H), 6.15–6.17 (d, 1H, PD C2-H), 5.92 (s, 1H, PD C4-H), 4.97–5.00 (d, 1H, PD C21-H), 4.84–4.87 (d, 1H, PD C21-H´), 4.27–4.38 (m, 3H, glycine C–H_2, PD C11-H). MALDI-TOF-MS m/z: 418.54 ($[M+ H]^+$).

CS and GP were conjugated by carbodiimide coupling to prepare CS-GP conjugate. CS (120 mg) was dissolved in 10 mL water, and 2.5 mL THF containing 30 mg GP was added. WSC (500 mg) and NHS (300 mg) were added to the solution, and the resultant mixture was stirred at room temperature for 4.5 h. The solution was chromatographed with a Sephadex G50 gel column [2.8 cm (inner diameter) ×19 cm (length)] using 0.1 M NaCl aqueous solution as the elution solvent. The eluted solution was fractionated at 10 mL each. The high MW fractions were collected, and the mixture was dialyzed against water at 4 °C. The final solution was lyophilized. The amount of the product (CS-GP) was 120 mg.

2.5. Measurement of PD Content in CS-GP

PD, CS and CS-GP were dissolved in a mixture of phosphate-buffered saline (PBS) at pH 7.4 and methanol (7 : 3, v/v) at concentrations of 12.5, 622 and 528 μg/mL, respectively. The PD content of CS-GP was calculated by comparing the net absorbance at 246 nm of the conjugated PD, obtained from UV absorption profiles of CS and CS-GP, with the absorbance at 246 nm of PD alone.

In addition, CS-GP (1.2 mg) was dissolved in 0.4 mL water, and PBS (1.2

mL) and methanol (0.533 mL) were added. To this solution, 0.1 M NaOH aqueous solution was added at the ratio of 1 : 1 (v/v). The resultant solution was incubated at 45 °C under horizontal shaking at 60 rpm. Aliquot samples (50 μL) were withdrawn at 10, 20, 25 min after the start of incubation. Immediately after sampling, 0.1 M acetate buffer of pH 4 (150 μL) was added to stop the hydrolysis. After the addition of 150 μL of the mobile phase of high performance liquid chromatography (HPLC), the resultant solution was analyzed by HPLC for the regenerated PD. The content of PD in CS-GP was estimated from the maximal amount of regenerated PD.

2.6. *In Vitro* Conversion Examination for GP in Various pH Media

GP was dissolved in a mixture of methanol and aqueous buffer (1 : 3, v/v) to obtain solutions at a concentration of 60 μg/ml. As to the aqueous buffers, 0.1 M acetate buffer of pH 4, 1/15 M phosphate buffer at pH 6 and 8, and PBS (pH 7.4) were used. Each solution (0.5 mL) was incubated at 37 °C under horizontal shaking at 60 rpm. At appropriate time points, aliquot samples (50 μL) were withdrawn. To this sample, 0.1 M acetate buffer of pH 4 (150 μL) was added to stop the hydrolysis of GP. After the addition of 150 μL of the HPLC mobile phase, the resultant solution was analyzed by HPLC for both GP and PD.

2.7. *In Vitro* Conversion Examination of CS-GP in Various pH Media

After CS-GP (1.2 mg) was dissolved in 0.4 mL water, aqueous buffer (1.2 mL) and methanol (0.533 mL) were added. Regarding the aqueous buffers, the same buffers as stated above in the GP incubation were used. Each solution (0.5 mL) was incubated at 37 °C under horizontal shaking at 60 rpm. At appropriate time points, aliquot samples (50 μL) were withdrawn. These samples were treated in the same manner as stated in the incubation of GP, and analyzed similarly by HPLC.

2.8. *In Vitro* Conversion Examination of GP and CS-GP in PBS–Plasma Mixture

A blood sample was taken from Wistar rats via the jugular vein using a heparinized syringe. Plasma was obtained by centrifugation of the blood and subsequent collection of the supernatant. A mixture of PBS and the plasma (10 : 3, v/v) was used as the incubation medium. GP and CS-GP were dissolved in the medium at concentrations of 7.7 and 230 μg/mL, respectively. Each solution (1.95 mL) was incubated at 37 °C under horizontal shaking at 60 rpm. At appropriate time points, aliquot samples (100 μL) were withdrawn. To each sample (100 μL), saturated NaCl aqueous solution (100 μL) and 6 % (v/v) phosphoric acid aqueous solution (100 μL) were added. Then, 4 mL of the mixture of *t*-butyl-methyl ether and n-pentane (3 : 2, v/v) was added, and the solution was shaken vigorously. After centrifugation of the mixture at 1400×g for 10 min, 3 mL of the resultant supernatant was taken and evaporated to dryness under nitrogen gas. To the residue, 100 μL of the HPLC mobile phase was added, and 20 μL of the resultant solution was injected on the HPLC column to analyze the concentration of PD.

2.9. Preliminary Anti-inflammatory Examination Using Rats with Adjuvant-Induced Arthritis

CS-GP was examined in order to identify the pharmacological potential. Namely, *in vivo* potential was investigated based on the extent of the anti-inflammatory effect and its duration using Lewis rats with adjuvant-induced arthritis. First, arthritis was induced as follows. Heat-killed *Mycobacterium tuberculosis* M37Ra (20 mg) was suspended in 4 mL liquid paraffin. The suspension (100 μL) was injected intracutaneously into the pad of the right hind paw of each rat. CS-GP saline solution (0.14–0.18 mL) was then injected intravenously via the jugular vein at 2 mg PD equivalent (equiv.)/kg on the day when the paw swelling reached the plateau. That is, CS-GP was administered 16

and 17 d after injection of the adjuvant; that is, total dose= 2×2 mg PD equiv./kg. On appropriate days after adjuvant injection, non-treated and CS-GP-injected rats were weighed and the volume of both hind paws investigated, in which the volume of the hind paw was measured by immersing it into water and reading the buoyant force.

In addition, for the preliminary study of the PD therapeutic potential, PD was investigated for the suppressive effect on the paw volume. Namely, PD solution in 30 % (w/v) PEG400 solution in saline was injected intravenously via the jugular vein at 2.5 mg PD equiv./kg on the day when the paw swelling reached a maximal level. That is, PD was administered 15 and 16 d after injection of the adjuvant; that is, total dose= 2.5×2 mg PD equiv./kg/mL. On appropriate days after adjuvant injection, non-treated and CS-GP-injected rats were weighed and the volume of both hind paws investigated in a similar manner as above.

2.10. HPLC Assay

As the HPLC apparatus, a Shimadzu LC-6AD pump equipped with a Shimadzu SPD-10AV VP UV-VIS detector and a Shimadzu C-R7A Chromatopac was used. An YMC Pack ODS-AM column (6 mm inner diameter×150 mm length; YMC Co., Ltd., Kyoto, Japan) was used as the analytical column. The detector was set at a wavelength of 246 nm, and a 26 % (v/v) 2-propanol aqueous solution containing 0.1 % (v/v) trifluoroacetic acid was used as the mobile phase. The HPLC assay was conducted at room temperature. The injection volume was set at 20 μL. The concentration of GP and PD was determined by the absolute calibration curve method. For samples obtained by extraction with organic solvent, only PD was analyzed by the absolute calibration method using standard samples treated in the same manner as the tested samples.

2.11. Statistical Analysis

For statistical analysis, the unpaired *t*-test was used, and significant difference was set as $p < 0.05$.

3. Results and Discussion

3.1. Chemical Characteristics of GP and CS-GP

A flow chart for the preparation of CS-GP is illustrated in Fig. 1. For Tr-GP, it was observed that the chemical shifts of protons at the C21 of PD (4.04–4.09, 4.47–4.51 ppm) changed to those of 4.72–4.75 and 5.02–5.05 ppm. This change in the chemical shifts of C21-H_2 to a low field indicated ester formation between the carboxy group of Tr-G and the hydroxyl group at the C21 position of PD. Furthermore, the signals in the high field of 0–3 ppm were observed to be derived from protons derived from PD (data not shown). In addition, the binding ratio between Tr-G and PD was calculated to be 1 : 1 (mol/mol) from their signal integrated intensities. Mass spectra also supported the structure of Tr-GP. Furthermore, ^{13}C-NMR spectra DEPT135°, DEPT90°, heteronuclear multiple bond connectivity (HMBC) and heteronuclear multiple quantum coherence (HMQC) (data not shown) supported the chemical structure of Tr-GP in Fig. 1.

GP was obtained by acidic hydrolysis of Tr-GP. In GP, the ester formation at the C21 position of PD was confirmed from the ^{1}H-NMR spectra, and the signals at 0–3 ppm was observed to be derived from PD (data not shown). Also, the binding ratio between glycine and PD was confirmed to be 1 : 1 (mol/mol) from the signal integrated intensities. ^{13}C-NMR spectra indicated that the compound contained 23 carbons, and DEPT135° revealed two methylene carbons at 66.36 and 50.81 ppm, which were obviously derived from C21 and glycine methylene carbon, respectively. In addition, HMQC showed that C21 was coupled with the protons at 4.97–5.00 and 4.84–4.87 ppm and that the

glycine methylene carbon was directly combined to the protons at 4.27–4.38 ppm. Mass spectra also supported the structure of GP. From these data, GP was identified as a glycine ester of PD at the C21 position as shown in Fig. 1.

CS-GP was prepared by carbodiimide coupling using WSC and NHS. The ^{1}H-NMR spectrum showed that CS-GP was formed by the combination of GP and CS. GP had a similar absorption curve in shape to that of PD, with the maximal wavelength at 246–247 nm in the mixture of PBS and methanol (7 : 3, v/v) (data not shown). The UV absorption profiles were compared among PD, CS-GP and CS (Fig. 2) for the drug content estimation. The PD content in CS-GP was calculated to be 2.24 % (w/w) from the net absorbance of the conjugated PD at 246 nm. The results by HPLC analysis after the alkaline hydrolysis of CS-GP gave a near content, which was approximately 80 % of the value estimated by UV absorbance. Furthermore, the content calculated by the UV absorbance was also consistent with that estimated from integrated intensities of the proton signals in the ^{1}H-NMR spectrum of CS-GP. Finally, the UV absorption measurement was employed to determine the PD content of CS-GP because it was the simplest and clearest approach. The MW distribution of CS-Na was checked using the ultrafilter (Amicon Ultracel®, MW cutoff: 30,000, 50,000, 100,000) produced by Millipore Corp. (Billerica, U.S.A.). The filtrate eluted with the ultrafilter was measured spectrophotometrically at 246 nm. As a result, 80 %, 70 % and 14 % of CS-GP was eluted with the ultrafilters with MW cutoff of 100,000, 50,000 and 30,000, respectively, which suggested that MW of CS-GP was mainly distributed at 30,000–50,000. This supported that the original MW of CS (MW 30,000–80,000), reported by the supplier, was preserved well in CS-GP.

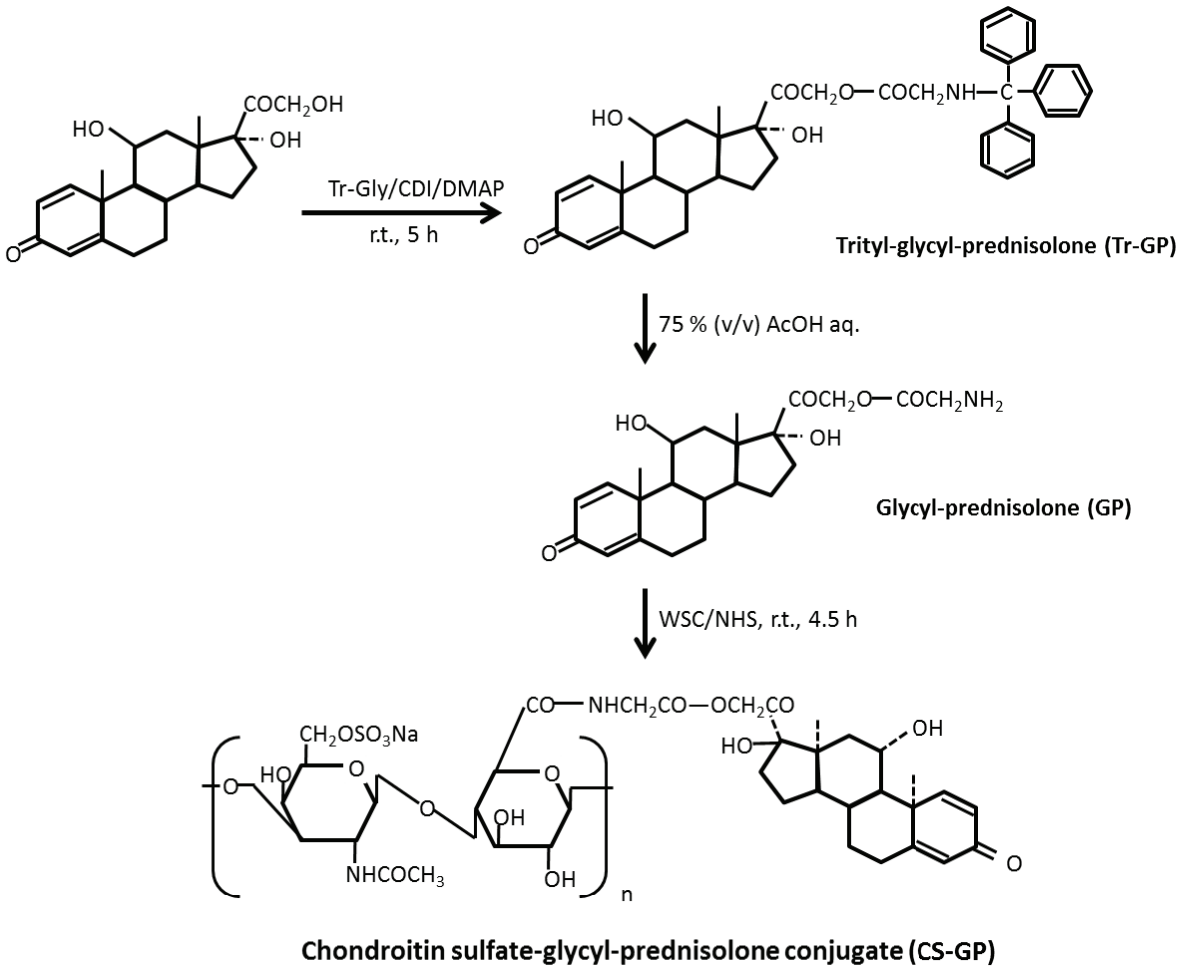


Fig. 1. Synthetic Procedures and Chemical Structures of Tr-GP, GP and CS-GP

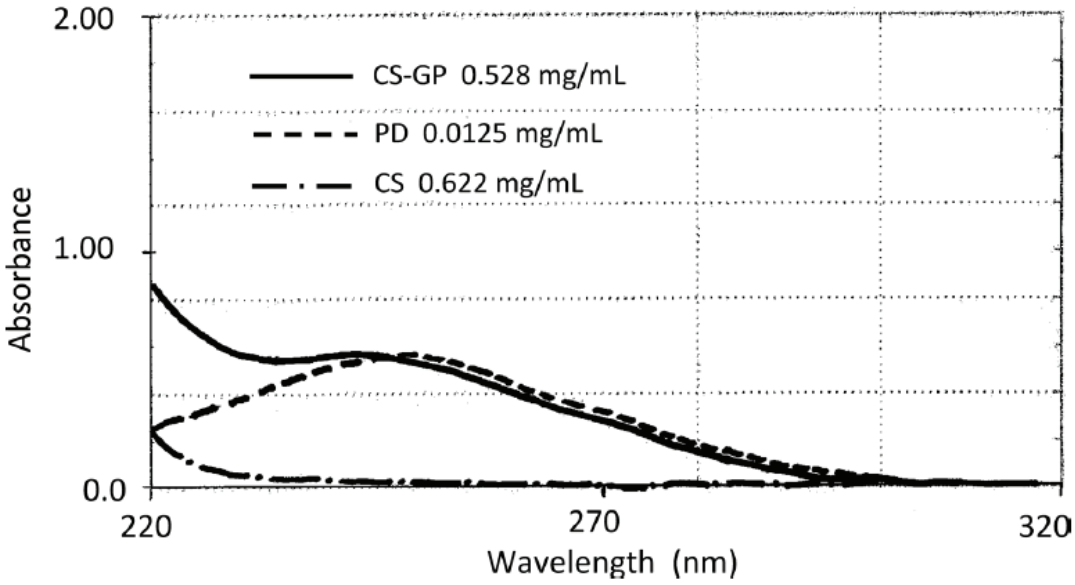


Fig. 2. UV Absorption Spectra of PD, CS and CS-GP

3.2. Hydrolytic Conversion of GP at Different pH Media

As it took some time for the powder of PD and GP to completely dissolve in the aqueous buffer alone, methanol was added at the degree not to influence the pH of the buffer, which allowed the rapid dissolution of the whole powder of GP and PD. In HPLC analysis, the retention times of GP and PD were 7.6–7.7 and 10.2–10.3 min, respectively (Fig. 3); therefore, GP and PD could be determined simultaneously. The degradation profiles of GP were obtained as shown in Fig. 4a. The stability of GP fell with the increase of pH of the media. At the same time, PD was released by the degradation of GP. The detected PD amount displayed a good mass balance with the degraded GP amount at acidic pH, but the mass balance became lower at weakly alkaline conditions, particularly at pH 8. Although GP was decomposed almost completely at pH 8 by 1 h incubation, the appearing PD was only 11.4 and 12.8 % at 1 and 4 h, respectively (Fig. 4b). Based on these observations, the conversion process was suggested as shown by the scheme in Table 1. The conversion rate was analyzed according that scheme.

First, the stability of PD was checked independently using the same incubation media. PD was stable at pH 4 and 6, but degraded slowly at pH 7.4 and somewhat fast at pH 8 (Fig. 5). As the remaining amount did not change greatly at these pH values, it was difficult to decide whether the degradation follow the pseudo-zero order or pseudo-first order kinetics. In the previous paper,[23] PD was found to be degraded at pH 9 obviously in the mono-exponential decline manner (remaining percentage: 78.6 % at 7 h, 40.5 % at 24 h, 19.1 % at 48 h). Therefore, from the analogy to the stability in the weakly basic pH, the degradation profile of PD ($P_{PD}(t)$) was considered to follow the pseudo-first order kinetics in the present study. Namely, it could be expressed by Eq. 1. Also, the conversion of GP was analyzed with the pseudo-first order kinetics according to the scheme in Table 1. The equations for

the remaining GP ($G(t)$) and appearing PD ($P_{GP}(t)$) were expressed as shown in Eqs. 2 and 3.

$$P_{PD}(t) = P_{PD}(0) \times \exp(-h_0 t) \quad (1)$$

$$G(t) = G(0) \times \exp(-(h_1 + h_2)t) \quad (2)$$

$$P_{GP}(t) = G(0) \times h_1 \times \frac{\exp(-(h_1 + h_2)t) - \exp(-h_0 t)}{h_0 - h_1 - h_2} \quad (3)$$

The analysis was conducted by fitting the equation to the plots represented by the mean observed values. The h_0 values were calculated by fitting Eq. 1 to the observed profiles in Fig. 5. The h_1 and h_2 values were obtained by fitting Eqs. 2 and 3 to the observed profiles in Figs. 4a, b, respectively, with the simultaneous fitting method using the MULTI program,[60] in which the h_0 values were fixed to the calculated values obtained by Eq. 1. The obtained values for the parameters are shown in Table 1. When the individual profile of each incubation sample, not being the mean profile, was processed in a similar manner, minus values were obtained in some cases, in particular, the stability parameter of PD itself. Furthermore, the variation was fairly large in some case, especially, the stability parameter of PD itself. Considering these points, the fitting was conducted to the observed mean profile, expressed in percentage. The half-lives of GP and PD were calculated with ln $2/(h_1+h_2)$ and ln $2/h_0$. The calculated curves were superimposed with various lines in Figs. 4 and 5. The calculated profiles were well-fitted to the observed ones, supporting the conversion of GP followed the pseudo-first order kinetics. The peculiarity of GP conversion was observed at pH 8. Namely, GP degraded quickly at pH 8, but PD appeared to a slight extent, indicating abnormality in mass balance. In fact, many peaks, considered to exhibit various degradation products, were observed in the HPLC at pH 8. The glycine moiety appeared to promote the decomposition of GP at the parts other than the ester bond, though the detailed

mechanism was unknown. The calculated conversion profiles were well-fitted to the observed ones, even at pH 8. The large h_2 values at pH 8 explained less conversion of GP to PD.

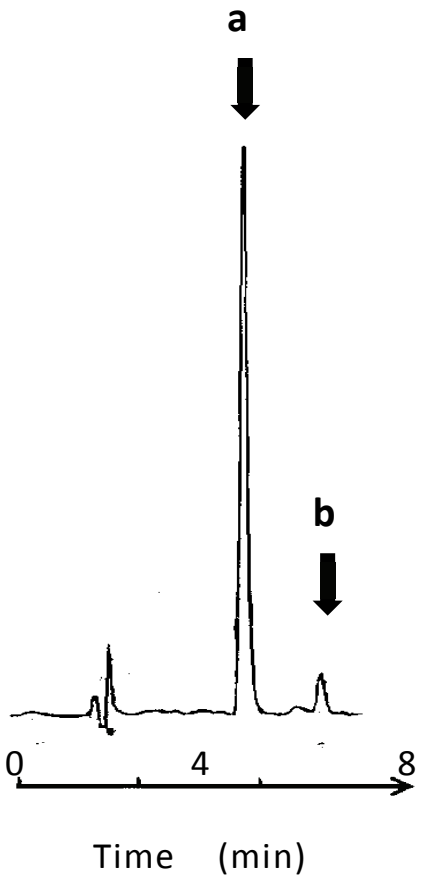


Fig. 3. HPLC Chromatogram in Conversion Studies of GP at 1 h after the Incubation at pH 4 and 37 °C

Peak a: GP, peak b: PD.

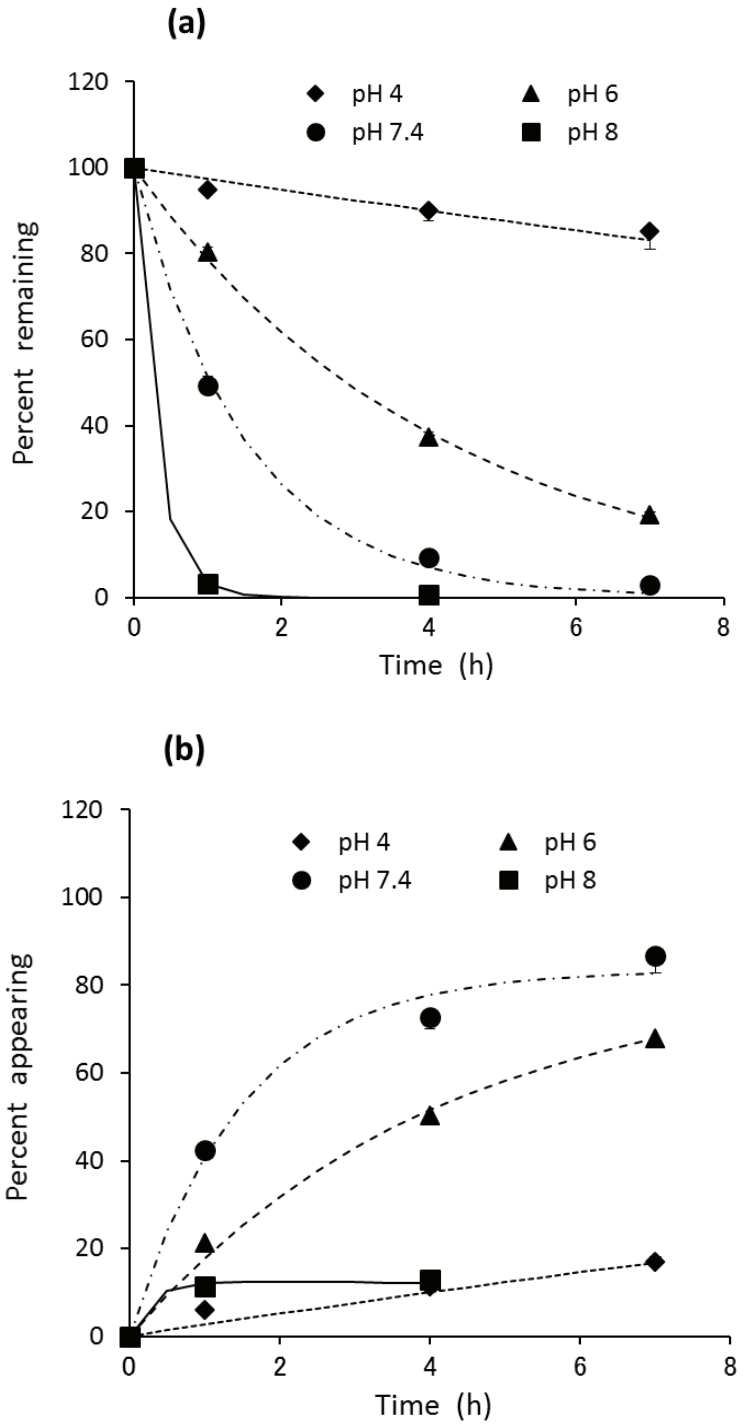


Fig. 4. Conversion Profiles of GP in Different pH Media at 37 °C

(a) Remaining GP (%), (b) appearing PD (%). Each result is expressed as the mean ± S.D. (n=3). The dotted, broken, dashed-dotted and solid lines are calculated curves for the observed profiles (mean values) at pH 4, 6, 7.4 and 8, respectively.

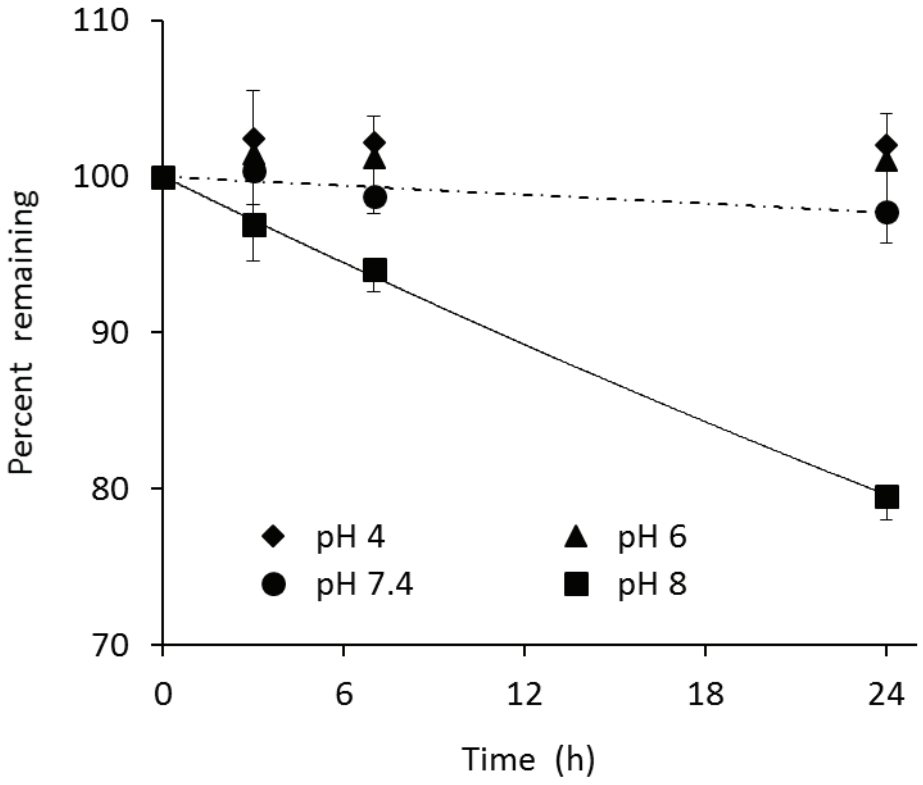
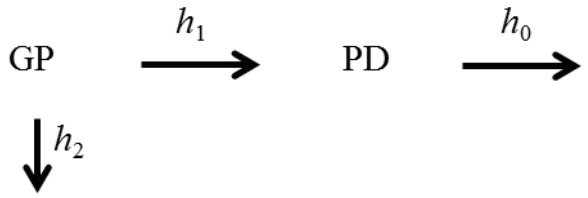


Fig. 5. Decomposition Profiles of PD in Different pH Media at 37 °C

Each result is expressed as the mean ± S.D. (n=3). Dashed-dotted and solid lines are calculated curves for the observed profiles at pH 7.4 and 8, respectively.

Table 1. *In Vitro* Conversion Rate Constants for PD and GP in Different pH Media at 37 °C



pH	Rate constant (h^{-1})			Half-life (h)	
	h_0	h_1	h_2	PD	GP
4	0.0	0.0263	0.0	—	26.4
6	0.0	0.200	0.0400	—	2.89
7.4	0.000967	0.559	0.108	716	1.04
8	0.00979	0.429	2.98	70.8	0.203

For analysis, the mean values in Figs. 4 and 5 were used.

3.3. Hydrolytic Conversion of CS-GP at Different pH Media

The conversion analysis for CS-GP was performed in a way similar to that in the above GP conversion except that a mixture of water, aqueous buffer and methanol (1 : 3 : 1.33, v/v) was used as a solvent. GP and PD existing during the incubation period were investigated by HPLC. As a result, no GP was detected in any incubation. The generated PD was obtained as shown in Fig. 6. The release rate of PD became faster with the increase in pH. Overall, the conversion to PD was much slower in CS-GP than GP. In particular, the release pattern at pH 8 was crucially different between CS-GP and GP. CS-GP released PD gradually and much more effectively at pH 8 than GP. The difference in release patterns was considered to be due to the structural difference. That is, the amino group of GP was presumed to make the compound more unstable because of its high reactivity, while, in CS-GP, the GP part appeared to be more stable due to the poor reactivity of the amide form. As the composition of the incubation media is slightly different from that in GP, the PD stability was checked. As a result, the degradation rate was found to be similar to but a little different from that in the media used for GP incubation (Fig. 7). No difference in pH was observed between CS-GP medium and GP medium. Therefore, the small difference in the composition between media might cause the slight difference in the stability of PD. The conversion scheme was set as shown in Table 2. The degradation profile of PD in the used media ($P_{PD2}(t)$) was analyzed by the pseudo-first order kinetics as state above, and was expressed with Eq. 4. When the conversion of CS-GP was analyzed with the pseudo-first order kinetics based on the scheme in Table 2, the equation for the appearing PD ($P_{CS\text{-}GP}(t)$) was expressed as shown in Eq. 5.

$$P_{PD2}(t) = P_{PD2}(0) \times \exp(-k_0 t) \quad (4)$$

$$P_{CS\text{-}GP}(t) = PT_{CS\text{-}GP} \times k_1 \times \frac{\exp(-(k_1 + k_2)t) - \exp(-k_0 t)}{k_0 - k_1 - k_2} \quad (5)$$

in which $PT_{CS\text{-}GP}$ was an initial content of PD in the CS-GP. The rate constant and half-life were determined in the same manner as for GP. The parameter values were calculated as shown in Table 2. The calculated curves were superimposed with various lines in Figs. 6 and 7. The k_1 value was smaller than the h_1 value at each pH. The k_2 value was much smaller than the h_2 value at pH 7.4 and 8, which indicated that the degradation rate and manner should be very different between GP and CS-GP at these pH conditions. These results suggested that CS-GP should exhibit a gradual and effective release of PD at physiological pH, but the release rate of PD would become lower at the inflammatory pH, which is known to be weakly acidic, pH 6–7.4.[61)]

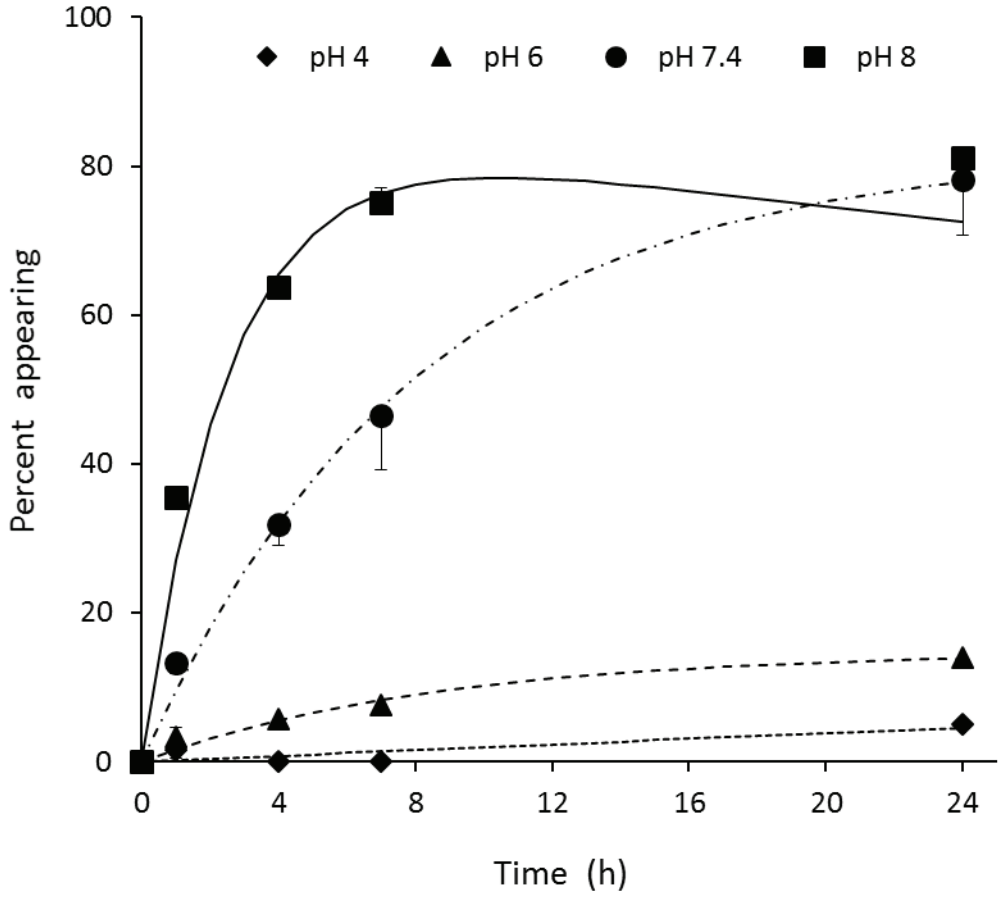


Fig. 6. Conversion Profiles of CS-GP to PD in Different pH Media at 37 °C

Each result is expressed as the mean ± S.D. (n=3). Dotted, broken, dashed-dotted and solid lines are calculated curves for the observed profiles at pH 4, 6, 7.4 and 8, respectively.

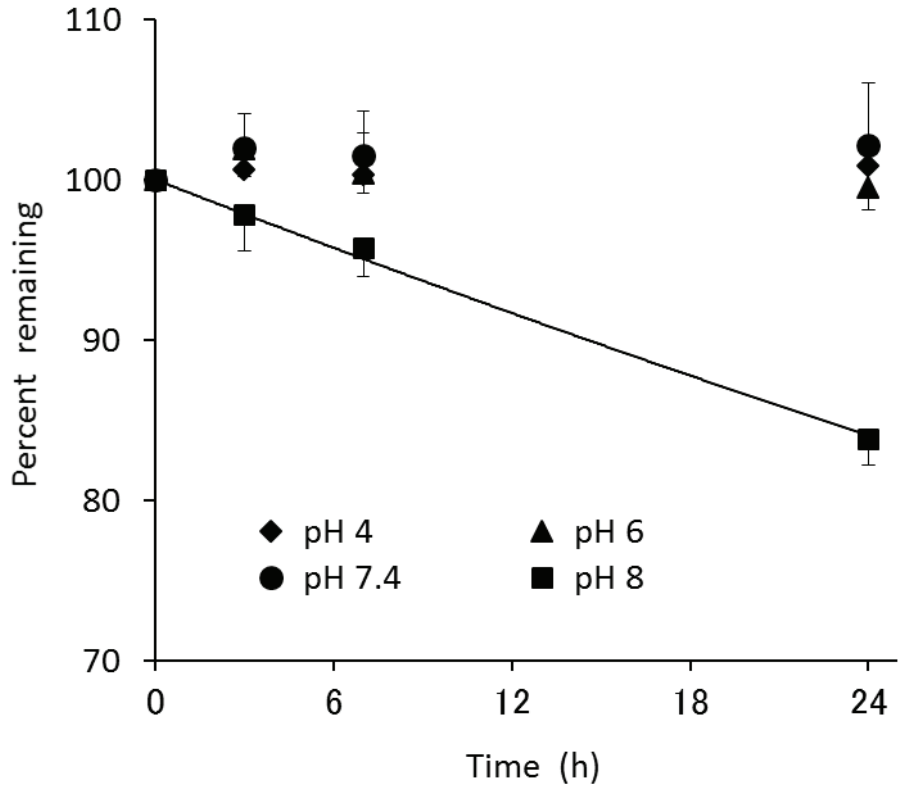
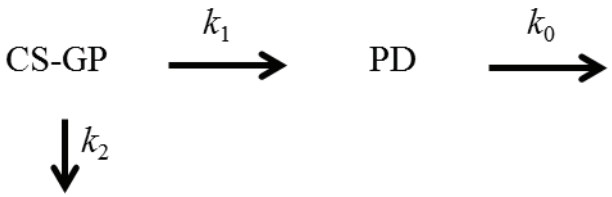


Fig. 7. Decomposition Profiles of PD in Different pH Media at 37 °C

Each result is expressed as the mean ± S.D. (*n*=3). The solid line is the calculated curve for the observed profiles at pH 8.

Table 2. *In Vitro* Conversion Rate Constants for PD and CS-GP in Different pH Media at 37 °C



pH	Rate constant (h^{-1})			Half life (h)	
	k_0	k_1	k_2	PD	CS-GP
4	0.0	0.00195	0.0	—	355
6	0.0	0.0175	0.102	—	5.80
7.4	0.0	0.102	0.0222	—	5.58
8	0.00724	0.328	0.0596	95.7	1.79

For analysis, the mean values in Figs. 6 and 7 were used.

3.4. Release of PD from GP and CS-GP in the PBS–Plasma Mixture

In order to elucidate more clearly the stability under the physiological conditions, effect of rat plasma on the release rate of PD was examined for GP and CS-GP. The release profiles are shown in Fig. 8. The release rate was accelerated in GP to a fair extent by the addition of rat plasma. Namely, more than 75 % of PD was released within 2 h, while approximately 50 % of PD was released without plasma. As to CS-GP, the release rate of PD was increased to a small extent by the addition of rat plasma; a slow release was maintained, and the release ratio was less than 50 % at 6 h even by the addition of rat plasma.

The activity of carboxy esterase is known to be fairly high in rat plasma.[62] The hydrolysis of GP was accelerated appreciably by the addition of rat plasma. On the other hand, the release rate from CS-GP was accelerated to a small extent. Probably, in CS-GP, the ester bond might be insusceptible to the enzymatic hydrolysis due to the bulky CS backbone. The results suggested that CS-GP should function as a prodrug showing slow release of PD in the systemic or physiological conditions, which was adequate because the conjugate should be stable before delivered to the target site.

Those findings for the stability and drug release of CS-GP suggested that PD should be released gradually systemically (approximately pH 7.4) and the diseased part (approximately pH 6–7.4[61]). According to the targeting of macromolecular conjugates to the inflammatory sites such as tumor tissues based on the high permeability of neovascular vessels, the conjugates with several tens of thousands of MW are known to be distributed well to the target site within a fairly short period (several h).[58, 59] Therefore, CS-GP, being moderate in size (30,000–50,000), is expected to be distributed fairly fast to the inflammatory diseased site such as rheumatoid arthritis. Although CS-GP was not stable completely in the systemic circulation conditions, it was considered to be delivered to the inflammatory site and to release drug gradually, leading to

the promotion of effectiveness.

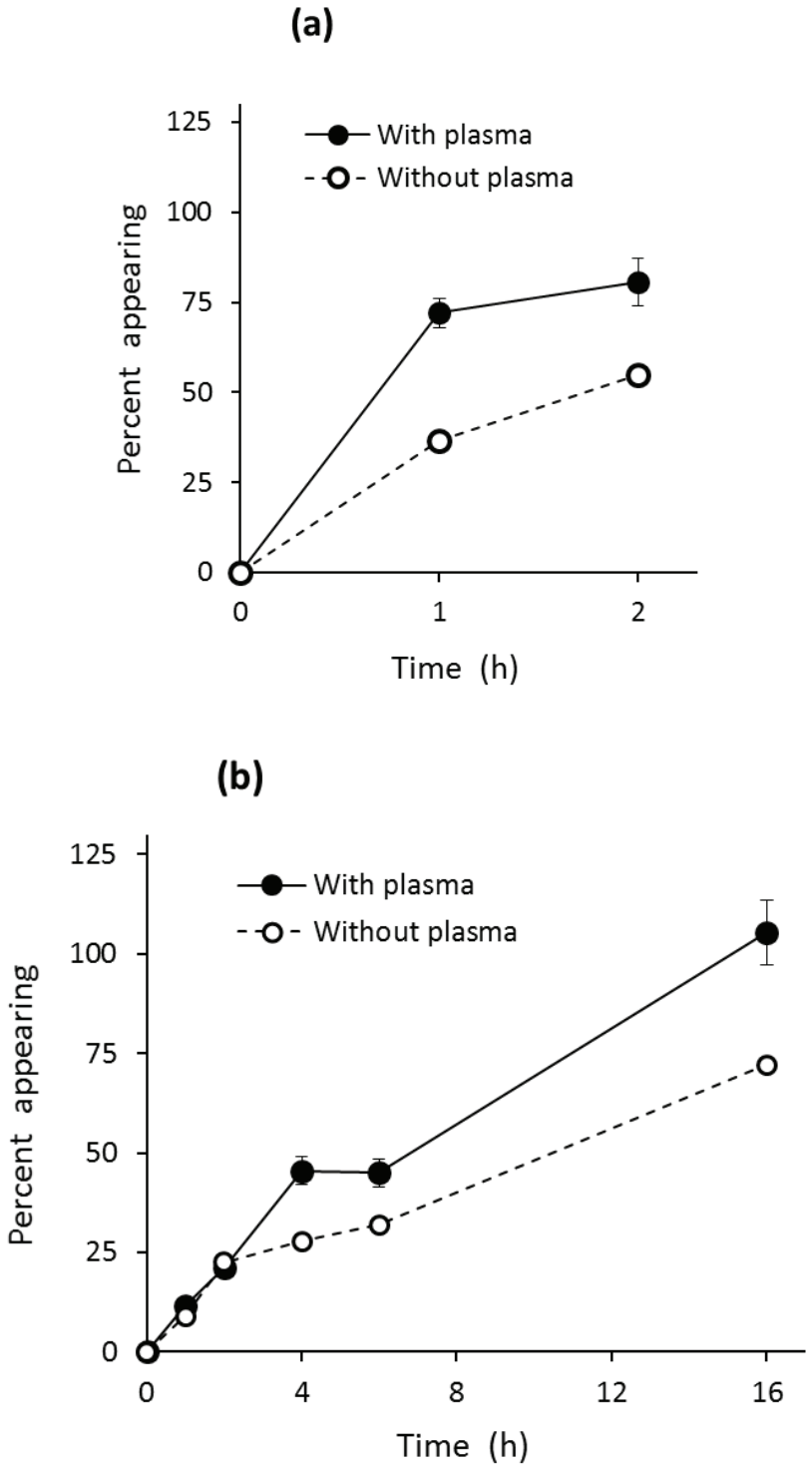


Fig. 8. Release Profiles of PD from GP (a) and CS-GP (b) in PBS with or without Rat Plasma at 37 °C

With plasma: the results, obtained using the PBS–rat plasma (10 : 3, v/v) mixture as a medium, are expressed as the mean ± S.D. (n=3). Without plasma: the results, obtained using PBS as a medium, are expressed by one experiment.

3.5. Preliminary Studies for Anti-inflammatory Effect of CS-GP

In this study, CS-GP was examined for its pharmacological effectiveness *in vivo* for a preliminary evaluation. The volumes of both hind paws were measured on the adequate time (d), and the first dosing was conducted on the day when the paw volume reached plateau, and the second dosing was performed on the next day. Higaki et al. evaluated the drug potential by comparing the inflammatory volumes between after and immediately before treatment.[6] Therefore, the drug pharmacological potential was evaluated in a similar concept. Namely, the drug effect was investigated from the volume ratio (VR) obtained by the comparison between the paw volume after treatment ($V(t)$) and that immediately before the first dosing (V(1st dosing)) as follows.

$$\text{Volume ratio } (VR) = V(t) / V(\text{1st dosing}) \quad (6)$$

in which t was the time (d) after injection of the adjuvant. In addition, the change in body weight was checked in order to check the animal conditions including toxic side effect. Namely, the weight ratio (WR) was calculated in the following equation.

$$\text{Weight ratio } (WR) = W(t) / W(\text{1st dosing}) \quad (7)$$

in which $W(t)$ is the body weight t d after injection of the adjuvant and W(1st dosing) is that just before the first drug administration.

One day after injection of the adjuvant, swelling and redness appeared in the adjuvant-injected hind paw (right paw), while no such change was observed in the opposite hind paw (left paw); however, from 10d after injection of the adjuvant, disseminated arthritis was observed in both hind paws. For both hind paws, the paw volume reached a maximal level (plateau) 15 or 16 d after injection of the adjuvant. The VR value was observed to decrease to a fair extent in both hind paws after the dosing of CS-GP (2 mg PD eq/kg×2 d), while it was maintained at almost the constant level around the value of 1.0 for the control (Fig. 9). When the VR values were compared between CS-GP and the control,

they were significantly lower with CS-GP. This demonstrated that CS-GP was pharmacologically effective against the adjuvant-induced arthritis in rats. In addition, the comparison of the *VR* values between PD (2.5 mg/kg×2 d) and control was performed separately. As shown in Fig. 10, PD exhibited no suppression of the paw volume, and the *VR* values were hardly different between PD and the control. This suggested that PD should not be effective against the present arthritis model. As to the change in body weight, the *WR* value increased a little in CS-GP (Fig. 9), while it was not changed with PD (Fig. 10). Furthermore, the change in body weight was not different between PD and control (Fig. 10), which suggested that the intravenous dosing of PD should not lead to the increase in the body weight. van den Hoven et al. reported that liposomal glucocorticoids exhibited improved therapeutic effect and that the body weight regain was observed in conjunction with the improvement.[6] They considered the remission of inflammation to be the main reason for the body weight recovery.[6] Furthermore, it was stated that the body weight loss was induced by the arthritis itself and the toxic side effect of glucocorticoids.[6] In the treatment with CS-GP (2 mg PD equiv./kg×2), the change in body weight was not marked. Probably, the slight weight increase in CS-GP was due to the remission of the inflammation.

The present *in vivo* studies were performed as a preliminary study to get the rough outline of the pharmacological potential of CS-GP. That is, PD was administered at a little greater dose. In addition, the *in vivo* present results of CS-GP and PD were obtained separately at the different periods. In the *in vivo* study of CS-GP, the plateau of the right paw swelling tended to retard a little, which was recognized from the fact that the *VR* value increased on 17 d. Therefore, the 1st dosing time was conducted on the 16 d for CS-GP, while that was done on the 15 d for PD. The suppressive effect against the arthritis based on the paw volume ratio indicated that CS-GP appeared to be more potent than

PD alone (Figs. 9, 10). Probably, the design concept, stated at i)–iv) in the introduction, appeared to be completed to a fair extent. However, the present *in vivo* results were preliminarily obtained. For more precise and detailed evaluation of the CS-GP efficacy, further refined dosing studies, including time schedules and check of the contribution of CS will be elucidated in Chapter 2.

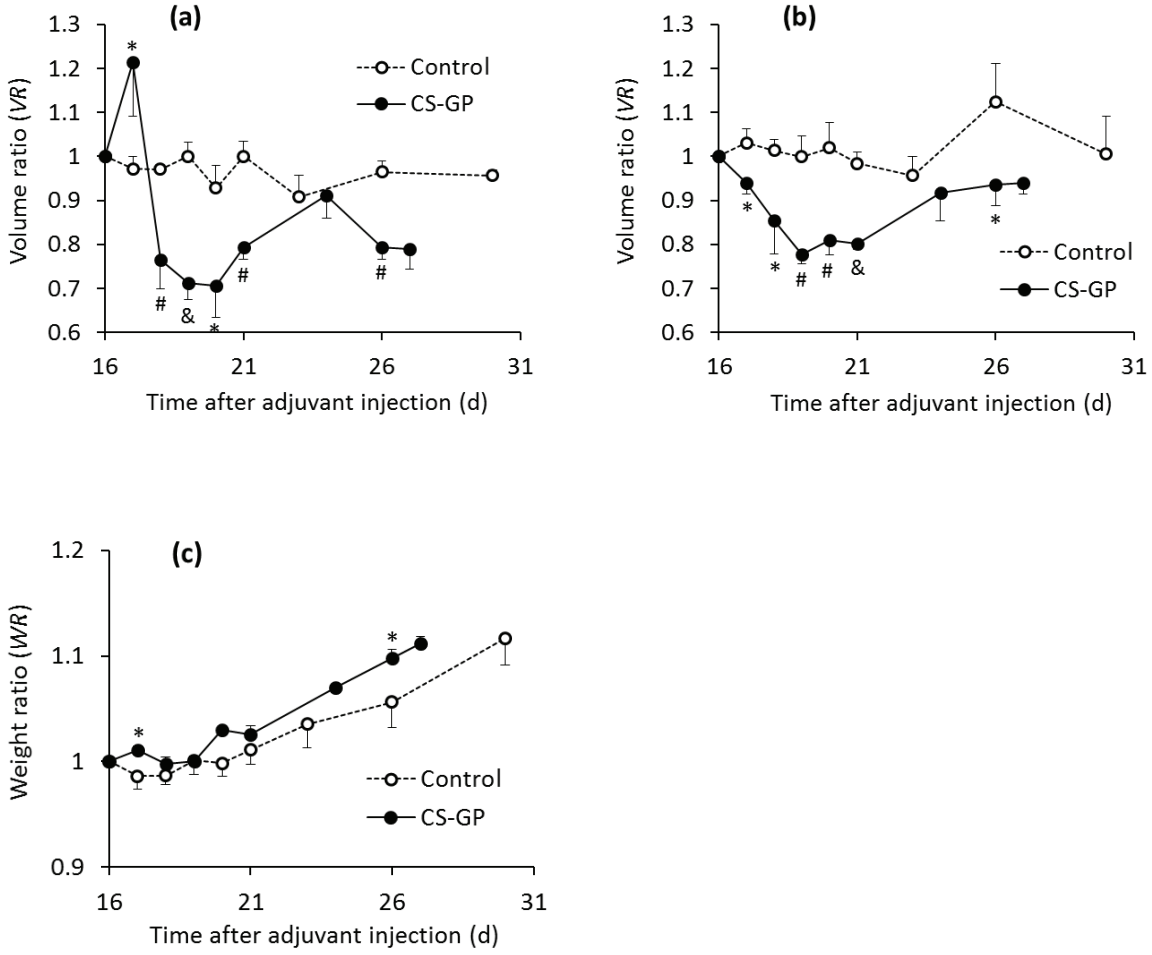


Fig. 9. Effect of CS-GP against Adjuvant-Induced Arthritis in Rats

CS-GP was administered intravenously consecutively on the 16 d and 17 d (total dose: 2 x 2 mg PD equiv./kg). (a)Right hind paw (injected paw); (b) left hind paw (opposite paw); (c) change in body weight. Each result is expressed as the mean ± S.E. (n=3). * $p < 0.05$, # $p < 0.01$, & $p < 0.001$ vs. control.

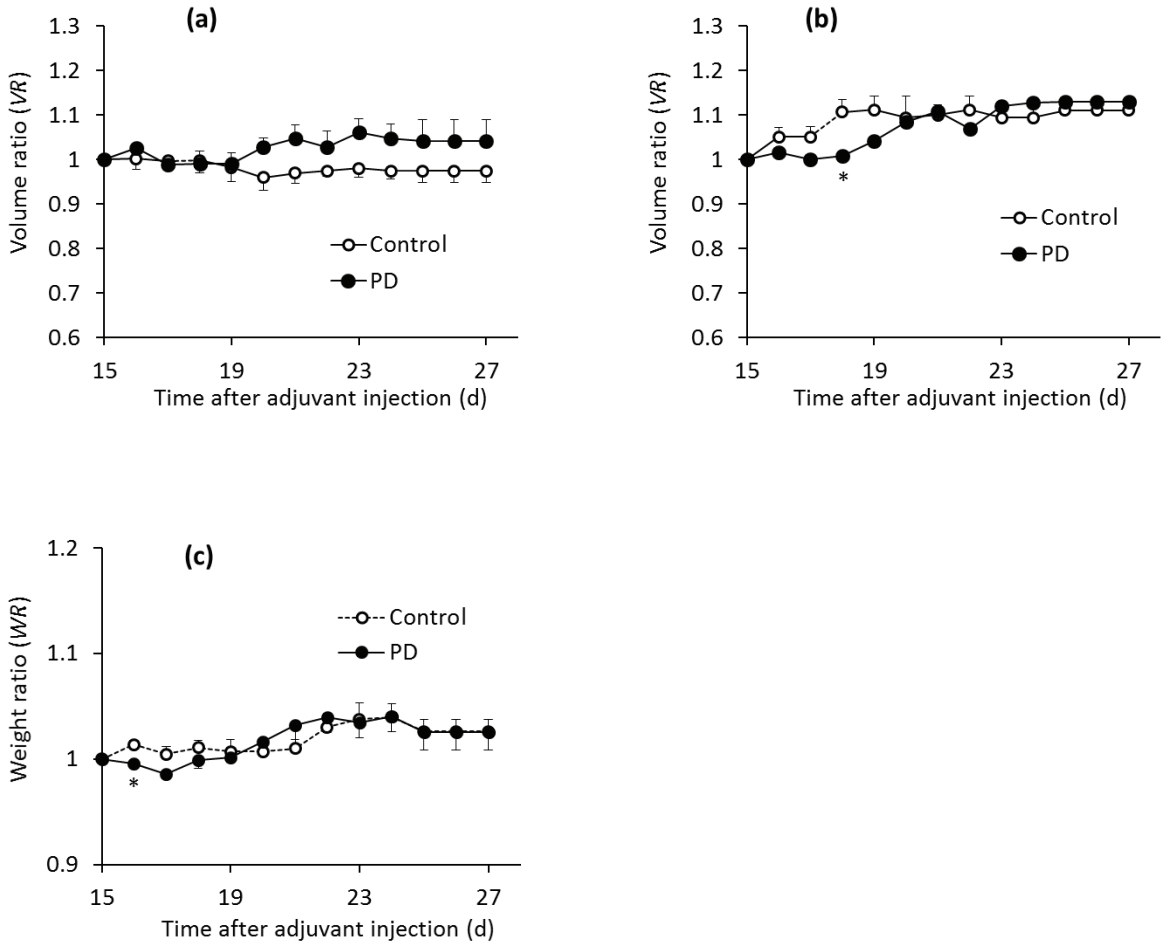


Fig. 10. Effect of PD against Adjuvant-Induced Arthritis in Rats

PD was administered intravenously consecutively on the 15 d and 16 d (total dose: 2.5 x 2 mg PD equiv./kg). (a) Right hind paw (injected paw); (b) left hind paw (opposite paw); (c) change in body weight. Each result is expressed as the mean ± S.E. (n=4). * $p < 0.05$ vs. control.

4. Conclusion

The conjugate of PD with CS, named CS-GP, was prepared using glycine as a linker. Synthesis of GP was performed by the two-step method. CS-GP was prepared by coupling between CS and GP with water-soluble carbodiimide. Overall, GP was less stable than CS-GP. For GP, PD released well in the buffer at pH 6–7.4, but not at pH 8 due to rapid composition other than conversion to PD. On the other hand, PD was released more slowly in CS-GP. CS-GP released PD efficiently at a moderate rate at physiological pH. All the conversion profiles could be analyzed with pseudo-first order kinetics models. The calculated rate constants supported the slower release of PD from CS-GP. The addition of rat plasma accelerated the release rate of PD from CS-GP to a small extent, suggesting that PD should be released gradually under the physiological conditions. The pharmacological tests using rats with adjuvant-induced arthritis revealed that CS-GP should have a good anti-inflammatory potential, while PD hardly exhibited effectiveness. At the same time, CS-GP did not display toxic side effects related with the body weight loss. Thus, it was elucidated that CS-GP behaved as a macromolecular prodrug of PD and showed good effectiveness against arthritis. The more detailed evaluation of the efficacy for CS-GP will be reported in the next chapter 2.

Chapter 2

In Vivo Evaluation of Chondroitin Sulfate-Glycyl-Prednisolone for Anti-arthritic Effectiveness and Pharmacokinetic Characteristics

1. Introduction

In chapter 1, PD commonly used for the treatment of RA was chosen as an anti-inflammatory agent, and a CS-GP conjugate with glycine as a linker was prepared. The *in vitro* characteristics and pharmacological potential using rat adjuvant-induced arthritis were examined. As a result, it was suggested that CS-GP could be a prodrug of PD in several *in vitro* study and preliminary rat arthritis model.

As glucocorticoids are highly potent and fast-acting[10, 11], they are often used in the treatment of refractory inflammatory diseases such as RA, ulcerative colitis and Crohn's disease; however, their chronic use often leads to severe systemic side effects like diabetes, osteoporosis and adrenal failure, resulting in the decrease of patients' QOL.[16, 63] At the same time, these toxic side effects prevent the agents from displaying sufficient action in many clinical uses.

Recently, a lot of attempts have been made to improve such drawbacks of conventional anti-inflammatory drugs.[14, 64, 65] For RA, various drug delivery systems have been developed for conventional anti-inflammatory agents.[6,16,51,52,66] In particular, a targeted delivery system is an important method to improve therapeutic usefulness because it enables a high drug concentration at the target site while reducing drug distribution in other areas. Such an excellent delivery system is expected to display higher potency at lower doses or frequencies, resulting in the reduction of toxic side effects.

However, there are several issues in applying delivery system to clinical treatment. In fact it is known that immunological reactions can occur with protein carriers and pegylated liposomes.[51, 67] Since nano-sized carrier systems are distributed in various organs and tissues, non-degradable large carriers might remain there, suggesting the problem of bioaccumulation.

CS, a glycosaminoglycan, is commercially available and used clinically.[68,69,70] CS is water-soluble, non-toxic and can be administered

intramuscularly and intravenously. It is susceptible to substantial excretion into urine; the excreted CS-related molecules are partly compounds of high molecular weight, similar to the original polymer, and partly degraded forms such as oligosaccharides and inorganic sulfate ions.[25, 54, 71] These pharmacokinetic features suggest that CS should behave as a polymer, showing marked retention in the systemic circulation.

For the reasons set forth above, in this chapter 2, CS-GP conjugates with different drug contents were prepared and characterized *in vitro*, and the *in vivo* efficacy was investigated in detail from comparison with PD alone, the carrier and their mixture using rats with adjuvant-induced arthritis. In addition, the pharmacokinetic characteristics of the conjugate were examined and used for evaluation of efficacy and toxicity.

2. Materials and Methods

2.1. Materials and Instruments

Chondroitin sulfate C (CS) sodium salt (CS-Na; MW40,000–80,000, derived from shark cartilage), with N and S of 2.6 and 6.6 %, respectively, and a ratio of 6-sulfate to 4-sulfateof 9 : 1, was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Prednisolone (PD), *N,N*_-carbonyldiimidazole (CDI), 4-dimethylaminopyridine (DMAP), *N*-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (WSC) were purchased from the same company. *N*-Tritylglycine (Tr-G) was purchased from Sigma Chemical Company (St. Louis, MO, USA). Heat-killed and desiccated *Mycobacterium tuberculosis* M37Ra was obtained from Difco Laboratories (Detroit, MI, USA) and used as an adjuvant. All other chemicals were of reagent grade.

^{1}H and ^{13}C NMR spectra were measured with a JEOL Lambda-500 (500 MHz) spectrometer, in which TMS was used as a reference with a chemical shift

of 0 ppm. UV–Vis. absorption spectra were recorded using a Beckman DU640 spectrophotometer. MS spectra were obtained with a JEOL JSM600 mass spectrometer for EI-MS and with an AXIMA–CRF PLUS mass spectrometer (Shimadzu Corp., Kyoto, Japan) for MALDI-TOFMS.

2.2. Animals

Male Wistar rats (7 weeks old; weighing 200–210 g) were purchased from Tokyo Laboratory Animals Science Co., Ltd., (Tokyo, Japan). Female Lewis rats (8 weeks old, 150–160 g) were obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan). They were bred on the breeding diet MF supplied by Oriental Yeast Co., Ltd., (Tokyo, Japan) with water ad libitum, at 23±1 ^{o}C and relative humidity of 60±5 %. They were used for the experiments several days after purchase. The experimental protocol was approved by the Committee on Animal Research of Hoshi University, Japan. The animal experiments were performed in compliance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, Japan.

2.3. Preparation of Chondroitin Sulfate-Glycyl-Prednisolone Conjugate (CS-GP)

GP (Fig. 11) was synthesized as described above,[34] although with partial modification. Briefly, the Tr-G ester of PD, named Tr-GP, was synthesized in the first step, in which purification was conducted without column chromatography, different from the previous method. Tr-G (477 mg, 1.5 mmol) and CDI (243 mg, 1.5 mmol) were dissolved in 10 mL tetrahydofuran at 0 ^{o}C, and stirred for 30 min at 0 ^{o}C. DMAP (15 mg, 0.12 mmol) and PD (270 mg, 0.75 mmol) were added to the solution, and the mixture was stirred at room temperature for 4.5 h. After the solvent was evaporated, Tr-GP was obtained by washing the residue with methanol. The obtained compound exhibited the same results in the TLC,

^{1}H NMR, ^{13}C NMR, and EI–MS measurements as previously.

GP was obtained by detritylation of Tr-GP using aqueous acetic acid. Namely, 75 % (v/v) aqueous acetic acid (10 mL) was added to Tr-GP (300 mg) and heated at 75 ^{o}C to dissolve Tr-GP completely. As soon as the precipitate began appearing, the mixture was cooled on ice, and the white precipitate was removed by filtration. After the solvent of the filtrate was evaporated, the residue was dissolved in a mixture of chloroform and methanol (15 : 1, v/v), and GP was separated by silica gel column chromatography with a mixture of chloroform and methanol (15:1, v/v). The obtained compound showed the same results in TLC, ^{1}H NMR, ^{13}C NMR, and MALDI-TOF-MS measurements as previously.

CS-GP conjugates (Fig. 11) were prepared by the carbodiimide coupling of CS and GP with different formulations. CS (120 mg) was dissolved in 10 mL water, and 2.5 mL tetrahydrofuran (THF) containing GP (15, 30 and 60 mg) was added. WSC (500 mg) and NHS (300 mg) were added to the solution, and the resultant mixture was stirred at room temperature for 4.5 h. The solution was chromatographed with a Sephadex G50 gel column [2.8 cm (inner diameter) × 19 cm (length)] using 0.1 M NaCl aqueous solution as the elution solvent. The eluted solution was fractionated at 10 mL each. The high MW fractions were collected, and the mixture was dialyzed against water at 4 ^{o}C. The final solution was lyophilized to yield CS-GP.

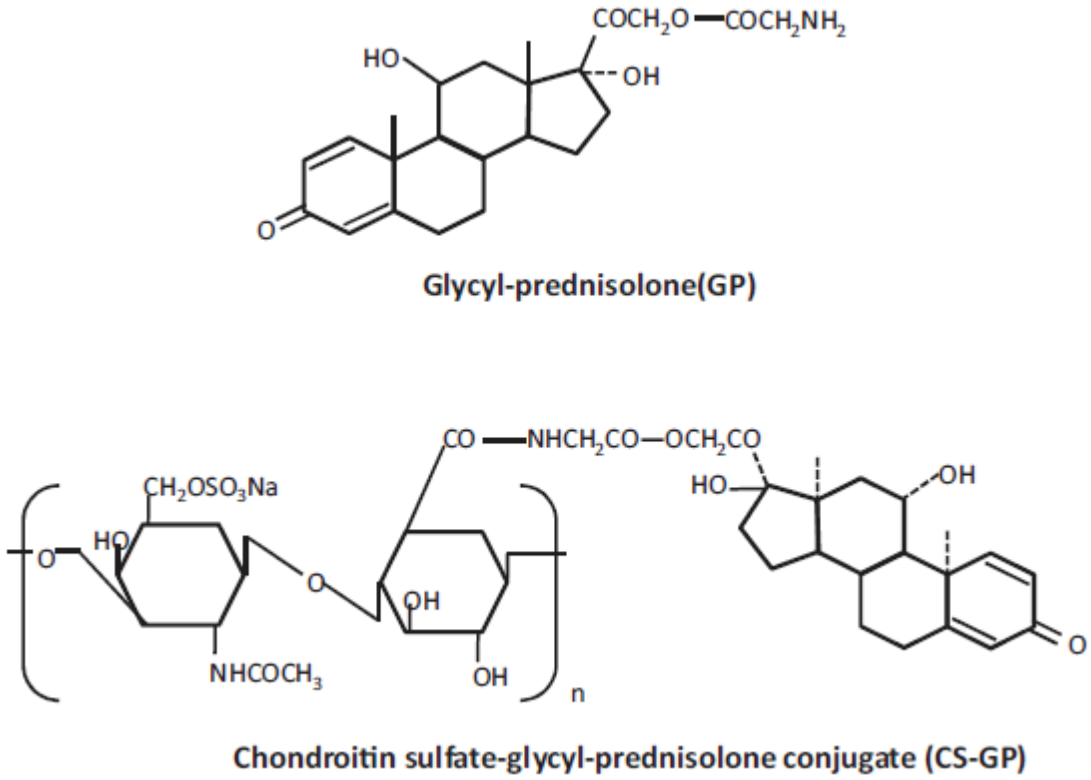


Fig. 11. Structures of Glycyl-Prednisolone (GP) and Chondroitin Sulfate-GP Conjugate (CS-GP)

2.4. *In Vitro* Analysis of CS-GP

The content of CS-GP was measured as shown previously.[34] Briefly, PD, CS and CS-GP were dissolved in a mixture of phosphate-buffered saline (PBS) at pH 7.4 and methanol (7 : 3, v/v) at concentrations of 12, 500 and 500 μg/mL, respectively. The net absorbance at 246 nm of the conjugated PD was obtained by the subtraction of CS absorbance (246 nm) from the absorbance (246 nm) of CS-GP, and was compared with the absorbance of PD alone at 246 nm in order to calculate the PD content.

In vitro release characteristics were investigated for CS-GP conjugates with different PD contents. After the conjugate (1.2 mg) was dissolved in 0.4 mL water, aqueous buffer (1.2 mL) and methanol (0.533 mL) were added. Regarding the aqueous buffers, 1/15 M phosphate buffer adjusted to pH 6.0 and phosphate-buffered saline with pH 7.4 (PBS) were used. Each solution (0.5 mL) was incubated at 37 ^{o}C under horizontal shaking at 60 rpm. At appropriate time points, aliquot samples (50μL) were withdrawn. Immediately after sampling, 150 μL of 1/10 M acetate buffer (pH 4.0) was added to suppress the release of PD. Then, 150 μL of the HPLC mobile phase was added, and the mixture was analyzed by HPLC.

2.5. Animal Experiments Using Rats with Adjuvant-Induced Arthritis

CS-GP was investigated for detailed effectiveness by comparison with PD alone, CS alone and the mixture of PD and CS. Rat models with adjuvant-induced arthritis were made by the known method,[72] and the inhibitory effect of each preparation was compared. The *in vivo* experiment procedure was performed as in Fig. 12. Namely, heat-killed M. tuberculosis M37Ra was suspended in liquid paraffin at 5 mg/mL. The suspension (100 μL) was injected intracutaneously into the pad of the right hind paw of each Lewis rat. The volume of the hind paws reached a plateau around 14 and 15 days after adjuvant

injection. PD solution (2.5 mg/mL) was made using 50 % (w/v) PEG400 saline as a solvent. CS-GP with high PD content (CS-GP(H)) was used in this experiment. CS-GP was dissolved in saline at 2.5 mg PD equiv./mL. CS was dissolved in saline at the same concentration as that of CS-GP. The mixture of CS and PD in saline contained the same concentrations of PD and CS as those in CS-GP, respectively. The preparations were injected intravenously via the jugular vein at 2.5 mg PD equiv./kg 14 and 15 days after injection of the adjuvant; that is, total dose = 2.5 × 2 mg PD equiv./kg. As to PD, the treatment was additionally conducted in a similar manner at twice the dose (total dose = 5 × 2 mg/kg). CS alone was administered similarly with the same volume of CS–GP preparation. No treatment was done for the control group. On appropriate days after adjuvant injection, the rats were weighed and the volume of each hind paw was measured by immersing it in water and reading the buoyancy.

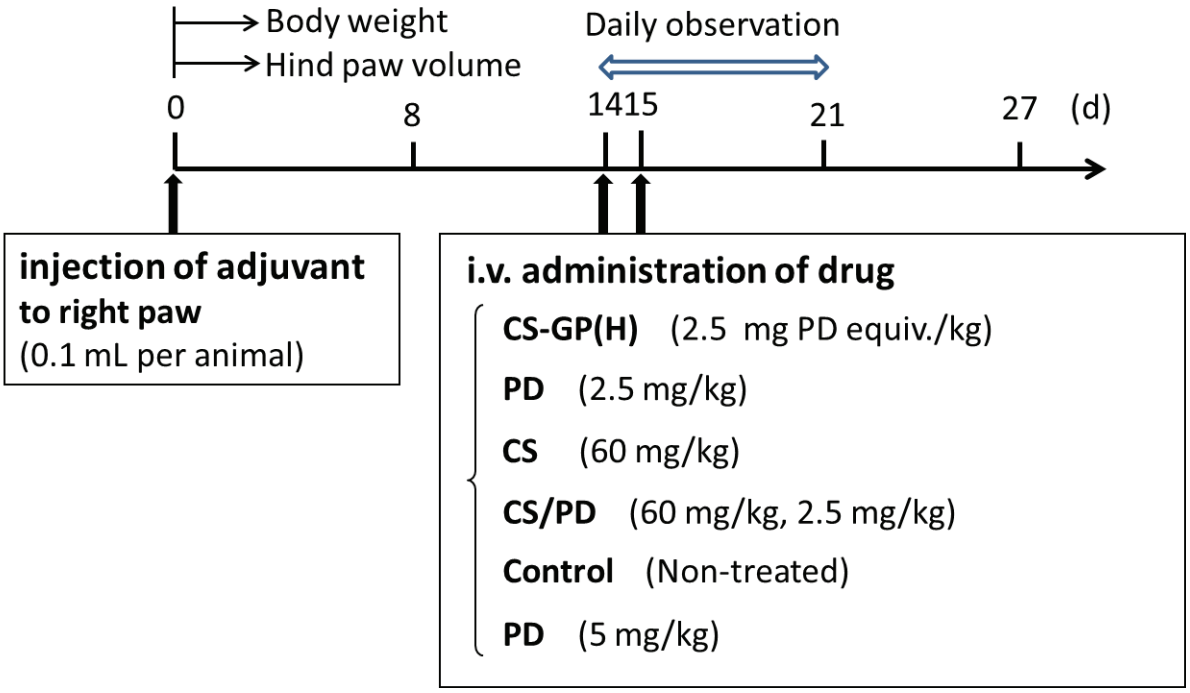


Fig. 12. Production of Arthritis Model and Schedules of Dosing and Observation

2.6. Evaluation of Therapeutic Efficacy and Toxic Side Effect

The animals were examined for body weight and both hind paws from just before adjuvant injection until the end of the efficacy experiment. The therapeutic efficacy was evaluated from two methods. One was performed by investigating a swelling ratio to the initial paw volume. Namely, the ratio of the paw volume at t-th day ($V(t)$) to the volume just before adjuvant injection ($V(0)$), expressed as $V(t)/V(0)$, were compared among each preparation. The $V(t)/V(0)$ values were considered to reflect the inflammation degree of the animals. As the other index of efficacy, the ratio of the paw volume at t-th day ($V(t)$) to the volume just before drug administration ($V(14)$), expressed as $V(t)/V(14)$, was calculated and compared among the dosing preparations. The $V(t)/V(14)$values were considered to represent the suppressive effect of each preparation against the inflammation. At the same time, the animals were checked for changes in body weight, which were used as an overall toxic index. Change in body weight was examined in two modes. The ratio of the body weight at t-th day ($W(t)$)to the weight just before adjuvant injection ($W(0)$), expressed as $W(t)/W(0)$, was investigated throughout the efficacy experiment. In addition, the ratio of the body weight at t-th day ($W(t)$) to the weight just before drug administration ($W(14)$), expressed as $W(t)/W(14)$,was calculated to check the overall toxic side effect of each preparation.

2.7. Investigation of Pharmacokinetic Characteristics of CS-GP in Rats

PD was dissolved in 50 % (w/v) PEG400 saline solution and injected intravenously into Wistar rats via the jugular vein at 2.5 mg/kg/mL. In this pharmacokinetic examination, CS-GP(H) was also used. CS-GP was dissolved in saline and administered intravenously to Wistar rats via the jugular vein at 2.5 mg PD equiv./kg/mL. At 0.25, 0.5, 1, 3, 7 and 24 h after administration, blood samples (0.35 mL) were withdrawn and centrifuged at 1500×g for 10 min to

obtain plasma. Saturated NaCl aqueous solution (100 μL), 6 % (w/v) phosphoric acid (100 μL) and 4 mL of the mixture of *t*-butylmethyl ether and pentane (3 : 2, v/v) were added to the plasma (100 μL) and shaken vigorously100 times. After the mixture was centrifuged at 1500×g for 10 min, the supernatant (3 mL) was taken and dried under nitrogen gas. The residue was dissolved with 100 μL HPLC mobile phase and analyzed for PD concentration by HPLC. For CS-GP, the obtained PD concentration corresponded to the concentration of free PD.

As for the samples for CS-GP administration, the plasma concentration of total PD (= free PD + conjugated PD) was investigated as follows. Fifty microliters of plasma sample, obtained as stated above, and 50 μL of 0.1 M NaOH aqueous solution were mixed and then incubated at 45 ^{o}C by shaking horizontally at 60 rpm for 10 min. Then, 150 μL of 0.1 M acetate buffer of pH 4 was added to the mixture. To the resultant mixture, saturated NaCl aqueous solution (200 μL), 6 % (w/v) phosphoric acid (200 μL) and 8 mL of the mixture of *t*-butylmethyl ether and pentane (3 : 2, v/v) were added and shaken vigorously 100 times. After the mixture was centrifuged at 1500×g for 10 min, the supernatant (3 mL) was taken and dried under nitrogen gas. The residue was dissolved in 100 μL HPLC mobile phase and analyzed for PD concentration by HPLC. This PD concentration was the concentration of total PD, and the concentration of conjugated PD was calculated by subtracting the concentration of the above free PD from that of the total PD.

Immediately after blood sampling at 24 h, the animals were sacrificed by extra anesthesia with ether inhalation, and the liver, lung, kidney and spleen were excised and washed with saline. After each organ was weighed, the same amount of saline was added, and it was homogenized with a glass homogenizer with a Teflon pestle under ice cooling. The homogenate was treated as stated above in the measurement of the plasma concentration. Namely, to the homogenate (200 μL), saturated NaCl aqueous solution (200 μL), 6 % (w/v)

phosphoric acid (200 μL) and 8 mL of the mixture of *t*-butylmethyl ether and pentane (3 : 2, v/v) were added and shaken vigorously 100 times. After the mixture was centrifuged at 1500×g for 10 min, the supernatant (6 mL) was taken and dried under nitrogen gas. The residue was dissolved in 200μL HPLC mobile phase and analyzed by HPLC. For CS-GP, the obtained PD concentration corresponded to the concentration of free PD.

In addition, the organ concentration of total PD (= free PD + conjugated PD) was investigated in a similar manner to that for the plasma concentration measurement. Namely, the homogenate (100 μL) and 0.1 N NaOH aqueous solution (100 μL) were mixed and incubated at 45 ^{o}C by shaking horizontally at 60 rpm for 10 min. Then, 300 μL of 0.1 M acetate buffer of pH 4 was added to the mixture. To the resultant mixture, saturated NaCl aqueous solution (400 μL), 6 % (w/v) phosphoric acid (400 μL) and 16 mL of the mixture of *t*-butylmethyl ether and pentane (3 : 2, v/v) were added. After the resultant mixture was shaken vigorously 100 times, it was centrifuged at 1500×g for 10 min. The organic phase (6 mL) was taken and dried under nitrogen gas. The residue was dissolved in 200 μL HPLC mobile phase and analyzed by HPLC.

2.8. HPLC Assay

As the HPLC apparatus, a Shimadzu LC-6AD pump was used with a Shimadzu SPD-10AV VP UV–VIS detector set at a wavelength of 246 nm and a Shimadzu C-R7A plus Chromatopac. A YMC Pack ODS-AM column (6 mm inner diameter × 150 mm length; YMC Co., Ltd., Kyoto, Japan) was used as the analytical column. A 26 % (v/v) 2-propanol aqueous solution containing 0.1 % (v/v) trifluoroacetic acid was used as the mobile phase. The HPLC assay was performed at room temperature, with the flow rate set at 1 mL/min and an injection volume of 20 μL. The PD concentration was determined by the absolute calibration curve method.

2.9. Statistical Analysis

Statistical analyses were performed with one-way ANOVA followed by Dunnett's test, and the significant difference was set as $p< 0.05$.

3. Results and Discussion

3.1. *In Vitro* Characteristics of CS-GP Conjugates with Different Drug Contents

Synthesis was performed in the same manner as in a previous study[34] except that the separation of Tr-GP was conducted only with methanol washing and no use of silica gel column chromatography. ^{1}H NMR, ^{13}C NMR, DEPT135°, DEPT90°, HMBC and HMQC spectra and mass spectra supported a glycine ester of PD at the C21 position (Fig. 11) was synthesized (data not shown). Also, the ^{1}H NMR spectrum showed that CS-GP was formed by the combination of GP and CS, and the PD content calculated from UV absorption spectra was consistent with that estimated from integrated intensities of the proton signals in the ^{1}H NMR spectrum of CS GP (data not shown).

The conjugates with different PD contents were prepared only by changing the amount of GP added. As a result, the PD content rose with the increase in the GP amount (Table 3). The amounts of the conjugates yielded were almost the same because their drug contents were small. The CS-GP conjugates with low, intermediate and high PD content were named CS-GP(L), CS-GP(M) and CS-GP(H), respectively. Their release characteristics were analyzed at pH 6 and 7.4. The results are shown in Fig. 13. The release rates were relatively similar among all the conjugates at both pH 6 and 7.4. It was suggested that ester hydrolysis should occur basically independently of the PD content. Also, this meant that the conjugate with greater drug content would release more PD according to the amount of the conjugate. PD was released gradually over 24 h under physiological pH, while the PD release rate fell at

acidic pH. The inflammatory joint space was acidified and its pH sometimes neared 6.[52, 61,73)] In the previous study, the effect of plasma on the release rate of PD from CS-GP was small; therefore, when ignoring the enzymatic effect on the release, the actual release was expected to be caused between release curves of pH 6 and 7.4 under disease conditions.

Table 3

Preparation Conditions of CS-GP and the Yield and Drug Content

Formulation	CS (mg)	GP (mg)	WSC (mg)	NHS (mg)	Yielded CS-GP (mg)	PD content (%, w/w)
CS-GP(L)	120	15	500	300	128	1.36
CS-GP(M)	120	30	500	300	125	2.01
CS-GP(H)	120	60	500	300	121	4.34

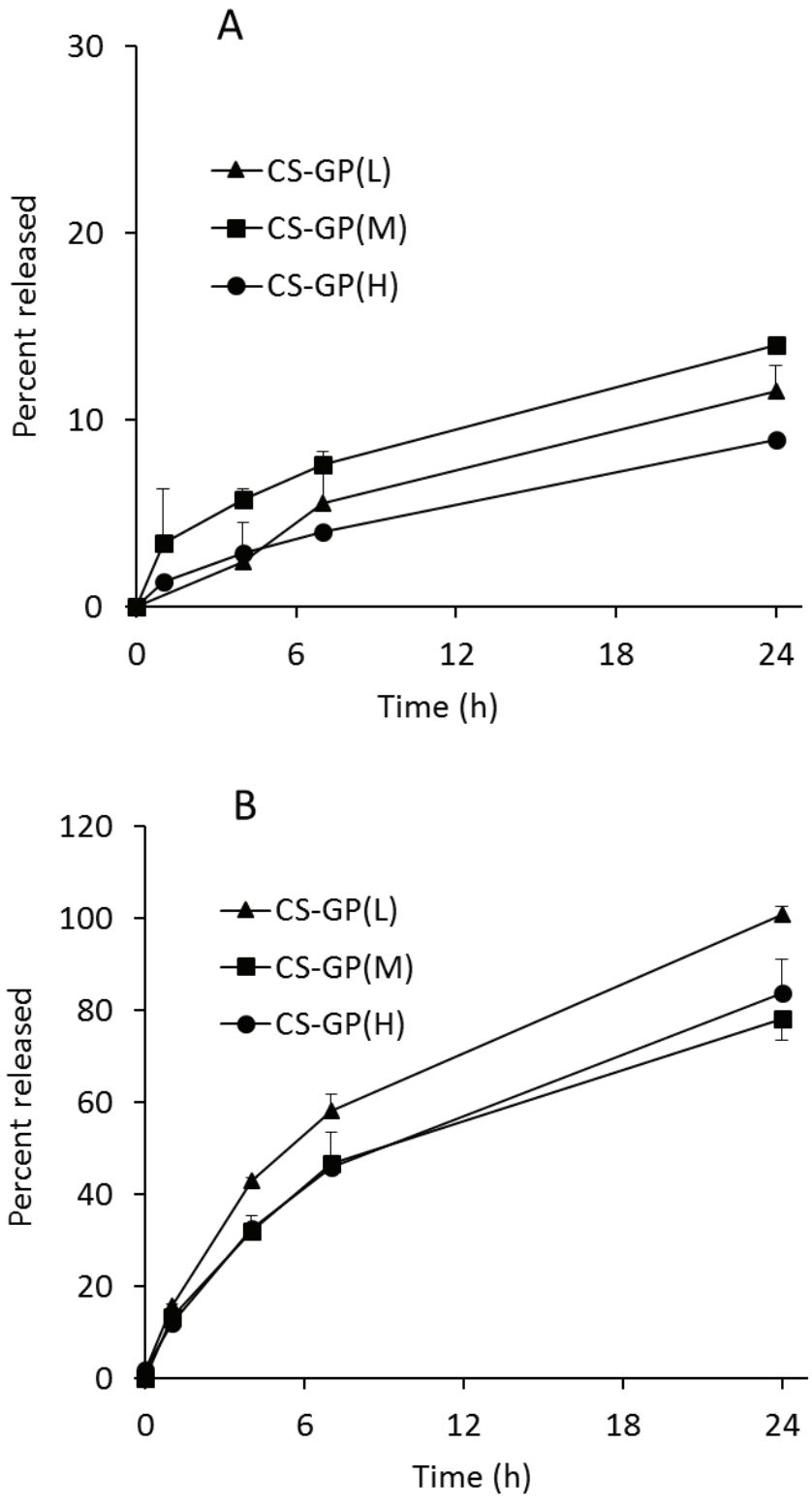


Fig. 13. Release Profiles of PD from CS-GP Conjugates with Different Drug Contents under the Conditions of pH 6 (A) and 7.4 (B) at 37 °C (mean ± SD, n = 3)

3.2. Therapeutic Effectiveness of CS-GP

CS-GP, PD alone, CS alone and PD/CS mixture were investigated for their anti-inflammatory potency. At the same time, the animals were checked for changes in body weight, which were used as an overall toxic index. PD (2.5 mg/kg), CS-GP (2.5 mg PD equiv./kg), CS, PD (2.5 mg/kg)/CS and PD (5 mg/kg) were expressed as PD (2.5), CS-GP (2.5), CS, PD (2.5)/CS and PD (5) in Figs. 14–17, and the control (no treatment) was expressed as control. The change in body weight is shown in Fig. 14. For each group, the body weight changed in a similar pattern to disease progression. Even after injection on 14 d and 15 d, a decrease in body weight was hardly observed in each preparation, indicating that all the preparations had low toxicity under the present dosing conditions. The therapeutic effect of each preparation was examined from suppression of the swelling of the hind paws. The inflammation extent was evaluated from swelling ratio of paw volume to the initial one ($V(t)/V(0)$) and from change in paw volume after the dosing of preparations ($V(t)/V(14)$). The profiles of the $V(t)/V(0)$ values were obtained as shown in Figs. 15 and 16. Swelling was reduced more obviously by the preparations in the right paw (Fig. 15). As to a right hind paw, CS-GP exhibited a significant reduction of paw volume against the control for a long period, while other preparations did not. PD alone, CS alone and the PD/CS mixture tended to decrease swelling but the significant effect was obtained only at some time points; PD (2.5 mg/kg) exhibited a significant reduction against the control on the 15 d ($p< 0.05$), and the swelling was significantly lower in PD (5 mg/kg) than the control on 15 d, 17 d–19 d ($p< 0.05$). CS is known to exhibit anti-inflammatory actions on adjuvant arthritis in rats, although the effect appears to be low, particularly in therapeutic use[74]. Also, CS tends to display an elevated therapeutic effect against osteoarthritis in combination with other agents, such as glucosamine sulfate.[37] In the present experiment, the tendency to suppress swelling was

observed with CS, but the effect was not significant except for on 19 d. The mixture of CS and PD was also not significantly effective against swelling; CS and PD appeared not to act synergistically.

The suppressive effect of each preparation on the swelling of the left hind paw was observed to a lesser extent (Fig. 16). Only CS-GP suppressed the swelling of the left hind paw significantly against the control ($p< 0.05$ on 17 d and 18 d). Although PD alone, CS alone and the PD/CS mixture tended to decrease the swelling of the left hind paw as compared with the control, none of them was significantly effective.

Change in paw volume and body weight was evaluated from the other mode; effect of each preparation on the inflamed paws and body conditions was estimated from the comparison between after and just before dosing. Namely, the ratio of the paw volume on the t-th day ($V(t)$) to that on 14 d ($V(14)$), $V(t)/V(14)$, was calculated and compared among the preparations. Also, the ratio of the body weight on the t-th day ($W(t)$) to that the just before dosing ($W(14)$), $W(t)/W(14)$, was evaluated as overall toxic side effect. The results are shown in Fig. 17. Change in body weight after dosing was not significantly different among each preparation (Fig. 17A). As to a right hind paw, only CS-GP significantly reduced the volume ratio ($V(t)/V(14)$) as compared with the control (Fig. 17B). On the other hand, the $V(t)/V(14)$ values calculated for the left hind paws were significantly lower in PD (2.5 mg/kg), PD (5 mg/kg) and CS-GP (2.5 mg PD equiv./kg) than the control across multiple days (Fig. 17C). In particular, CS-GP showed the lower $V(t)/V(14)$ values than other preparations for a long period, and tended to be superior in paw volume reduction to PD (5 mg/kg). The profiles given by $V(t)/V(0)$ values were somewhat different from those shown by $V(t)/V(14)$ values. The reason was that the $V(14)$ values were varied to some extent among the preparations, while the $V(0)$ values were almost the same, 1.3 or 1.4 mL, for all the preparations. However, both the analyses using $V(t)/V(0)$

and $V(t)/V(14)$ values suggested that CS-GP should be the most suppressive against the inflammation of both hind paws. The results in change in body weight indicated that CS-GP should not be toxic at the present dosing conditions.

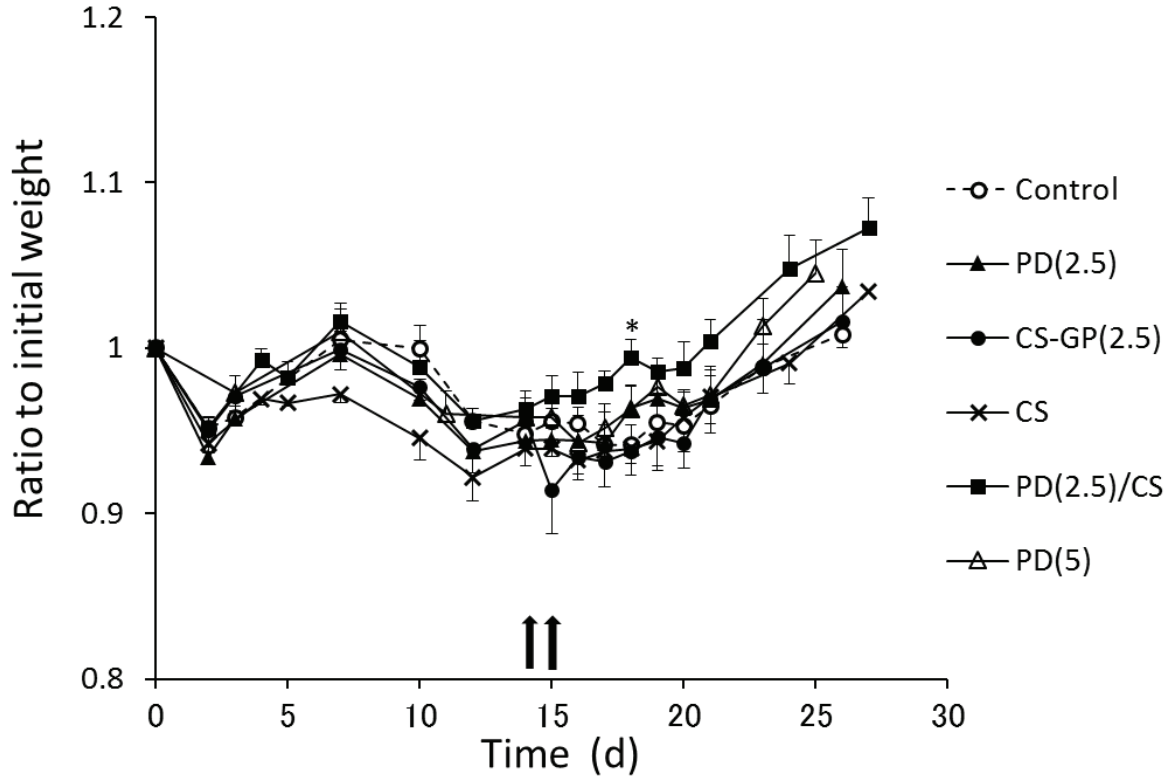


Fig. 14. Change in Body Weight after Adjuvant Injection into Rats (mean ± SE, n = 3)

*p< 0.05 vs. control (Dunnett's test). The arrow shows the dosing time.

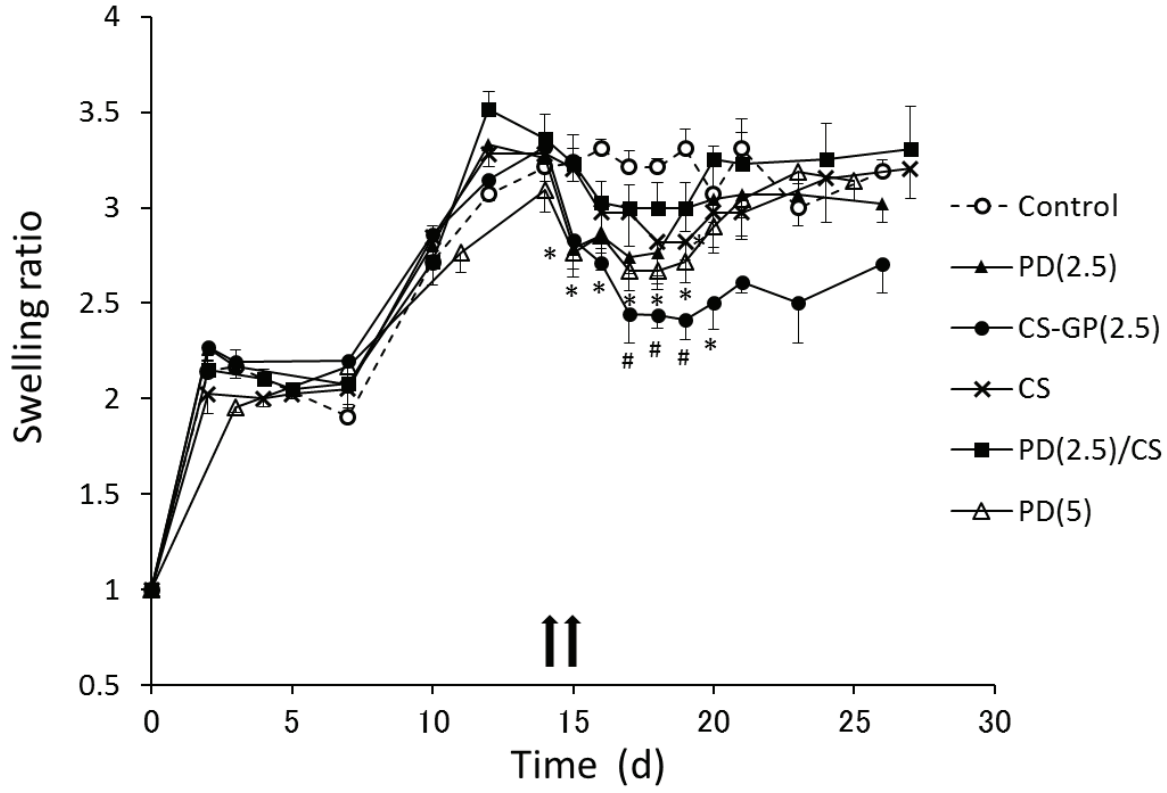


Fig. 15. Swelling of Right Hind Paw after Adjuvant Injection into Rats, and Therapeutic Effect of Each Preparation against Arthritis (mean ± SE, n = 3)

*p< 0.05, #p< 0.01 vs. control (Dunnett's test). The arrow shows the dosing time.

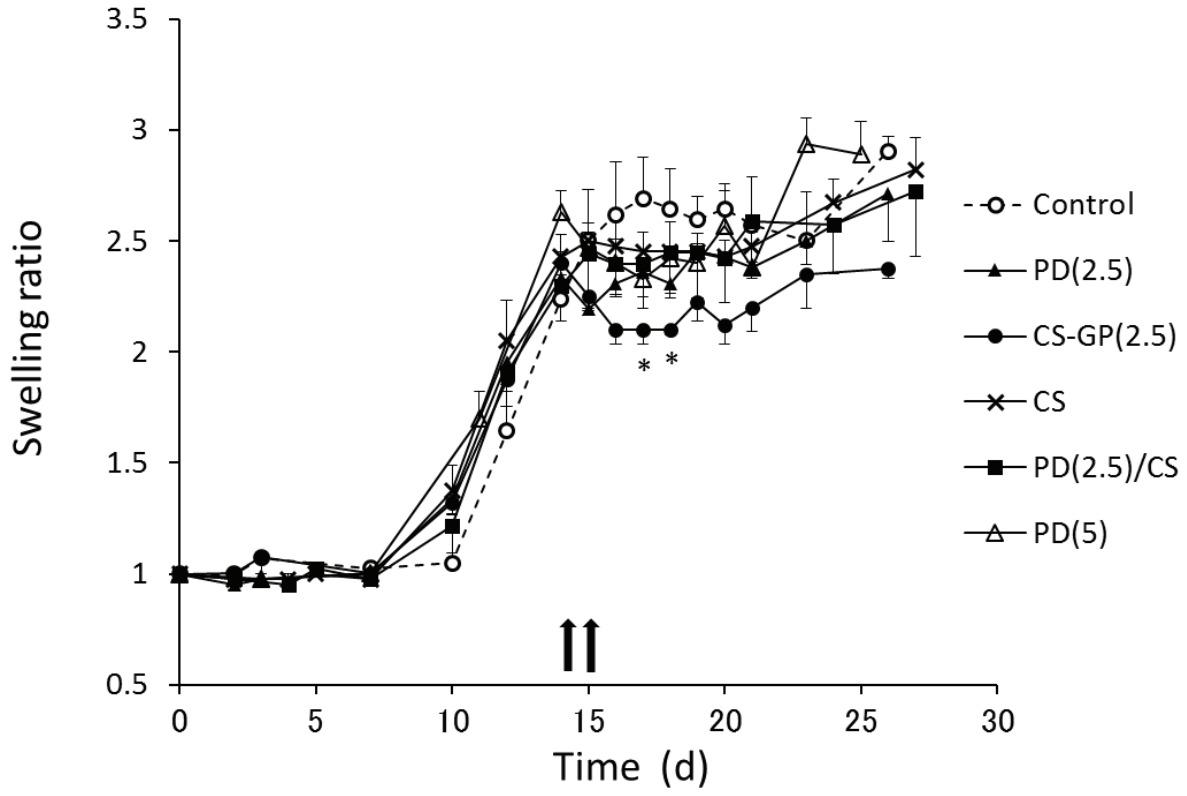


Fig. 16. Swelling of Left Hind Paw after Adjuvant Injection into Rats, and Therapeutic Effect of Each Preparation against Arthritis

(mean ± SE, n = 3)

*p< 0.05 vs. control (Dunnett's test). The arrow shows the dosing time.

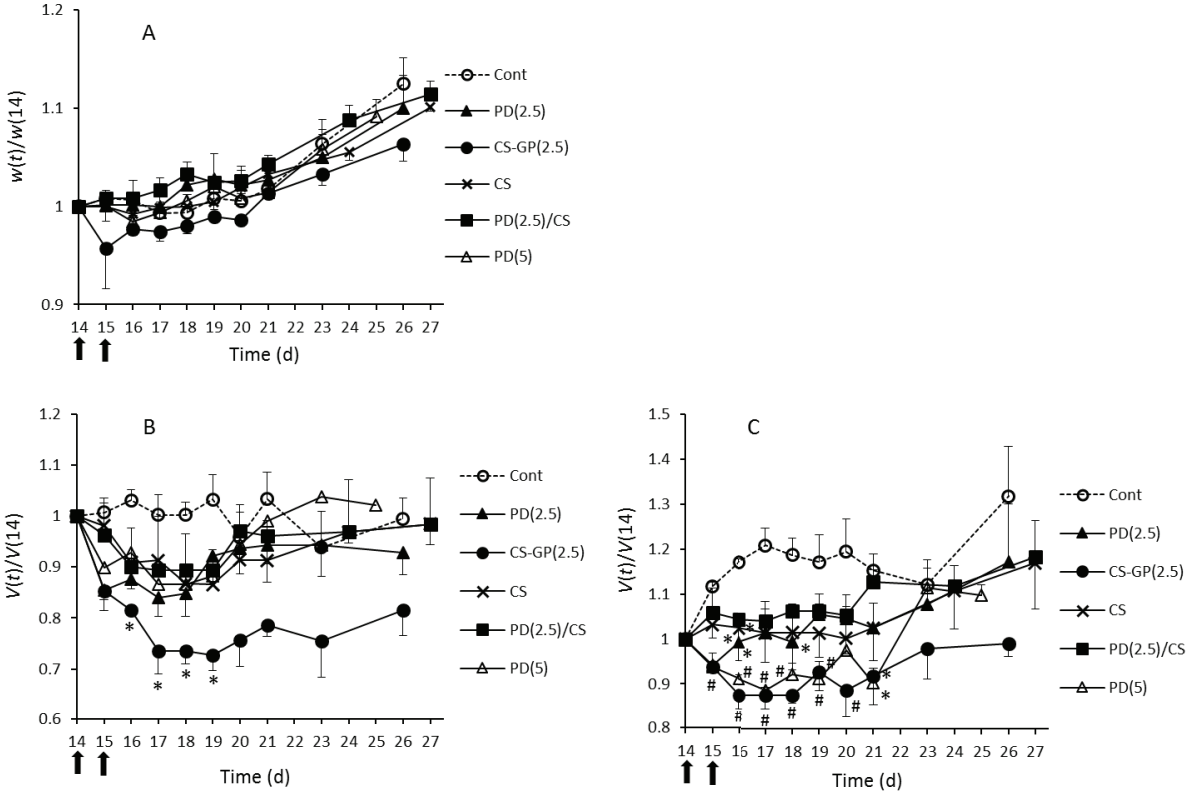


Fig. 17. Effect of Each Preparation on Body Weight (A), Right Paw Volume (B) and Left Paw Volume (C), expressed as the Ratio to the Value Just before I.V. Administration (mean ± S.E., n=3)

* $p< 0.05$, # $p< 0.05$ vs. control (Dunnett's test). The results were calculated based on the data of Figs. 14 – 16. The arrow shows the dosing time.

3.3. Pharmacokinetics and Biodistribution

The pharmacokinetic features of CS-GP and PD were investigated after i.v. injection into normal rats at 2.5 mg PD equiv./kg. The plasma concentration-time profiles were obtained as shown in Fig. 18. After i.v. injection of PD alone, the plasma concentration declined rapidly until 1 h, and then was eliminated slowly (Fig. 18A). For CS-GP, both free and total (= free + conjugated) PD concentrations were assessed after i.v. administration at 2.5 mg PD equiv./kg. The conjugated PD concentration is shown with a broken line (Fig. 18B). The total PD concentrations were 20.2, 19.2, 14.2 and 3.9 μg/mL at 0.25, 0.5, 1 and 3 h, respectively, after the injection, which were approximately 10 times larger than the free PD concentrations, respectively. This was based on the high plasma retention of the conjugated PD. The pharmacokinetic parameters were calculated according to the trapezoidal rule using the program MULTI.[60] The plasma concentration at 0 h was calculated by the extrapolation of the initial 2–3 points to time 0, and used for pharmacokinetic analysis. The pharmacokinetic parameters were obtained as shown in Table 4. The AUC(0–24 h) values of the total PD and conjugated PD were significantly greater than that of PD alone, being approximately 20 and 19 times larger. For free PD, no significant difference in AUC(0–24 h) was observed between CS-GP and PD alone, although CS-GP tended to display a greater AUC(0–24 h) value for free PD as compared with PD alone. Although the blood sample was quickly cooled in the pharmacokinetic experiments, it took some time (5 min or more) for the next steps, including centrifugation and treatment with other solutions. Therefore, during those operations, additional release might have occurred to some extent; in particular, the influence would be greater at an earlier time. This might be because the free PD generated from CS-GP tended to show a higher concentration and larger AUC (0–24 h) value, though both the AUC (0–24 h) values were not significantly different.

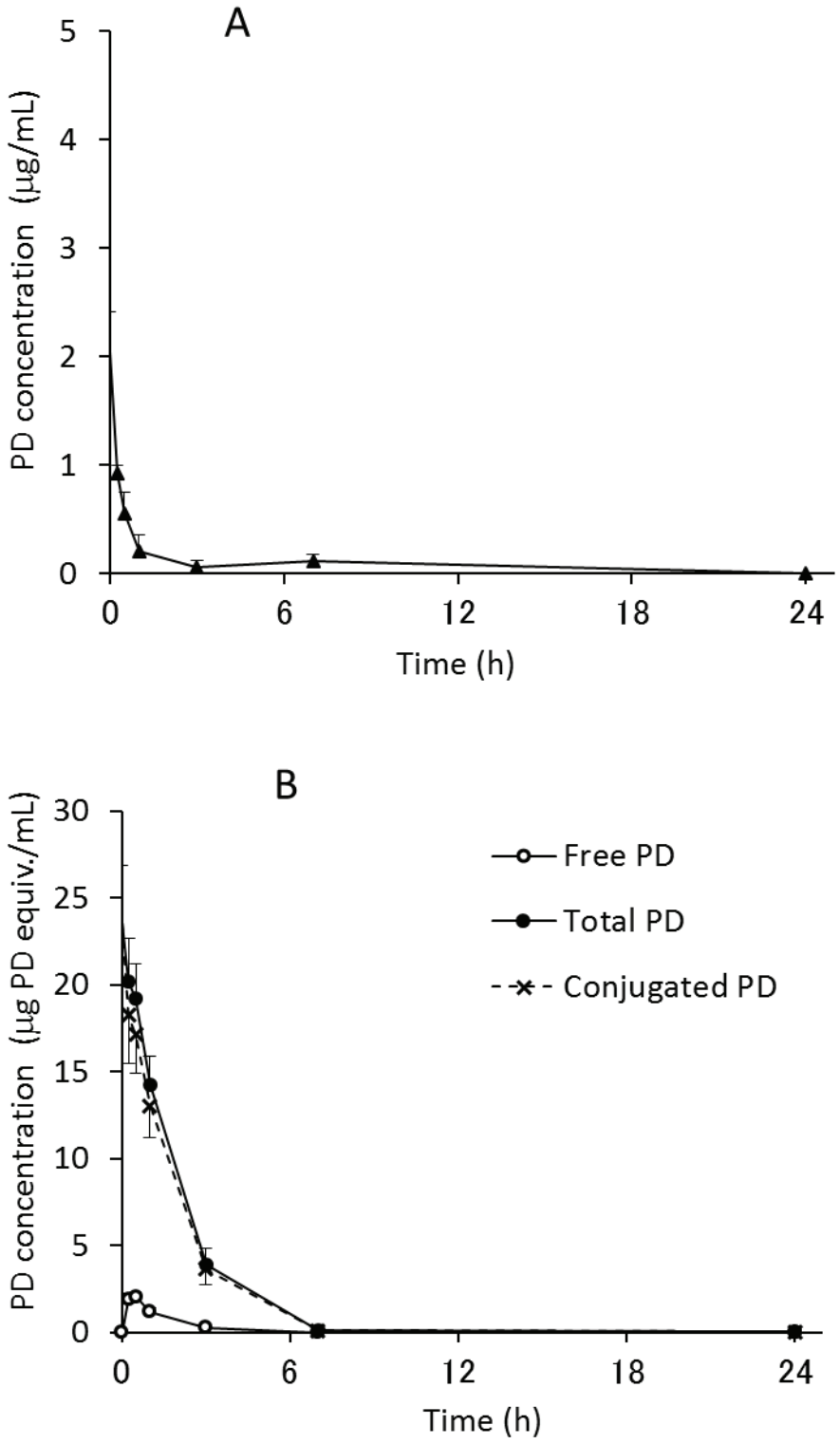


Fig. 18. Plasma Concentration–Time Profiles after I.V. Administration of PD Alone (A) and CS-GP (B) to Normal Rats (mean ± SE, n = 3)

Table 4

Pharmacokinetic Parameters for PD Alone and CS-GP after I.V. Administration to Normal Rats (mean ± SE, n = 3).

Drug	Species of PD	AUC(0-24 h) (μg·h/mL)	MRT(0-24 h) (h)	VRT(0-24 h) (h^2)
PD alone		2.30 ± 0.65	3.37 ± 1.39	6.20 ± 3.15
CS-GP	Total	46.21 ± 5.84 #	1.54 ± 0.11	4.01 ± 2.12
	Free	3.60 ± 0.59	1.27 ± 0.04	1.00 ± 0.03
	Conjugated	42.62 ± 6.06 #	1.56 ± 0.11	4.19 ± 2.21

# $p < 0.01$ vs. PD alone (Dunnett's test).

The pharmacokinetic results demonstrated that CS-GP enhanced the systemic retention of PD extensively, which was due to the much higher localization of CS-GP in the blood circulation. Although CS-GP did not prolong the systemic retention of PD so much, the AUC value increased to a great extent. This high localization of CS-GP in the systemic circulation was considered to facilitate the accumulation of the drug at the inflammatory site based on EPR effect.[7] Furthermore, it was found out that CS-GP did not maintain the plasma PD level for long because of the pharmacokinetic characteristics of CS,[25,54,71] which is not retained so long in the blood circulation.

The drug distribution was investigated in several organs 24 h after i.v. injection. The distribution profiles were obtained as described in Fig. 19. For PD alone, PD was detected in only the kidney and liver. The kidney concentration was lower than 0.4 μg/g, and the concentration was even less in the liver. For CS-GP, free and total concentrations were examined. PD and CS-PD were detected in only the liver and lung. Total PD concentrations in the liver and lung were 2.2 and 0.2 μg/g, respectively, which were larger than the PD concentrations observed in PD alone. The conjugated PD was observed more than free PD in the liver, but the conjugated PD was not found in the lung. However, this drug distribution extent was very low; even in the liver, being the largest tissue, the total distributed amount for CS-GP was 20.3 μg PD equiv. per rat (<5 % of dose). These low organ distributions were probably due to the biological features of CS, which is metabolized and excreted relatively easily. Non-accumulation in organs suggested that CS should be a suitable carrier for the drug delivery system.

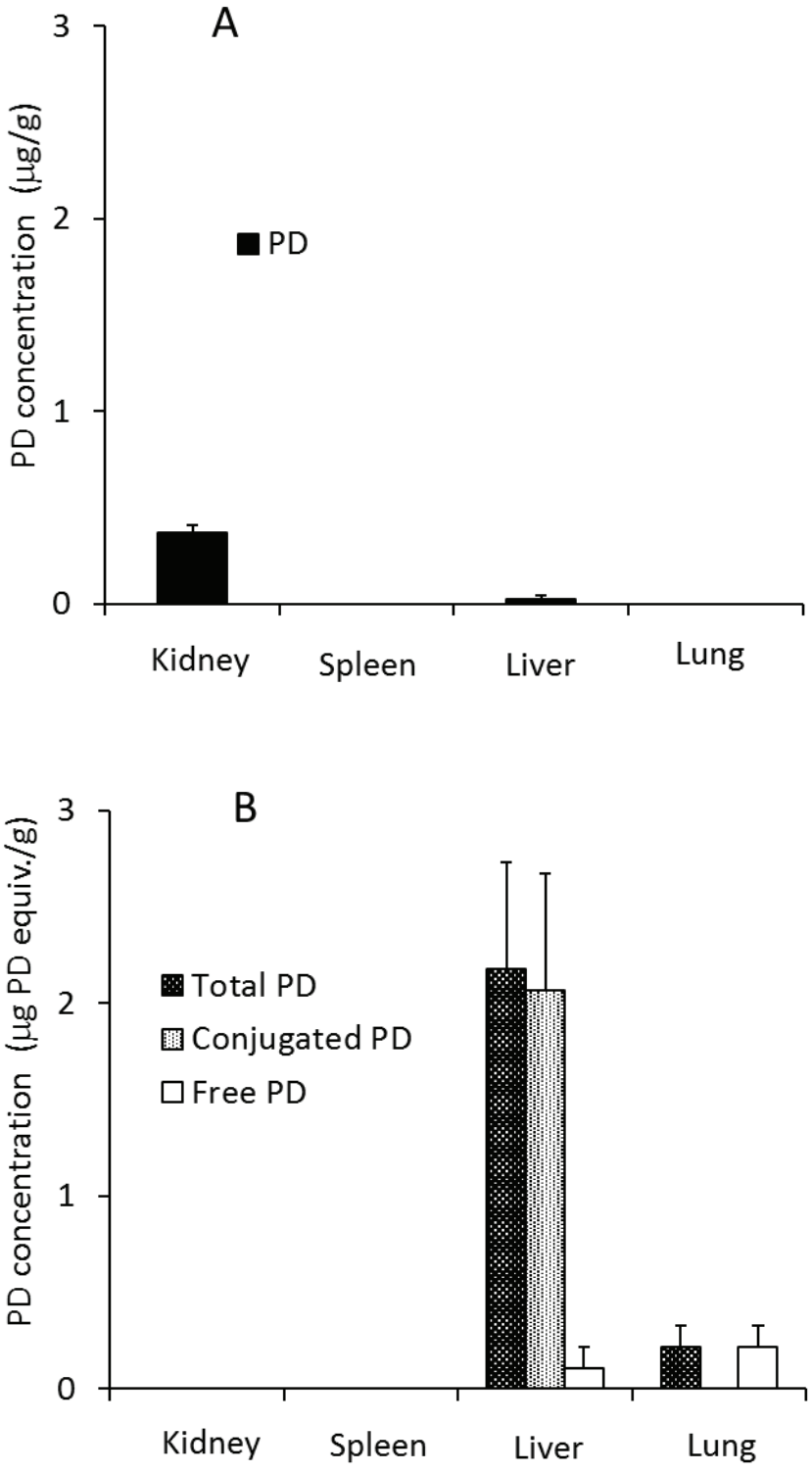


Fig. 19. Organ Distribution at 24 h after I.V. Administration of PD Alone (A) and CS-GP (B) to Normal Rats (mean ± SE, n = 3)

4. Conclusion

CS-GP conjugates with different PD contents, and 1.4–4.3 % (w/w) was prepared based on the method reported previously and investigated for release at pH 6 and 7.4 at 37 °C. At each pH, the release rate was not so different irrespective of the drug content. PD was released gradually and fairly rapidly at the physiological pH 7.4, while it was released slowly at the severely inflammatory pH 6, suggesting that CS-GP would release PD gradually in the body and more slowly in the diseased part. The effectiveness of CS-GP was investigated *in vivo* using rats with adjuvant-induced arthritis. Efficacy was evaluated by the comparison of anti-arthritic effects among CS-GP (2.5 mg PD equiv./kg), PD alone (2.5 and 5 mg/kg), CS alone and the PD (2.5 mg/kg)/CS mixture. CS-GP showed the most suppressive effect against arthritic swelling in both hind paws and reduced the paw volume significantly than the control for a fairly long period, but other preparations were less effective. The pharmacokinetic characteristics of CS-GP were investigated after i.v. injection into normal rats. CS-GP showed much higher plasma levels, resulting in a much greater AUC value, indicating that CS-GP showed a high retentive property in the systemic circulation. In addition, CS-GP exhibited no localization in tested major organs. These results suggested that CS-GP should be highly effective, less toxic and suitable for a targeting delivery system for the treatment of RA.

Chapter 3

Chondroitin Sulfate-Glycyl-Prednisolone Conjugate as Arthritis Targeting System: Localization and Drug Release in Inflammatory Joints

1. Introduction

In chapter 2, the conjugate of chondroitin sulfate-glycyl-prednisolone (CS-GP), was found to act as a macromolecular prodrug of PD and to promote anti-arthritic effect,[35] in which efficacy was evaluated using the rats with adjuvant-induced arthritis.[17, 72] In the pharmacokinetics in healthy rats, it was suggested that good retention of CS-GP in the plasma should facilitate the drug distribution to the arthritis site based on EPR effect. As the used CS has a molecular weight (MW) of only several tens of thousands,[34] its EPR effect might not be very high.

Therefore, in this chapter 3, the pharmacokinetic features and the extent of the drug localization to the arthritis site were examined using rats with adjuvant-induced arthritis in order to elucidate the mechanism of the promoted efficacy by CS-GP. At the same time, the passive targeting ability of CS-GP was evaluated by comparison of the drug localization between arthritic and healthy rats. Furthermore, the MW situation of CS-GP in the systemic circulation was investigated after i.v. administration.

2. Materials and Methods

2.1. Materials

Chondroitin sulfate C (CS) sodium salt (CS-Na), derived from shark cartilage and containing 2.6 % N and 6.6 % S, was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and the ratio of 6-sulfate to 4-sulfate in CS-Na was 9 : 1, that is, 6-sulfate was greater, and the average MW was 40,000–80,000. *N*, *N*'-Carbonyldiimidazole (CDI), 4-dimethylaminopyridine (DMAP), *N*-hydroxysuccinimide (NHS) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (WSC) were also obtained from the same manufacturer. *N*-Tritylglycine (Tr-G) was purchased from Sigma Chemical Company (St. Louis, MO, USA). Heat-killed and

desiccated *Mycobacterium tuberculosis* M37Ra was obtained from Difco Laboratories (Detroit, MI, USA) and used as an adjuvant. All other chemicals were of reagent grade. Sephadex G-50 fine powder was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden), and used for gel chromatography. Amicon Ultra-0.5 mL centrifugal filters with molecular weight (MW) cutoffs of 30 K and 100 K were obtained from Merck Millipore, Ltd. (Darmstadt, Germany), and used for estimation of molecular weight distribution.

2.2. Animals

Lewis female rats (8 weeks old, 150-160 g) were obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan). They were bred on the breeding diet MF supplied by Oriental Yeast, Co., Ltd. (Tokyo, Japan) with water ad libitum at 23±1 °C and relative humidity of 60±5 %. They were used for the experiments a few days after purchase. The experimental protocol was approved by the Committee on Animal Research of Hoshi University, Japan. The animal experiments were performed in compliance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, Japan.

2.3. Preparation and Chemical Characterization of CS-GP

Chondroitin sulfate-glycyl-prednisolone (CS-GP) were produced according to the previously-reported procedure.[34, 35] The production scheme is shown in Fig. 20. In the first step, glycyl-prednisolone (GP) was synthesized as follows. Tr-G (477 mg, 1.5 mmol) and CDI (243 mg, 1.5 mmol) were dissolved in 10 mL tetrahydofuran at 0 °C and stirred for 30 min at 0 °C. DMAP (15 mg, 0.12 mmol) and PD (270 mg, 0.75 mmol) were added to the solution and stirred at room temperature for 4.5 h. After evaporation of the solvent, the ester of PD with Tr-G, called Tr-GP, was obtained by washing the residue with methanol. The chemical structure of Tr-GP was confirmed by TLC, ^{1}H-NMR, ^{13}C-NMR,

and EI-MS measurements as reported previously. Then, Tr-GP (300 mg) underwent detritylation by heating in 75 % (v/v) aqueous acetic acid (10 mL) at 75 ^{o}C. As soon as the white precipitate began appearing while heating, the mixture was cooled on ice. After the white precipitate was removed by filtration, the filtrate was evaporated and the resultant residue was chromatographed with a silica gel column with a mixture of chloroform and methanol (15 : 1, v/v) as elution solvent. The fractions corresponding to GP were collected and evaporated to dryness. The obtained compound, GP, was confirmed for its chemical structure by TLC, ^{1}H-NMR, ^{13}C-NMR, and MALDI-TOF-MS measurements, as reported previously.

CS-GP was prepared by carbodiimide coupling as follows. CS (240 mg) was dissolved in 20 mL water, and 5 mL tetrahydrofuran (THF) containing GP (60 mg) was added. WSC (1 g) and NHS (0.6 g) were added to the solution, and the resultant mixture was stirred at room temperature for 4.5 h. The solution was chromatographed with a Sephadex G50 gel column [2.8 cm (inner diameter) x 25 cm (length)] using 0.1 M NaCl aqueous solution as the elution solvent. The eluted solution was fractionated at 15 mL each. The high molecular weight fractions were collected and dialyzed against water at 4 °C. The final solution was lyophilized to yield CS-GP, which was shown to contain PD at 3.9 % (w/w) from the UV absorbance at 246 nm in the purified water/phosphate-buffered saline with pH 7.4/methanol solution (0.4/2/0.533, v/v). CS-GP in powder form was kept stable at room temperature in a desiccator with dry silica gel, in which free PD was little found even in half a year.

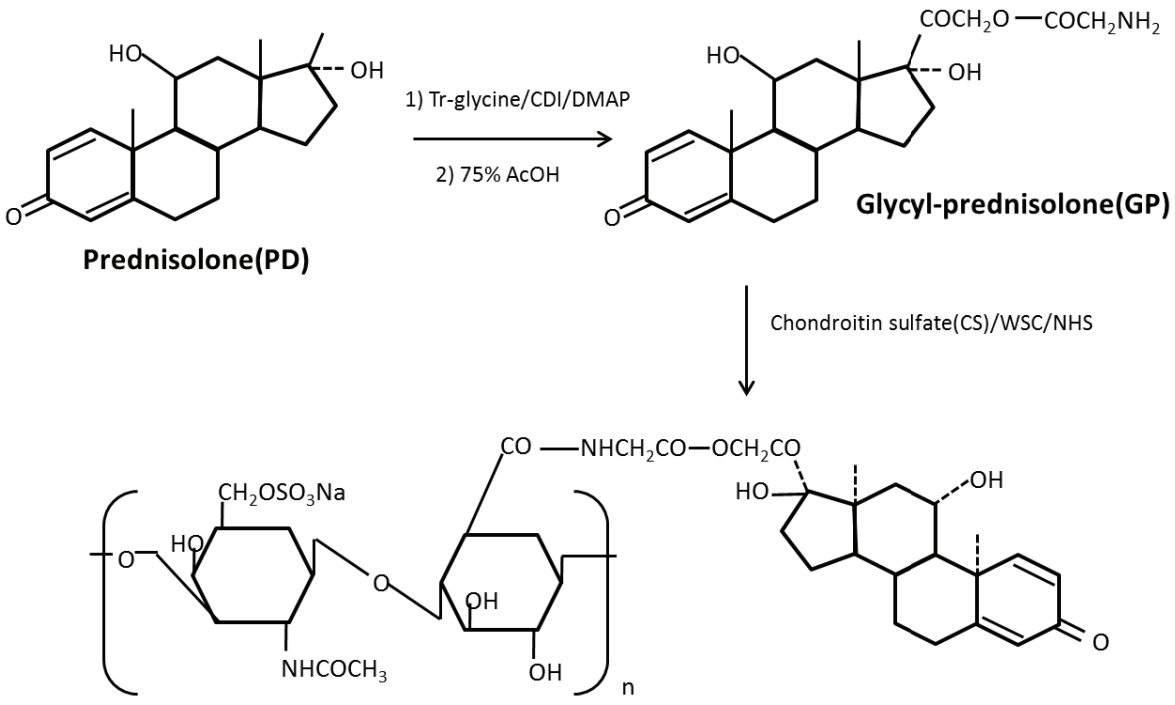


Fig. 20. Synthesis of Chondroitin Sulfate-Glycyl-Prednisolone Conjugate (CS-GP).

2.4. *In Vitro* Release Studies at Inflammatory Joint pH

The *in vitro* release of PD from CS-GP was examined using a mixture of purified water/buffer/methanol (0.4/1.2/0.533, v/v), in which 1/15 M phosphate buffers of pH 6.5 and 7 (typical pH of inflamed tissue media) were used as the buffer solution. The mixture containing CS-GP (2.13 mL) was incubated at 2.61 μg PD equiv./ mL at 37 ^{o}C under horizontal shaking at 60 rpm. At appropriate time points, aliquot samples (50 μL) were withdrawn, and 1/10 M acetate buffer of pH 4 (150 μL) was added to stop the hydrolysis. After 150 μL of HPLC mobile phase was added, the mixture was injected onto HPLC to analyze the PD concentration in the incubation media.

2.5. Pharmacokinetic Experiment in Rats with Adjuvant-Induced Arthritis

Animal models with adjuvant-induced arthritis were made according to the method by Hirano et al. (1994).[17] Namely, heat-killed *Mycobacterium tuberculosis* M37Ra was suspended in liquid paraffin at 5 mg/mL. The suspension (100 μL) was injected intracutaneously into the footpad of the right hind paw of each Lewis rat. The volume of the hind paws reached a plateau around 14 days after the adjuvant injection. Dosing samples were prepared as follows. CS-GP was dissolved in saline at 2.5 mg PD equiv./mL, and PD was dissolved in 50 % (w/v) PEG400 saline solution at 2.5 mg/mL.

The pharmacokinetic experiment was performed according to the schedule in Fig. 21A. Namely, 14 d after adjuvant injection, the rats were administered CS-GP or PD solution at 2.5 mg PD equiv./kg/mL via the jugular vein. Blood sampling (each, 0.3 mL) was performed immediately before dosing and 15 min, 30 min, 1 h, 3 h and 7 h after drug administration.

For dosing of PD alone (PD solution), the blood sample was centrifuged at 1500xg for 10 min to obtain plasma. Saturated NaCl aqueous solution (100 μL),

6 % (w/v) phosphoric acid (100 μL) and 4 mL of the mixture of *t*-tributylmethyl ether and pentane (3 : 2, v/v) were added to the plasma (100 μL). The mixture was shaken vigorously 100 times and then centrifuged at 1500xg for 10 min. The supernatant (3 mL) was taken and dried under nitrogen gas. The residue was dissolved with 100 μL HPLC mobile phase and analyzed by HPLC to determine the concentration of PD in plasma.

For CS-GP, the plasma concentration of free PD and that of total (free + conjugated) PD were measured. After centrifugation of the blood sample, two plasma samples (each, 50 μL) were obtained; one sample (50 μL) was used for analysis of the free PD concentration, and the other (50 μL) was used for measurement of the total PD concentration. First, 50 μL of the plasma sample was treated in a similar manner to PD alone. Namely, saturated NaCl aqueous solution (50 μL), 6 % (w/v) phosphoric acid (50 μL) and 2 mL of the mixture of *t*-tributylmethyl ether and pentane (3 : 2, v/v) were added to the plasma (50 μL). After the mixture was shaken vigorously 100 times and centrifuged at 1500xg for 10 min, the resultant supernatant (1.5 mL) was taken and dried under nitrogen gas. The residue was dissolved in 100 μL HPLC mobile phase and analyzed by HPLC to obtain the free PD concentration.

Second, to another plasma sample (50 μL), 50 μL of 0.1 M NaOH aqueous solution was added, mixed and then incubated at 45 ^{o}C by shaking horizontally at 60 rpm for 10 min. Then, 150 μL of 0.1 M acetate buffer of pH 4 was added. Saturated NaCl aqueous solution (200 μL), 6 % (w/v) phosphoric acid (200 μL) and 8 mL of the mixture of *t*-tributylmethyl ether and pentane (3 : 2, v/v) were added to the whole resultant mixture and shaken vigorously 100 times. After the mixture was centrifuged at 1500xg for 10 min, the supernatant (3 mL) was taken and dried under nitrogen gas. The residue was dissolved in 100 μL HPLC mobile phase and analyzed by HPLC to determine the total drug concentration. The concentration of conjugated PD was calculated by subtracting the concentration

of the above free PD from that of the total PD.

2.6. Drug Distribution Studies at Inflammatory Joints in Rats with Adjuvant-Induced Arthritis

The drug distribution at the inflammatory joint was analyzed as shown in Fig. 21B. This was performed separately from the above pharmacokinetic experiment. On 14 d after adjuvant injection, CS-GP or PD solution, prepared in the same manner as in the pharmacokinetic study, was injected at 2.5 mg PD equiv./kg/mL into the jugular vein. At 1, 7 or 24 h after drug administration, a blood sample (0.3 mL) was taken from the jugular vein under anesthesia with ethyl ether. Immediately after, the animals were sacrificed by excessive anesthesia with ethyl ether inhalation. Then, both right and left hind paws were taken using scissors for bone resection so that the inflamed part around the ankle was taken with each neighboring side (each: approximately 0.5 cm). After the skin was removed from the taken leg part, the resultant tissue (1.0 g–2.0 g) was treated as follows to measure the drug concentration.

The obtained tissue containing flesh and bone was minced using scissors for bone resection and those for cutting meat in a small plastic box. After the obtained joint fragments (0.5–1 g) were put into a glass bottle, liquid nitrogen (3 mL) was added several times until the fragments were completely frozen. The frozen fragments were mixed well in the bottle and placed in an iron cylinder (1.5 cm ϕ x 2.5 cm long) which had been cooled in advance by dipping in liquid nitrogen. The frozen fragments were pressed manually with a pestle at 300 kg for 30 s. Thus, 1 or 2 disks were obtained for each inflamed tissue and were preserved at –80 ^{o}C until drug extraction for preparation of the HPLC sample. Small pieces of debris and liquid derived from the tissue fragments were collected by washing the plastic box, and the washings, total volume of which was set to be equal to the total weight of the above tissue disks, were put in the

glass bottle used for drug extraction; the final liquid was called wash liquid and was used for preparation of the HPLC sample.

The sample for HPLC analysis was prepared as follows. A portion of the above frozen disk (0.2 g) was cut out, and 0.2 mL of the above wash liquid and 0.2 mL of 0.1 M acetate buffer (pH 4) were added. After the mixture was stirred by a vortex mixer and sonicated at 20 kHz (100 kW) at 5 min, 1.8 mL acetonitrile was added, stirred by a vortex mixer and sonicated at 20 kHz (100 kW) at 5 min. Then, the mixture was left to stand at room temperature for 1.5 h, during which the whole tissue fragments were denatured almost completely. After centrifugation, 1.2 mL of the supernatant was withdrawn, and the organic solvent was evaporated at 30 ^{o}C under nitrogen gas. The remaining aqueous liquid underwent lyophilization. To the residue, 200 μL HPLC mobile phase was added, and the resultant solution was analyzed by HPLC to determine the tissue concentration of free PD.

The total (free + conjugated) PD concentration in the inflamed tissue was measured as follows. The frozen disk (0.2 g) and wash liquid (0.2 mL), prepared as above, were mixed, and 0.1 M NaOH aqueous solution (0.4 mL) was added. Then, the mixture was warmed to 45 ^{o}C for 8 min while shaking horizontally at 60 rpm. Then, 0.1 M HCl aqueous solution (0.4 mL) was added to neutralize the pH, and 3.6 mL acetonitrile was added. The mixture was stirred by a vortex mixer and sonicated at 20 kHz (100 W) for 10 min, then centrifuged at 1500xg for 10 min. After 2.4 mL of the supernatant was taken, the contained organic solvent was dried at 40 ^{o}C under nitrogen gas. Then, the remaining aqueous liquid was lyophilized. To the residue, 200 μL HPLC mobile phase was added, and the resultant solution was analyzed by HPLC to obtain the total drug concentration of the inflammatory joint.

Furthermore, the control study using healthy rats was performed to evaluate the passive targeting ability of CS-GP. That is, CS-GP was

administered intravenously to healthy rats at 2.5 mg PD equiv./kg. At 24 h after administration, the rats were sacrificed by excessive anesthesia with ethyl ether inhalation. Immediately after that, both right and left hind paws were taken using scissors for bone resection with each neighboring side (each: approximately 0.5 cm). The skin of the taken tissue was removed, and the resultant tissue (ca. 0.3 g) was analyzed for the total PD concentration in the same manner as described above. The obtained total PD concentrations were compared between adjuvant-induced arthritis and healthy rats.

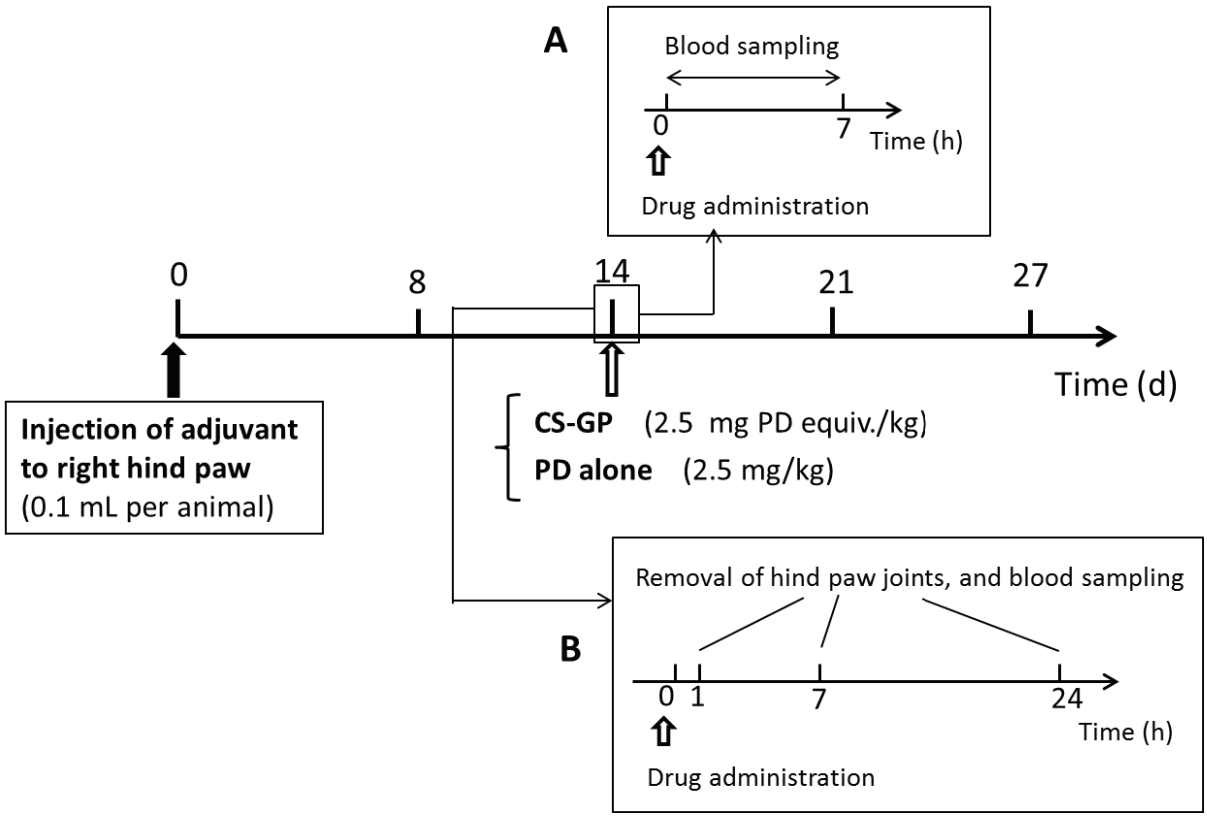


Fig. 21. Schemes of Studies on Pharmacokinetics (A) and Drug Distribution in the Inflammatory Joints (B) after I.V. Administration to Rats with Adjuvant-Induced Arthritis

2.7. Estimation of Physicochemical State of CS-GP in Systemic Circulation

The blood sample taken 1 h after i.v. injection of CS-GP was used for examination of its apparent molecular weight in the systemic circulation. First, the original molecular weight distribution of CS-GP was measured using ultrafilter membranes with molecular weight (MW) cutoffs of 30K and 100K (Amicon Ultra). Namely, 0.5 mg/mL CS-GP solution in phosphate-buffered saline at pH 7.4 was filtered using the ultrafilter membranes. The original solution and the filtrates were diluted with PBS and measured spectrophotometrically at 246 nm.

For the blood sample, the plasma was obtained after centrifugation. The MW distribution of CS-GP was estimated based on the total drug concentration in each eluted filtrate obtained after ultrafiltration, because the concentration of free PD is low in the plasma 1 h after i.v. injection. Namely, the examination was conducted as follows. The plasma (50 μL) and PBS (450 μL) were mixed and filtered using the ultrafilter with an MW cutoff of 100K. In addition, the plasma (25 μL) and PBS (450 μL) were mixed and filtered using the ultrafilter of 30K cutoff. The mixture before ultrafiltration and the filtrates were treated in the same manner as the plasma sample in the pharmacokinetic experiment. Namely, 50 μL of 0.1 M NaOH aqueous solution was added to each sample (50 μL) and then incubated at 45 ^{o}C by shaking horizontally at 60 rpm for 10 min. Then, 150 μL of 0.1 M acetate buffer of pH 4 was added. Saturated NaCl aqueous solution (200 μL), 6 % (w/v) phosphoric acid (200 μL) and 8 mL of the mixture of *t*-tributylmethyl ether and pentane (3 : 2, v/v) were added and shaken vigorously 100 times. After centrifugation, the supernatant (3 mL) was taken and dried under nitrogen gas. The residue was dissolved in 100 μL HPLC mobile phase and analyzed by HPLC to determine the total drug concentration. From the total PD amount in the sample or filtrate, the distribution of CS-GP in the individual MW range was estimated, because the free PD concentration was

much less than the total PD concentration 1 h after i.v. injection (see Fig. 23).

2.8. HPLC Assay

For the HPLC apparatus, a Shimadzu LC-6AD pump was used with a Shimadzu SPD-10AV VP UV-VIS detector set at a wavelength of 246 nm and a Shimadzu C-R7A plus Chromatopac (Kyoto, Japan). A YMC Pack ODS-AM column (6 mm inner diameter × 150 mm length; YMC Co. Ltd., Kyoto, Japan) was used as the analytical column. A 26 % (v/v) 2-propanol aqueous solution containing 0.1 % (v/v) trifluoroacetic acid was used as the mobile phase. The HPLC assay was performed at room temperature, with the flow rate set at 1 mL/min and an injection volume of 40 μL. The PD concentration was determined using the absolute calibration curve, which was obtained after treating fresh samples spiked with a known amount of the drug in the same way as the treatment of the test sample.

2.9. Statistical Analysis

Statistical analyses of more than 2 groups were performed with one-way ANOVA followed by Dunnett's test, and comparison of two groups was conducted with unpaired *t*-test. Significant difference was set as $p<0.05$.

3. Results and Discussion

3.1. *In Vitro* Release of PD from CS-GP at Inflammatory Joint pH

According to the literature on the medium pH at joints with rheumatoid arthritis, the pH is 7–7.4 in a moderate disease state, and falls to <7 as the disease condition worsens. In a severe disease state, the pH falls to 6–6.5. In the previous study, the release profiles were investigated at pH 6 and 7.4. Therefore, the release of PD was further examined at pH 6.5 and 7 in the present study. The release profiles are shown in Fig. 22, including the release profiles at pH 6 and

7.4, obtained previously. The release rate constants, obtained by pseudo-first order kinetic analysis, were 0.013 and 0.031 h^{-1}, respectively; that is, the 50 % release time periods were calculated to be 53 and 22 h at pH 6.5 and 7, respectively. Considering that the release rate of PD was dependent mainly on pH, not caused enzymatically, [34] PD was considered to be released slowly over one to several days in the inflammatory joint area. As the PD release was caused gradually at physiological pH 7.4 (approximately 50 % for 7 h), two-long systemic circulation might be unsuitable for the localization to the diseased area. In the case of CS-GP, the systemic retention was not so long as stated below in the plasma concentration study, probably due to the metabolic fate of CS itself. Therefore, the loss by release of PD in the systemic circulation was not a critical problem, though it would be desirable to suppress the drug release in the systemic circulation maximally.

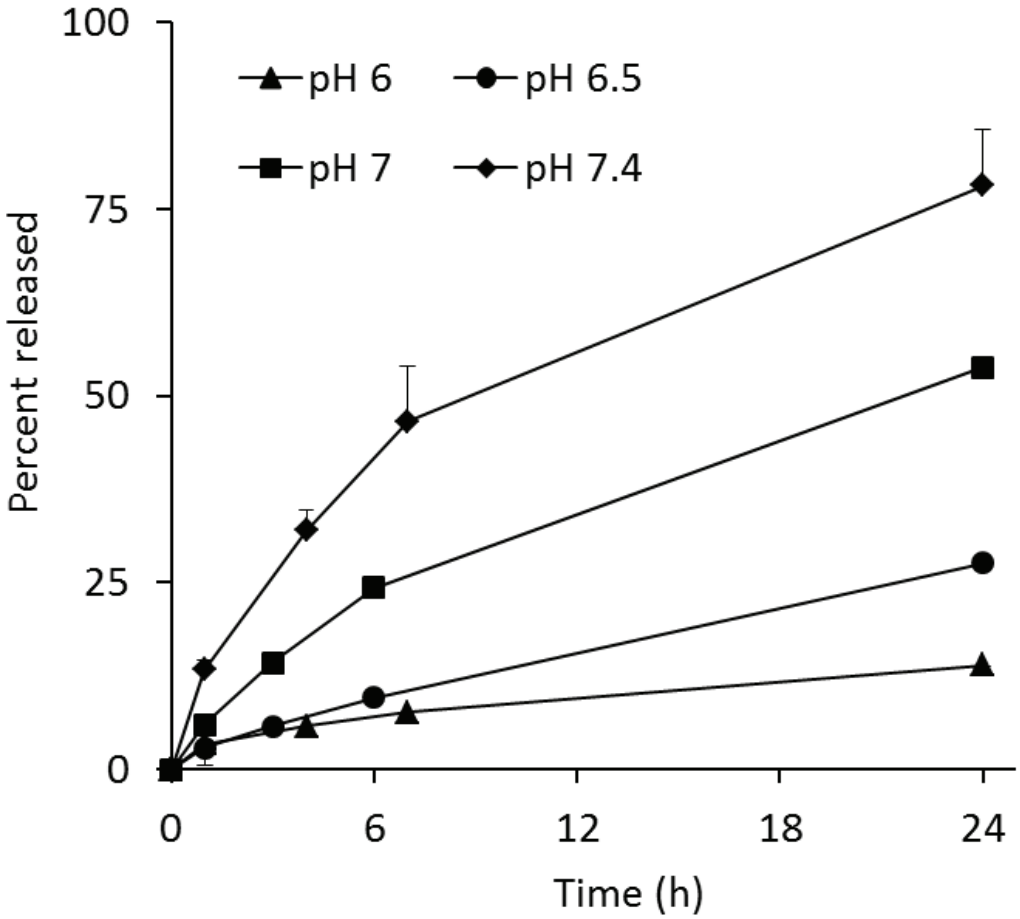


Fig. 22. *In Vitro* Drug Release from CS-GP during Incubation of Its Aqueous Solutions at Different pH Related to Inflammatory Joints.

Each point represents the mean ± S.D. (n=3).

3.2. Pharmacokinetics after I.V. Injection in Rats with Adjuvant-Induced Arthritis

After PD alone and CS-GP were intravenously administered to rats with adjuvant-induced arthritis, the plasma levels were investigated from 0.25–7 h. For PD alone, the plasma concentration immediately after i.v. injection (0 h) was calculated by extrapolation with the exponential curve obtained for the points of 0.25 and 0.5 h. Also, for CS-GP, the total PD concentration immediately after i.v. injection (0 h) was calculated in the same manner. The conjugated PD concentration (CS-GP(C)) was calculated by subtraction of the free PD level from the total level. For both administrations, the plasma concentration profiles were basically similar to those obtained previously in normal rats. [35] The results are shown in Fig. 23. For PD alone, the plasma concentration was eliminated in a bi-phasic manner, in which rapid elimination at 0–30 min and slow decline at 0.5–7 h were observed. For CS-GP, the total concentration was eliminated in a mono-exponential manner from 0.25 h to 7 h. At 0.25–3 h after administration, the total concentration was more than 10 times greater in CS-GP than in PD alone; a significant difference was observed from 0 h to 3 h in comparison of PD alone with both total and conjugated PDs ($p< 0.05$ or 0.01). The concentration of free PD was much less than the total concentration from 0 h to 7 h after administration of CS-GP, and not so different from that given by PD alone.

Thus, conjugated PD was found to contribute to the high plasma level of the total concentration of CS-GP. Considering that CS is eliminated by hepatic metabolism and urinary excretion generally with a half-life of several dozen min – a few h in human and animals,[25, 71, 75] CS-GP was considered to be concentrated well in the systemic circulation. Thus, it could be proposed that the high distribution of CS-GP in the systemic circulation should promote the localization of PD at the inflamed tissue due to EPR effect.[76] Table 5

summarizes the results in moment analysis of the plasma concentration-time profiles, in which the computer program MULTI was used.[60] The AUC value of the total PD was nearly 13 times significantly greater in CS-GP than in PD alone ($p< 0.01$). As the AUC value of the conjugated PD was 11 times significantly higher than the PD level in PD alone ($p< 0.01$), the conjugated PD was considered to raise the drug retention in the systemic circulation. The values of MRT and VRT were similar among all the types of drugs in Table 5 except for the VRT value of PD alone. The greater value of VRT in PD alone was due to the slow elimination in the later phase of the plasma concentration-time curve after administration of PD alone. On the other hand, after i.v. injection of CS-GP, the concentrations of the total and free dugs decayed in a more linear manner. However, the VRT values of total, free and conjugated PD given by CS-GP were not significantly different from that given by PD alone.

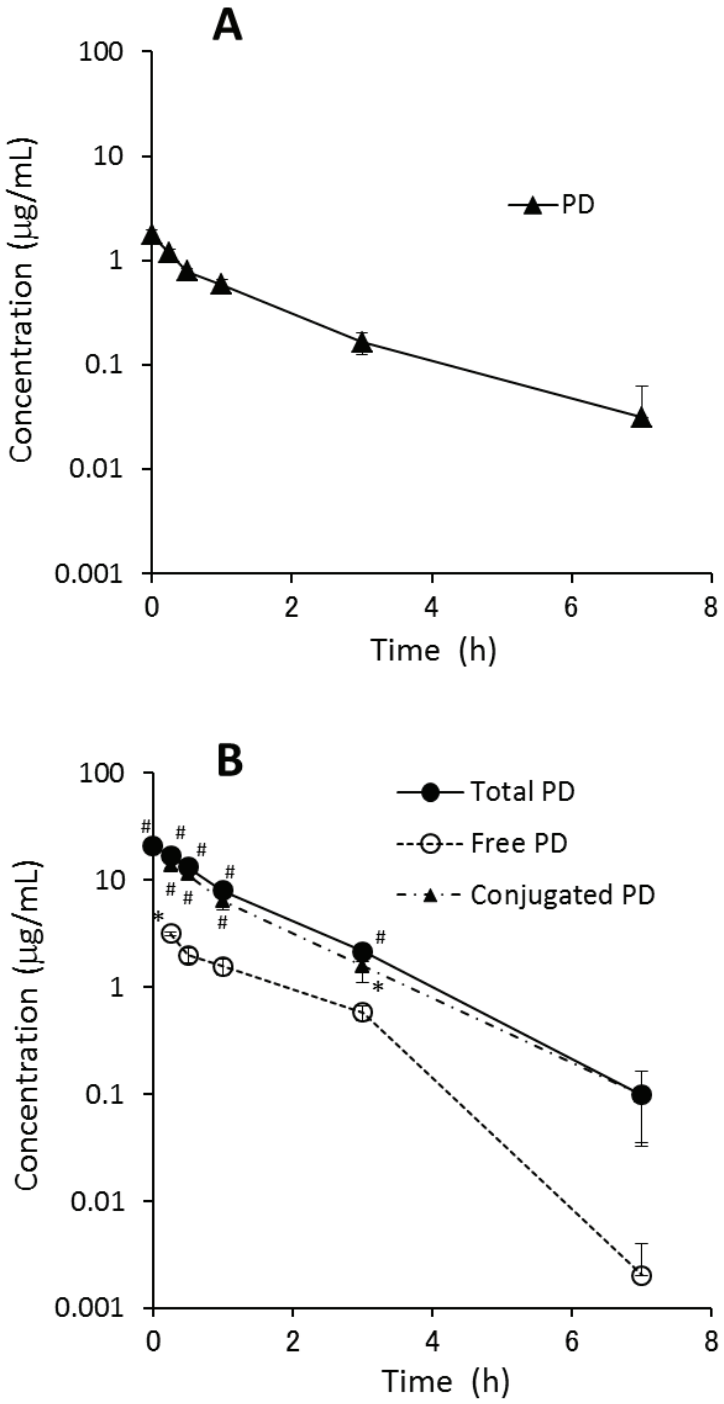


Fig. 23. Plasma Concentration-Time Profiles after I.V. Administration of PD Alone (A) and CS-GP (B) at the Dose of 2.5 mg PD equiv./kg in Rats with Adjuvant-Induced Arthritis.

Each point represents the mean ± S.E. (n=4). * $p< 0.05$, # $p< 0.01$ vs. PD alone (Dunnett's test).

Table 5. Pharmacokinetic Parameters Obtained from the Moment Analysis

Preparation	Drug species	AUC(0-7 h) (μg·h/mL)	MRT(0-7 h) (h)	VRT(0-7 h) (h^2)
PD alone	PD	2.10 ± 0.25	1.32 ± 0.22	1.80 ± 0.80
CS-GP	Total PD	28.37 ± 2.70 #	1.20 ± 0.03	1.37 ± 0.16
	Free PD	5.25 ± 0.27	1.48 ± 0.11	1.21 ± 0.11
	Conjugated PD	23.12 ± 2.82 #	1.12 ± 0.06	1.36 ± 0.17

The results are expressed as the mean ± S.E. (n=4). # $p < 0.01$ vs. PD (PD alone) (Dunnett's test).

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3.3. Drug Distribution Profiles in Inflammatory Joints after I.V. Injection in Rats with Adjuvant-Induced Arthritis

Inflammation was found to occur most severely near the heel in both right and left hind paws. Each inflammatory joint was taken by cutting the limb and foot close to the joint. The skin of the taken joint was peeled, and the resultant peeled inflammatory joint was used as a sample for measurement of the distributed drug amount. The right hind paw joint weighed 1.5–2.0 g, and the left hind paw joint weighed 1.0–1.6 g; that is, the right hind paw joint tended to be heavier than the left hind paw joint.

The amount of drug recovered from the inflammatory joint was determined by spiking the homogenate with a known amount of the drug and subsequent treatment for HPLC analysis. Namely, free PD was recovered by denaturation with acetonitrile under the condition of weakly acidic pH. The recovery ratio of free PD from the joint homogenate was 85 % (w/w). On the other hand, total PD was investigated by hydrolysis of the ester bond with a 0.1 N NaOH aqueous solution and subsequent treatment using acetonitrile. The recovery rate of the total (free + conjugated) PD for CS-GP was 49 % (w/w). The decrease in the recovery rate for the total PD was considered to be due to the use of 0.1 N NaOH at 45 ^{o}C in ester hydrolysis. Therefore, the stability characteristics of PD and CS-GP were investigated using 0.1 N NaOH aqueous solution at 45 ^{o}C (Fig. 24). As a result, PD remained at approximately 60 % after incubation in 0.1 N NaOH aqueous solution for 10 min. For CS-GP, nearly 30 % of PD was decomposed 10 min after incubation of the stability test in a 0.1 N NaOH aqueous solution. Treatment under an alkaline condition was a major reason in the decrease in the recovery of the total PD. The determination was conducted with the standard curve including these phenomena; that is, correction by the recovery rates of PD and CS-GP were taken into account in the results (see Fig. 25).

The drug concentration-time profiles in the inflammatory joint tissues were measured after i.v. administration to disease-model rats. Free PD concentration was measured after i.v. administration of both PD alone and CS-GP. The results are shown in Fig. 25. In addition, the distribution of conjugated PD was calculated by subtracting the free PD amount from the total PD amount, and is described in Fig. 25. At 1 h after i.v. administration, PD alone exhibited a higher PD level than the total PD level given by CS-GP, but the drug concentration was eliminated fairly rapidly. At 7 h after i.v. administration, the total and conjugated PD concentrations by CS-GP were higher than the PD concentration by PD alone; a significant difference was observed for the left hind paw joint ($p<0.01$). At 24 h, the total and conjugated PD levels were significantly higher than that by PD alone ($p<0.05$ for the right hind paw joint, $p< 0.01$ for the left hind paw joint). These total and conjugated PD levels tended to be rather higher at 24 h than at 7 h. This might be due to the following reasons; although the plasma level of CS-GP decreased to the fairly low level at 7 h after administration, CS-GP still remained in the systemic circulation to some extent. Therefore, CS-GP was considered to be possibly delivered to the inflammatory site even after 7 h. In addition, CS-GP seemed to be well-retained in the joint tissues once delivered there; CS-GP might interact with the joint tissue components, which was found from the preliminary studies in the mixing of CS-GP and the tissues (data not shown). Also, as the release of PD from CS-GP was slow at the acidic joint pH, CS-GP was considered to be kept long in the conjugate form at the joint. These features were presumed to contribute to the high localization of CS-GP in the inflammatory joint at 24 h.

After administration of CS-GP, free PD was observed at a low but almost constant level, which was 0.09–0.14 μg/mL for the right hind paw joint and 0.06–0.11 μg/mL for the left hind paw joint. At 24 h after i.v. administration, the free PD concentrations were similar for both CS-GP and PD alone. Since the pH

of an inflammatory joint tends to be weakly acidic, the drug release was considered to be caused slowly. As CS-GP remained in inflammatory joints at much higher level at 24 h, the free PD level was expected to be retained longer because of the drug supply from the conjugate distributed in the inflammatory tissue. On the other hand, the drug was eliminated faster for PD alone. Reportedly, the effective concentration of PD for immunological suppression was shown to be several dozen–nearly one hundred ng/mL.[77,78] CS-GP showed good localization to inflammatory joint, prolonged retention there and sustained release of free PD, leading to higher efficacy of CS-GP than PD alone, which was reported previously.[35]

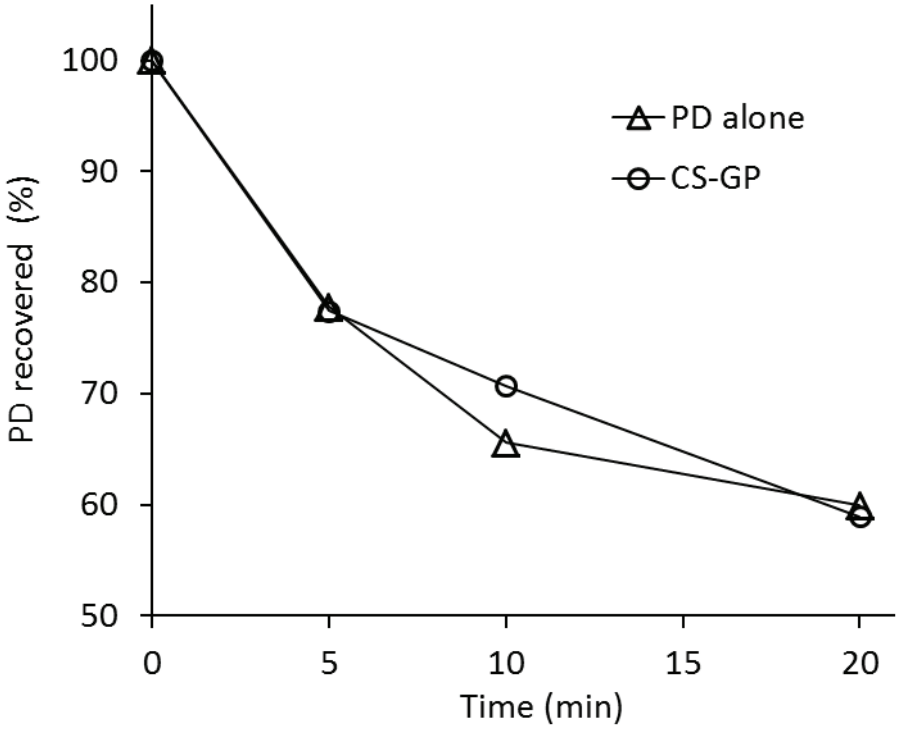

Fig. 24. Ratio of PD Recovered after Incubation of 0.1 M NaOH Aqueous Solution with PD Alone (△) and CS-GP (○) at 45 °C (n=1)

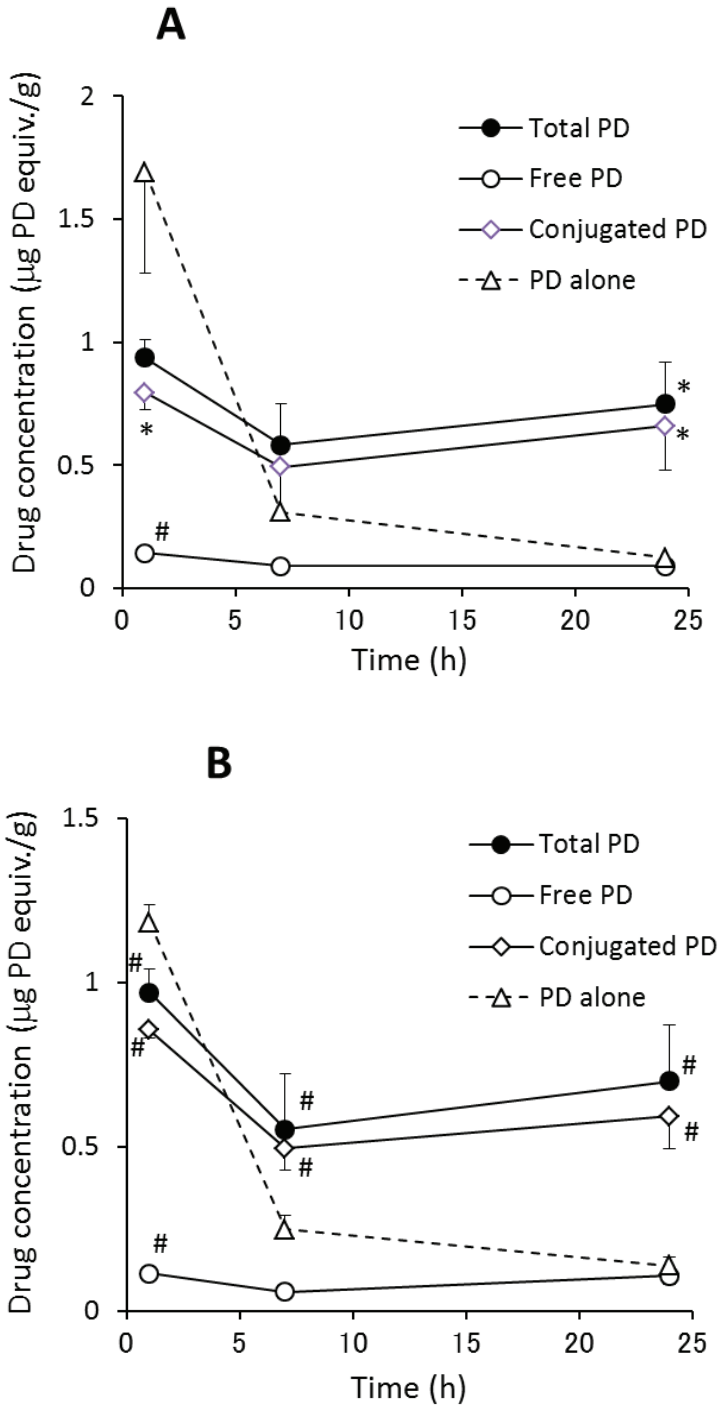

Fig. 25. Distributed Drug Concentration in the Right (A) and Left (B) Hind Paws after I.V. Administration of PD Alone and CS-GP at the Dose of 2.5 mg PD equiv./kg to Rats with Adjuvant-Induced Arthritis.

Each point represent the mean ± S.E. (n=3 for 1 and 7 h, 4 for 24 h).

* $p< 0.05$, $^{\#}$ $p< 0.01$ vs. PD alone (Dunnett't test).

3.4. Joint Drug Distribution in Adjuvant-Induced Arthritis and Healthy Rats

In order to evaluate the passive targeting ability of CS-GP, the concentrations of the total (conjugated + free) PD distributed into the joint tissue at 24 h after i.v. administration were compared between adjuvant-induced arthritis and healthy rats. The results are shown in Fig. 26. The total PD concentration in the joint tissue was found to be higher in adjuvant-induced arthritis rats than healthy rats. The total PD concentration of the left hind paw was significantly greater in adjuvant-induced arthritis rats than in healthy rats ($p< 0.05$). As to the right hind paw, the mean total PD level was more than twice greater in adjuvant-induced arthritis rats than in healthy rats, though the significant difference was not found ($p> 0.05$), which was probably due to the large variation of the data in adjuvant-induced arthritis rats. These results suggested that CS-GP could give better localization of the drug in the arthritic tissue than in the normal one, which supported that CS-GP should exhibit a good passive targeting ability based on EPR effect.

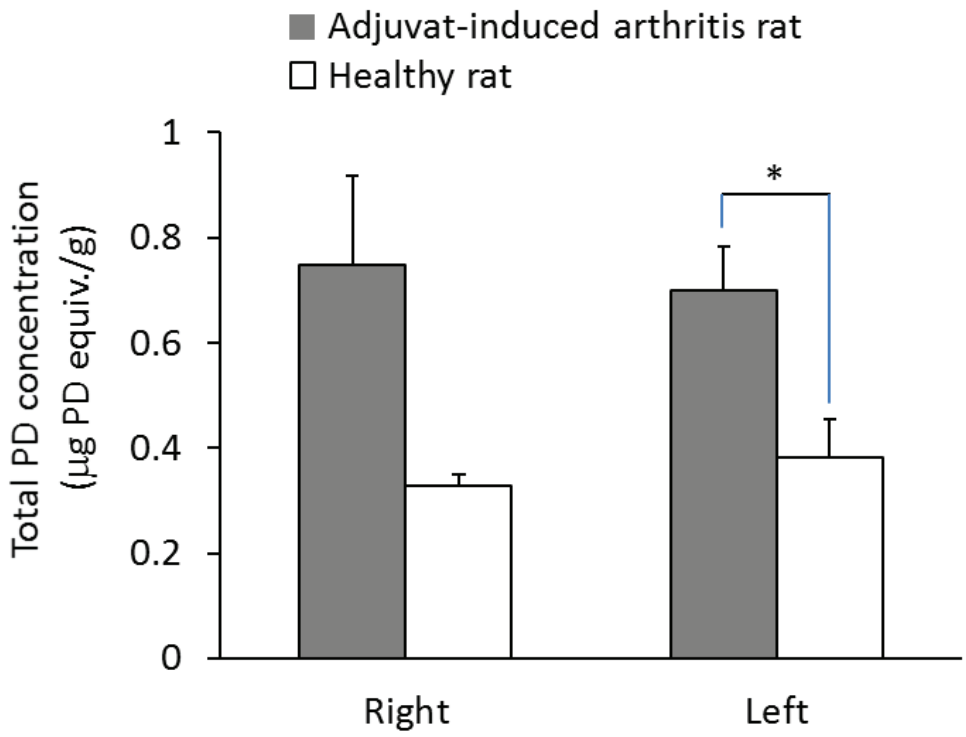

Fig. 26. Distributed Drug Concentration in the Right (A) and Left (B) Hind Paws at 24 h after I.V. Administration of PD Alone and CS-GP at the Dose of 2.5 mg PD equiv./kg to Adjuvant-Induced Arthritis Rats (■) and Healthy Rats (□)

Each column represent the mean ± S.E. (n=4 for adjuvant-induced arthritis rats, 3 for healthy rats). * $p < 0.05$ (unpaired *t*-test).

3.5. Physicochemical State of CS-GP in Systemic Circulation

The plasma sample collected 1 h after i.v. injection of CS-GP was diluted with PBS so that the resultant mixture could pass smoothly through the ultrafiltration membrane (Amicon Ultra). Namely, the plasma sample was diluted to 10-fold volume for the membrane with MW 100K cutoff, and to 20-fold volume for membrane with MW 30K cutoff. The diluted plasma and its filtrates obtained by ultrafiltration were analyzed for their concentration of total PD. The amount of CS-GP was calculated from the content of total PD in the MW range (<30K, 30K–100K, 100K<). The results were obtained as shown in Fig. 27B. The MW distribution of original CS-GP was investigated in a similar manner after CS-GP was dissolved in PBS, and is shown in Fig. 27A. CS-GP in PBS was mainly distributed in the MW range of 30K – 100K, which was consistent with the average MW from the company product information.

The apparent MW of CS-GP in the plasma sample was higher than the MW of original CS-GP before administration. As to this reason, two possibilities could be proposed. One was that the part of CS with low MW was eliminated by the urinary excretion, resulting in the part of CS with high MW remained in the systemic circulation. The other possibility was that the apparent molecular weight of CS might increase in the systemic circulation. In the literature, CS is known to interact with a cationic protein, lysozyme.[79] Various plasma proteins, particularly cationic proteins, might be related to the interaction with CS. The interaction of CS with different plasma proteins including serum albumin will be clarified in the following report. The pharmacokinetics and MW change of CS have already been reported, and it is known to be present systemically or excreted in urine, partly with fairly high MW and partly with small fragments including sulfate ion.[54, 80, 81] Namely, CS was considered to undergo its own metabolism and elimination in the body, and CS-GP was expected to be subjected to the fate of CS. CS-GP obtained from the plasma at 1 h after the

intravenous injection was considered to have high MW to a considerable extent. Its MW distribution profile might imply the interaction of remaining high MW CS-GP with the plasma components. At that time, the increase in apparent molecular weight might be associated with the good localization and prolonged retention of CS-GP in inflammatory joints; generally, macromolecules with high MW display higher systemic retention and better localization at an inflammation site.[55, 82] The electrical properties of CS might function beneficially for passive targeting to arthritic sites. The interaction of CS and plasma components will be made clear in the future.

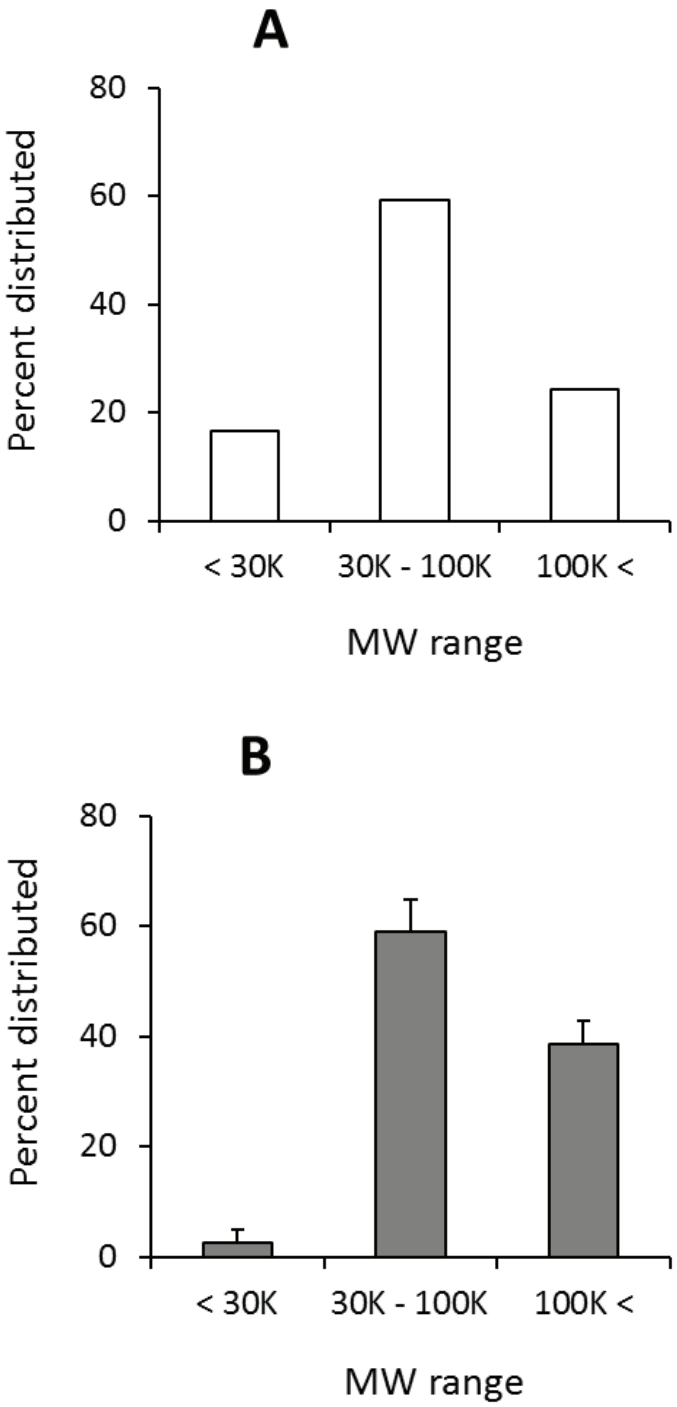

Fig. 27. Distribution of Apparent Molecular Weight of CS-GP in PBS (A) and Plasma Obtained 1 h after I.V. Administration (B)

Each column represents the mean ± S.E. (n=3) for B; for A, the original value is shown (n=1).

4. Conclusion

The pharmacokinetic features and drug distribution at the arthritic site of chondroitin sulfate-glycyl-prednisolone conjugate (CS-GP) were investigated to elucidate the mechanism of high and promoted efficacy. The release of PD from CS-GP at weakly acidic pH in rheumatoid arthritis was analyzed, in which PD was released slowly for several days or more. Pharmacokinetics and drug distribution studies were performed using rats with adjuvant-induced arthritis. From the plasma concentration-time profiles after i.v. administration, CS-GP exhibited very high retention in the systemic circulation, which suggested the passive targeting ability of CS-GP based on the EPR effect. More PD was distributed in inflammatory joints with PD alone than CS-GP at 1 h after i.v. administration, but the drug concentration was eliminated faster with PD alone. On the other hand, CS-GP exhibited prolonged joint distribution from 1 h until 24 h, and the regenerated free PD was also maintained at a fairly constant level. At 24 h after i.v. administration, the total and conjugated PD levels were much higher with CS-GP than PD alone. The good localization and prolonged retention of the drug were considered to be due to the EPR effect at the inflammatory site. The apparent molecular weight of CS-GP in the systemic circulation was observed to be high, which might be associated with the good delivery of CS-GP to the diseased site. The pharmacokinetics, biodistribution and physicochemical properties of CS-GP were considered to lead to its excellent anti-arthritic function.

SUMMARY

There are many effective drugs in clinical usage, but some are restricted for dosage or dosing period due to several adverse effects. Drug targeting therapy is a technique in which a drug is delivered to target tissues and remaining for appropriate time periods to maximize the efficacy and minimize the side effect. Although a lot of studies have been examined, some issues remain to apply clinically. For example, undesirable body distribution, residue to tissues and immunological reaction to drug carriers are reported. Therefore, more safe and efficient systems are required. The aim of this thesis is to develop a conjugate between prednisolone and chondroitin sulfate and examined the property and feasibility as a prodrug for the treatment of rheumatoid arthritis.

In chapter 1, a conjugate between prednisolone (PD) and chondroitin sulfate (CS) with glycine as a linker was prepared in order to obtain an effective macromolecular prodrug against inflammatory disease, especially rheumatoid arthritis. First, PD was converted to the *N*-trityl-glycine ester (Tr-GP), and the glycine ester of PD (GP) was obtained by detritylation of Tr-GP. Then, GP and CS were condensed with water-soluble carbodiimide to yield chondroitin sulfate-glycyl-prednisolone conjugate (CS-GP). The obtained conjugate had a PD content of 2.24 % (w/w). Conversion characteristics were investigated for GP and CS-GP to evaluate their potential as a prodrug. In the stability test of GP, PD was released well in the buffer at pH 6–7.4, but degraded rapidly at pH 8 without sufficient release of PD. As to CS-GP, PD was released more slowly than in GP, and the release rate rose with the increase in the medium pH. PD was released gradually from CS-GP over 24 h at a physiological pH. The conversion profiles of both GP and CS-GP almost followed pseudo-first order kinetics. The calculated conversion rate constants supported the gradual and effective release from CS-GP. The release rate of PD from GP and CS-GP was accelerated by the

addition of rat plasma, but the promotion of release from CS-GP was small, suggesting that PD should be released gradually from CS-GP in the systemic circulation. It was demonstrated from the preliminary pharmacological study using rats with adjuvant-induced arthritis that CS-GP had high anti-inflammatory potential against arthritis.

In chapter 2, CS-GP conjugates with different PD contents of 1.4, 2.0 and 4.3 % (w/w) were obtained, and their conversion characteristics were investigated at 37 ^{o}C under pH 6 and 7.4. Release profiles were similar among the conjugates, being slow at pH 6 (24 h: 9–14 %), and fairly fast at pH 7.4 (7 h: 46–58 %). In order to evaluate *in vivo* efficacy in detail, CS-GP, PD, CS and the PD/CS mixture were investigated for anti-inflammatory effects by i.v. administration to rats with adjuvant-induced arthritis. At 2.5 mg PD equiv./kg × 2 d, CS-GP significantly suppressed swelling of both hind paws, while other preparations were less effective. CS-GP (2.5 mg PD equiv./kg × 2 d) tended to be more effective than PD (5 mg/kg × 2 d). Change in body weight indicated that CS-GP was not toxic. After i.v. injection in normal rats, CS-GP showed much higher plasma levels than PD alone, and organ accumulation of CS-GP was not observed. CS-GP was suggested to be possibly useful for the treatment of rheumatoid arthritis.

In chapter 3, CS-GP was examined for its pharmacokinetic features and tropism for inflammatory joints using rats with adjuvant-induced arthritis in order to identify the mechanism of the potential enhancement. After intravenous injection (2.5 mg PD equiv./kg), CS-GP gave an AUC of the total (free + conjugated) drug much higher than PD alone. After intravenous administration, the drug distribution to the inflammatory joints was investigated between CG-GP and PD alone. For PD alone, the PD concentration was 1.2–1.7 μg/g at 1 h and fell to 0.12–0.14 μg/g at 24 h. On the other hand, CS-GP maintained the total concentration in the range of 0.55–0.97 μg/g for 1–24 h, and maintained

the free PD concentration at 0.06–0.14 μg/g for 1–24 h. In addition, after intravenous administration at the same dose, the drug concentrations at each joint tissue were compared between arthritic and healthy rats. The total PD concentration in the joint tissue was found to be higher in adjuvant-induced arthritis rats than healthy rats. The total PD concentration of the left hind paw was significantly greater in adjuvant-induced arthritis rats than in healthy rats ($p< 0.05$). As to the right hind paw, the mean total PD level was more than twice greater in adjuvant-induced arthritis rats than in healthy rats. Theses suggested that CS-GP could give better localization of the drug in the arthritic tissue than in the normal one, which supported that CS-GP should exhibit a good passive targeting ability based on EPR effect. Furthermore, CS-GP showed high MW in plasma, which might be associated with high plasma retention and good tropism to the inflammatory sites.

The conjugate between prednisolone and chondroitin sulfate via glycine linker (CS-GP), synthesized newly, was evaluated *in vitro* and *in vivo*. The resultant physicochemical characteristics, biological functions and pharmacokinetic features demonstrated the possible usefulness of CS-GP as an anti-arthritic delivery system.

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