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Anticoagulant Efficacy of Solid Oral Formulations Containing a New Heparin Derivative

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Abstract: The need for an efficacious and safe oral anticoagulant that does not require monitoring has been largely unmet. Many efforts have centered on preparing orally available heparin to improve patient compliance. In this study, novel orally active heparin derivatives (LHD), i.e. low molecular weight heparin (LMWH) conjugated with deoxycholic acid (DOCA), were evaluated *in vitro* and *in vivo* for their enhancement effect of oral heparin absorption. After oral administration of 10 mg/kg of water-soluble LHD, Ws-LHD1.5 showed optimum oral efficacy and its bioavailability was about 24% in rats. The oral absorption of LHD1.5 was also enhanced by several solubilizers, among which Poloxamer 407 provided the best results. When 5 mg/kg of LHD1.5 with Poloxamer 407 was orally administered to monkeys, the maximum anti-FXa activity in plasma was 0.26 ± 0.04 IU/mL and its bioavailability was 17.4%. In a rat thrombosis model, 5 mg/kg of orally administered LHD1.5 formulated with Poloxamer reduced thrombus formation by 63.9 ± 16.6%, which was higher than the efficacy of clinically used enoxaparin (49.4 ± 17.8% at 100 IU/kg, sc). Considering the oral absorption efficacy and therapeutic effect, the conjugation ratio was optimized as about 1.5 molecules of DOCA per mole of heparin. Therefore, LHD1.5 with Poloxamer 407 can be further formulated as a solid oral anticoagulant drug.

Keywords: Low molecular weight heparin; deoxycholic acid; oral delivery; anticoagulants; deep vein thrombosis

Introduction

Low molecular weight heparin (LMWH) is one of the most potent anticoagulants and is mainly used to treat and prevent deep vein thrombosis (DVT), which may lead to pulmonary embolism (PE) following arthroplasty and abdominal surgery.[1−3] However, its use is constrained by its parenteral method of administration. Hence, oral warfarin is usually prescribed instead for discharged patients; unfortunately, warfarin has numerous drug-to-drug interactions and common food effects that often result in unpredictable dosage responses, thus requiring continuous monitoring of coagulation. Considering the drawbacks of other oral anticoagulants and the unmet clinical needs for novel parenteral anticoagu-

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(1) Gallus, A. S. Anticoagulants in the prevention of venous thromboembolism. *Baillieres Clin. Haematol.* **1990**, *3*, 651–684.

(2) Weitz, J. I. Low-molecular-weight heparins. *Drug Ther.* **1997**, *337*, 688–698.

(3) Jack, H.; Sonia, S. A.; Jonathan, L. H.; Valentin, F. Guide to anticoagulant therapy: heparin. *Circulation* **2001**, *103*, 2994–3018.

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lants, research has focused on oral delivery of heparin, especially of LMWH for its improved pharmacokinetic and pharmacodynamic properties.[4−6]

Heparin is a hydrophilic macromolecular drug and has a negative charge in itself. This physicochemical property of heparin limits its membrane permeability after oral administration.[7] A variety of formulation strategies has been investigated to overcome the poor oral bioavailability of heparin, including microemulsion, polymeric nanoparticles, liposomes, polyion complex micelles and dendrons.[8−12] There have also been numerous attempts to enhance cell membrane permeability by using absorption enhancers or by increasing the drug lipophilicity with labrasol, sulfonated surfactants, EDTA acid, chitosan derivatives, thiolated polycarbophil, sodium caprate, sodium *N*-[10-(2-hydroxybezoyl)-amino]decanoate (SNAD), and sodium *N*-[8-(2-hydroxybezoyl)amino]caprylate (SNAC).[13−20] In our previous studies, we synthesized a chemical conjugate of LMWH and deoxycholic acid (DOCA), a bile component; this conjugate was successfully absorbed following oral administration to rats and mice.[21,22] The chemical conjugation of LMWH with DOCA was proven to permeate through the intestinal membrane via interaction with bile transporters on the membrane and lipophilicity was increased by the conjugated DOCA as a result.[23] In addition, the LMWH derivative showed more enhanced oral absorption when DMSO was introduced as a solubilizer to prevent self-assembled nanoparticle formation under aqueous condition.[24]

Up to now, we conjugated one or two molecules of DOCA to one molecule of LMWH; but since there are 7 to 8 carboxylic acid groups in one LMWH molecule for DOCA conjugation, more conjugation may increase more interaction with intestinal membrane and absorption. However, more DOCA conjugation can also cause a decrease in the anticoagulant activity of LMWH, so it is necessary that the optimum DOCA conjugation ratio for maximizing oral absorption and minimizing the reduction of anticoagulant activity should be defined. In addition, DMSO showed improved oral absorption by preventing the self-aggregation of hydrophobic DOCA conjugates, but this model solubilizer should be replaced with a pharmaceutically accepted solubilizer for obtaining a solid oral dosage form since DMSO

(4) Lever, R.; Page, C. P. Novel drug development opportunities for heparin. *Nat. Rev.* **2002**, *1*, 140–148.
(5) Goldberg, M.; Gomez-Orellana, I. Challenges for the oral delivery of macromolecules. *Nat. Rev.* **2003**, *2*, 289–295.
(6) Motlekar, N. A.; Youan, B. C. The quest for non-invasive delivery of bioactive macromolecules: a focus on heparins. *J. Controlled Release* **2006**, *113*, 91–101.
(7) Norris, D. A.; Puri, N.; Sinko, P. J. The effect of physical barriers and properties on the oral absorption of particulates. *Adv. Drug Delivery Rev.* **1998**, *34*, 135–154.
(8) Kim, S. K.; Lee, E. H.; Vaishali, B.; Lee, S.; Lee, Y. K.; Kim, C. Y.; Moon, H. T.; Byun, Y. Tricaprylin microemulsion for oral delivery of low molecular weight heparin conjugates. *J. Controlled Release* **2005**, *105*, 32–42.
(9) Jiao, Y.; Ubrich, N.; Marchand-Arvier, M.; Vigneron, C.; Hoffman, M.; Lecompte, T.; Maincent, P. In vitro and in vivo evaluation of oral heparin-loaded polymeric nanoparticles in rabbits. *Circulation* **2002**, *105*, 230–235.
(10) Hoffart, V.; Lamprecht, A.; Maincent, P.; Lecompte, T.; Vigneron, C.; Ubrich, N. Oral bioavailability of a low molecular weight heparin using a polymeric delivery system. *J. Controlled Release* **2006**, *113*, 38–42.
(11) Ueno, M.; Nakasaki, T.; Horikoshi, I.; Sakuragawa, N. Oral administration of liposomally-entrapped heparin to beagle dogs. *Chem. Pharm. Bull. (Tokyo)* **1982**, *30*, 2245–2247.
(12) Hayes, P. Y.; Ross, B. P.; Thomas, B. G.; Toth, I. Polycationic lipophilic-core dendrons as penetration enhancers for the oral administration of low molecular weight heparin. *J. Controlled Release* **2006**, *14*, 143–152.
(13) Ito, Y.; Kusawake, T.; Rama Prasad, Y. V.; Sugioka, N.; Shibata, N.; Takada, K. Preparation and evaluation of oral solid heparin using emulsifier and adsorbent for in vitro and in vivo studies. *Int. J. Pharm.* **2006**, *317*, 114–119.
(14) Windsor, E.; Cronheim, G. E. Gastrointestinal absorption of heparin and synthetic heparinoids. *Naturwissenschaften* **1961**, *190*, 263–264.
(15) Thanou, M.; Nihot, M. T.; Jansen, M.; Verhoef, J. C.; Junginger, H. E. Mono-N-carboxymethyl chitosan (MCC), a polyampholytic chitosan derivative, enhances the intestinal absorption of low molecular weight heparin across intestinal epithelia in vitro and in vivo. *J. Pharm. Sci.* **2001**, *90*, 38–46.
(16) Kast, C. E.; Guggi, D.; Langoth, N.; Bernkop-Schnürch, A. Development and in vivo evaluation of an oral delivery system for low molecular weight heparin based on thiolated polycarbophil. *Pharm. Res.* **2003**, *20*, 931–936.
(17) Pineo, G.; Hull, R.; Marder, V. Oral delivery of heparin: SNAC and related formulations. *Best Pract. Res., Clin. Haematol.* **2004**, *17*, 153–160.
(18) Berkowitz, S. D.; Marder, V. J.; Kosutic, G.; Baughman, R. A. Oral heparin administration with a novel drug delivery agent (SNAC) in healthy volunteers and patients undergoing elective hip arthroplasty. *J. Thromb. Haemostasis* **2003**, *1*, 1914–1919.
(19) Gonze, M. D.; Manord, J. D.; Leone-Bay, A.; Baughman, R. A.; Garrard, C. L.; Sternbergh, W. C., III; Money, S. R. Orally administered heparin for preventing deep venous thrombosis. *Am. J. Surg.* **1998**, *176*, 176–178.
(20) Rivera, T. M.; Leone-Bay, A.; Paton, D. R.; Leipold, H. R.; Baughman, R. A. Oral delivery of heparin in combination with sodium N-[8-(2-hydroxybezoyl)amino]caprylate: pharmacological considerations. *Pharm. Res.* **1997**, *14*, 1830–1834.
(21) Lee, Y. K.; Nam, J. H.; Shin, H. C.; Byun, Y. Conjugation of low-molecular-weight heparin and deoxycholic acid for the development of a new oral anticoagulant agent. *Circulation* **2001**, *104*, 3116–3120.
(22) Lee, Y. K.; Kim, S. K.; Lee, D. Y.; Lee, S. K.; Kim, C. Y.; Shin, H. C.; Moon, H. T.; Byun, Y. Efficacy of orally active chemical conjugate of low molecular weight heparin and deoxycholic acid in rats, mice and monkeys. *J. Controlled Release* **2006**, *111*, 290–298.
(23) Kim, S. K.; Kim, K. M.; Lee, S. K.; Park, K. S.; Park, J. H.; Kwon, I. C.; Choi, K.; Kim, C. Y.; Byun, Y. Evaluation of absorption of heparin-DOCA conjugates on the intestinal wall using a surface plasmon resonance. *J. Pharm. Biomed. Anal.* **2005**, *39*, 861–870.
(24) Kim, S. K.; Vaishali, B.; Lee, E. H.; Lee, S. K.; Lee, Y. K.; Kumar, T. S.; Moon, H. T.; Byun, Y. Oral delivery of chemical conjugates of heparin and deoxycholic acid in aqueous formulation. *Thromb. Res.* **2005**, *117*, 419–427.

is not allowed to be used for oral formulation and it is hard to control its remaining amount after the drying process.

The present study was aimed at evaluating novel orally active LMWH derivatives (LHD), i.e., LMWH conjugated with DOCA, with various conjugation ratios to determine the optimum DOCA conjugation ratio for increased oral absorption with the minimum reduction of anticoagulant activity. To enhance oral absorption, different solubilizer systems were also evaluated. Finally, the optimized LHD with solubilizer was subsequently prepared in a solid oral dosage form, and evaluation was carried out for its oral absorption in nonhuman primates as well as its preventive effect on thrombus formation in a rat model.

Experimental Section

Materials. Low molecular weight heparin (LMWH; Fraxiparin, 4500 Da) was obtained from Nanjing King-Friend Biochemical Pharmaceutical Company Ltd. (Nanjing, China). Deoxycholic acid (DOCA), *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*-hydroxylsuccinimide (HOSu), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), ethylenediamine, formamide, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). *N*,*N*-Dimethylformide (DMF) was obtained from Merck (Darmstadt, Germany). Coatest anti-Factor Xa assay kits were purchased from Chromogenix (Milano, Italy).

For oral formulations, lauroyl macrogolglycerides (Labrafil) and stearoyl macrogolglycerides (Gelucire 50/13) were obtained from Gattefossé (Lyon, France). Polyoxyl 35 castor oil (Cremophore EL), macrogol cetostearyl ether (Cremophore A25), polyethylene polyoxypropylene block copolymer (Poloxamer 407), and polyvinylpyrrolidone K30 were obtained from BASF Aktiengesellschaft (67056 Ludwigshafen, Germany). Polyoxyethylene 40 stearate (Myrj 52) and D-alpha tocopheryl polyethylene glycol 1000 succinate (TPGS) were purchased from Sigma chemical Co. (St. Louis, MO). Microcrystalline cellulose was obtained from Mingtai Chemical Co., Ltd. (Taoyuan Hsien, Taiwan). Lactose and cross-linked sodium carboxymethylcellulose were purchased from DMV international (Veghel, The Netherlands). Magnesium stearate was obtained from Faci Asia Pacific Pte Ltd. (Jurong Island, Singapore).

Synthesis of LMWH−DOCA. The chemical conjugate of LMWH and DOCA was synthesized by conjugating the amine group of *N*-deoxycholylethylamine (DOCA-NH_2) with the carboxylic groups of heparin as described in the previous study. In brief, DOCA (10 g) was reacted with DCC (8.4 g) and HOSu (4.7 g) in 89.99 mL of DMF to activate the carboxyl groups of DOCA. The activated DOCA was precipitated in acetonitrile and then filtered. The activated DOCA (10 g) was mixed with ethylenediamine (136 mL) solution in DMF (94 mL) and reacted for 5 h at room temperature to form DOCA-NH_2. In order to conjugate DOCA-NH_2 to LMWH, from which calcium salts had been removed, LMWH (300 mg) was dissolved in 65 mL of formamide, and EDAC was added to activate the carboxylic acid of LMWH. DOCA-NH_2 was coupled to the activated carboxylic acid of LMWH. The reaction mixture was incubated at room temperature for 12 h. The reaction mole ratios of DOCA-NH_2 to LMWH were 1:1, 1:2, 1:6, 1:10, and 1:20; 120 mol % of EDAC was added to the reaction moles of DOCA-NH_2. The reacted solution was then precipitated in ethanol, centrifuged and vacuum-dried. The product was then dissolved in water and lyophilized, and the LMWH−DOCA conjugates (LHD1, LHD1.5, LHD2, LHD2.5, and LHD3) were obtained as white powders.

Determination of the Amount of Conjugated DOCA. The chemical conjugation of LMWH−DOCA was confirmed through ^{1}H NMR of the amide bonds that formed between carboxylic groups of LMWH and the amine group of DOCA-NH_2. The anticoagulant activity was determined by Coatest anti-FXa chromogenic assay (Chromogenix, Milano, Italy), and the conjugation ratio of DOCA-NH_2 covalently attached to LMWH was determined spectrophotometrically after reaction with sulfuric acid. The alcoholic hydroxyls of DOCA were dehydrated easily in the presence of sulfuric acid (70−80%), and chromophores in the visible range were formed. The procedure described by Fini et al. for quantification of bile acids was applied with some modification.[25] LMWH−DOCA (500 μg) was weighed in a tube and dissolved in 25 μL of water and 25 μL of DMSO, and then 90 μL of water was added and mixed, followed by addition of 360 μL of sulfuric acid. The mixture was incubated at 80 °C for 3 min and then cooled in an ice bath. The absorbance was measured at 460 nm against a blank containing the same components as the sample but without the heparin derivative. The conjugation ratio of DOCA-NH_2 to LMWH was calculated from the standard curve of DOCA-NH_2.

The particle sizes of LMWH−DOCA and water-soluble LMWH−DOCA in deionized water were measured using an ELS-8000 (electrophoretic light scattering spectrophotometer, Otsuka Electronics Co., Ltd., Japan) as described in the previous study.[24]

***In Vivo* Experiments in Rats.** In order to prepare water-soluble LMWH−DOCA (Ws-LHD), LMWH−DOCA was dissolved in 10% aqueous DMSO solution and freeze-dried at −80 °C to obtain a white powder. The amount of DMSO bound to LHD was determined by calculating the weight change of the sample before and after being freeze-dried. On the other hand, oral formulations of LMWH−DOCA were prepared using various solubilizers such as Labrafil, Cremophore EL, Myrj 52, TPGS, Gelucire 50/13, Cremophore A25 and Poloxamer 407.

Male Sprague−Dawley rats (250−270 g) were fasted for 12 h before dosing. The rats were anesthetized with light diethyl ether, and test samples were administered either intravenously or orally. Each solution of LMWH (10 mg/kg), Ws-LHD (10 mg/kg) and LHD1.5 (10 mg/kg) with or

(25) Fini, A.; Fazio, G.; Roda, A.; Bellini, A. M.; Mencini, E.; Guarneri, M. Basic cholane derivatives. XI: comparison between acid and basic derivatives. *J. Pharm. Sci.* **1992**, *81*, 726–730.

without solubilizers (1 mg/kg) in water was administered to the animal by an oral gavage tube that was carefully passed down the esophagus into the stomach. To evaluate the absolute oral bioavailability of each material, LMWH (1 mg/kg), Ws-LHD (1 mg/kg) or LHD1.5 (1 mg/kg) solution was also prepared in saline and injected via tail vein. The total volumes of oral administration and intravenous injection were 400 and 150 μL, respectively, and the dose for each test material was weighed based on the actual weight of each test material regardless of its heparin content. The blood samples were then immediately centrifuged at 2500*g* at 4 °C for 15 min. The concentration of LMWH−DOCA was determined by FXa chromogenic assay.

After determining the area under the curve (AUC) of the concentration−time profile by the linear trapezoidal method, the absolute and relative bioavailabilities were calculated by following equations:

$$\text{absolute bioavailability (\%)} = \frac{AUC_{0-8h,oral,testmaterial}/dose_{oral,testmaterial}}{AUC_{0-8h,iv,testmaterial}/dose_{iv,testmaterial}} \times 100$$

where $AUC_{0-8h,oral,testmaterial}$ = area under the concentration−time curve from zero to 8 h after oral administration of each test material, $dose_{oral,testmaterial}$ = dose of oral administration of each test material, $AUC_{0-8h,iv,eachsample}$ = area under the concentration−time curve from zero to 8 h after intravenous administration of each test material, and $dose_{iv,testmaterial}$ = dose of intravenous administration of each test material.

$$\text{relative bioavailability (\%)} = \frac{AUC_{0-8h,oral,testmaterial}/dose_{oral,testmaterial}}{AUC_{0-8h,iv,LMWH}/dose_{iv,LMWH}} \times 100$$

where $AUC_{0-8h,oral,testmaterial}$ = area under the concentration−time curve from zero to 8 h after oral administration of each test material, $dose_{oral,testmaterial}$ = dose of oral administration of each test material, $AUC_{0-8h,iv,LMWH}$ = area under the concentration−time curve from zero to 8 h after intravenous administration of LMWH, and $dose_{iv,LMWH}$ = dose of intravenous administration of LMWH.

***In Vivo* Experiments in Monkeys.** In order to prepare tablets for monkey *in vivo* experiment, LHD1.5 (25 g), Poloxamer 407 (5.4 g), microcrystalline cellulose (6 g), lactose (6 g), cross-linked sodium carboxymethycellulose (5 g), and polyvinylpyrrolidone K30 (1.5 g) were mixed together. The mixture was then lubricated by adding 0.5 g of magnesium stearate and compressed to obtain a tablet.

Male cynomolgus monkeys (4.5−5.0 kg, Korea Research Institute of Chemical Technology (KRICT), Daejeon, Korea) were fasted for 12 h before drug administration. LHD1.5 in dosages of 5 (425 IU/kg) and 10 mg/kg (850 IU/kg) with 1.08 mg/kg and 2.16 mg/kg of Poloxamer 407, respectively, were prepared in tablets. Also, LHD1.5 (1 mg/kg) in PBS solution (pH 7.4) was administered intravenously in order to calculate its oral bioavailability. The weight of LHD1.5 was the weight of LHD derivative. All of the animal experiments were carried out in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals and approved by KRICT. Blood (450 uL) was collected from the vein and directly mixed with 50 μL of sodium citrate (3.8% solution), followed by centrifugation at 2500*g* at 4 °C for 15 min. The concentration of LHD1.5 was determined by FXa chromogenic assay.

Venous Thrombosis Model. An animal model of DVT was prepared as described in the literature. Sprague−Dawley rats (SD male rat, 250−280 g) were fasted for 12 h. SD rats received LMWH (enoxaparin; 100 IU/kg) by subcutaneous injection or 3 (255 IU/kg), 5 (425 IU/kg) and 10 mg/kg (850 IU/kg) of LHD1.5 with 0.65, 1.08, and 2.16 mg/kg of Poloxamer 407 by oral administration, respectively. After administration of enoxaparin and LHD1.5 via subcutaneous and oral routes, respectively, each animal was anesthetized with ketamine (45 mg/kg) and xylazine (5 mg/kg) by intramuscular injection. When anesthetized, both sides of the rat vena cava were exposed and separated from the surrounding tissue. Each end (2 cm) of vena cava was loosely tied and the branched blood vessels were completely tied with 2−0 silk thread. At 60 min after enoxaparin or LHD1.5 with Poloxamer 407 administration, 1 mL/kg human pooled plasma warmed to 37 °C was injected into the tail vein. Fifteen seconds later, the vena cava was ligated with 2−0 silk thread *in situ* to produce stasis. At 120 min after finishing the surgical operation, the veins were segregated and opened in a Petri dish filled with 3.8% sodium citrate. Thrombus formation was evaluated by measuring the wet weight of the thrombus.

Statistical Analysis. The cumulative data from animal experiments were expressed as mean ± SEM, and Student's unpaired *t* test was used for comparison between the groups. A value of $P < 0.05$ was considered as statistically significant.

Results

Characterization of LMWH−DOCA. The LMWH−DOCA conjugates presented amide linkages between heparin and DOCA-NH_2 as confirmed by the new amide bond peak from 7 to 8 ppm in the NMR spectra. The DOCA conjugation ratios of LHD1, LHD1.5, LHD2, LHD2.5 and LHD3 were 0.9 ± 0.0, 1.4 ± 0.0, 2.2 ± 0.0, 2.7 ± 0.1 and 3.3 ± 0.2, respectively, and the weight percentage of the conjugated DOCA-NH_2 ranged from 8.3% to 22.8%. However, the anticoagulant activity of LMWH−DOCA, as evaluated by anti-FXa chromogenic assay, decreased as the DOCA-NH_2 conjugation ratio increased and decreased to 20 IU/mg for LHD3. For the synthesized LMWH derivatives, deoxycholic acids that were chemically conjugated to LMWH could reduce the binding affinity of LMWH to antithrombin III, thereby lowering their anticoagulant activities. In addition, since various LHDs have different amounts of LMWH because of the weight of conjugated DOCA-NH_2, LHDs can show decreased anticoagulant activities. After the DMSO binding, the anticoagulant activity also decreased as the conjugation ratio increased, but its anticoagulant activity decreased by 10−20% on the same material. This phenomenon may have been caused by the increased weight of

Table 1. Characterization of LMWH−DOCA Conjugates

	reaction mole ratio of EtDOCA to LMWH	conjugation ratio (no. of EtDOCA in a heparin molecule)[a]	wt % of EtDOCA in a conjugated material[a]	anticoagulant act. (anti-FXa act., IU/mg)[a]	wt % of bound DMSO on water-soluble LMWH−DOCA[a]	anticoagulant act. of water-soluble LMWH−DOCA (anti-FXa act., IU/mg)[a]
LMWH	0	0	0	100		
LHD1	1	0.9 ± 0.0	8.3 ± 0.1	97 ± 1.6	18.4 ± 1.4	78 ± 0.4
LHD1.5	2	1.4 ± 0.0	12.5 ± 0.3	72 ± 0.1	20.1 ± 2.4	58 ± 0.6
LHD2	6	2.2 ± 0.0	17.7 ± 0.0	43 ± 2.4	14.3 ± 2.3	34 ± 0.6
LHD2.5	10	2.7 ± 0.1	19.9 ± 0.2	27 ± 2.4	15.7 ± 1.6	20 ± 0.1
LHD3	20	3.3 ± 0.1	22.8 ± 1.4	20 ± 2.2	17.6 ± 1.3	3 ± 0.5

[a] Mean ± SEM ($n = 3$).

Table 2. Particle Size of LMWH−DOCA and Water-Soluble LMWH−DOCA

	particle size (nm)[b]		particle size (nm)[b]
LMWH	nd[a]		
LHD1	257 ± 6	Ws-LHD1	nd
LHD1.5	153 ± 2	Ws-LHD1.5	nd
LHD2	177 ± 8	Ws-LHD2	242 ± 13
LHD2.5	322 ± 3	Ws-LHD2.5	276 ± 7
LHD3	517 ± 27	Ws-LHD3	357 ± 30

[a] Not detected. [b] Mean ± SEM ($n = 3$).

conjugated materials due to the bound DMSO because the weight % of bound DMSO corresponded to the reduction in the anticoagulant activity (Table 1).

LMWH−DOCA formed self-assembled nanoparticles in water because the conjugated hydrophobic DOCA molecules were gathered inside against the aqueous environment. The particle size of the conjugated materials ranged from 150 to 500 nm, as shown in Table 2, but there was no direct relationship between particle size and the DOCA conjugation ratio. Ws-LHD1 and Ws-LHD1.5, which were bound with DMSO by secondary interactions, were completely dissolved in water, but particle formation still occurred in the case of Ws-LHD2 because of increased conjugation ratio of hydrophobic DOCA.

In Vivo **Experiments in Rats.** Figure 1 shows the pharmacokinetic parameters of water-soluble LMWH−DOCA conjugates after oral administration in rats. The maximum oral anticoagulant activities of Ws-LHD1, Ws-LHD1.5, Ws-LHD2, Ws-LHD2.5 and Ws-LHD3 occurred at about 1.5 h after administration and were 0.25 ± 0.05, 0.30 ± 0.05, 0.30 ± 0.06, 0.20 ± 0.01, and 0.19 ± 0.01 IU/mL, respectively. Their effective areas under the curve (AUCs) and the maximum effective concentrations (E_{max}) first increased and then decreased as the DOCA conjugation number increased. Ws-LHD1.5 showed high oral absorption and the maximum anticoagulant activity among the conjugates. The calculated oral bioavailabilities of Ws-LHD1, Ws-LHD1.5, Ws-LHD2, Ws-LHD2.5 and Ws-LHD3 were 12.4, 24.1, 18.0, 39.8, and 22.9%, respectively, and the relative oral bioavailabilities calculated based on the anticoagulant activity of LMWH were 11.8, 14.3, 13.3, 8.1, and 10.9%, respectively. Ws-LHD2.5 showed maximum oral bioavailability, but its activity-based oral bioavailability was about 8% because of its low anticoagulant activity.

The pharmacokinetic parameters of LHD1.5 with solubilizers after oral administration to rats are shown in Figure 2. The oral bioavailabilities of LHD1.5−Labrafil, LHD1.5−Cremophore EL, LHD1.5−Myrj 52, LHD1.5−TPGS, LHD1.5−Gelucire 50/13, LHD1.5−Cremopore A25, and LHD1.5−Poloxamer 407 were 9.1, 14.7, 17.3, 17.7, 12.3, 8.0, and 22.9%, respectively.

Figure 3 shows the plasma anti-FXa activity profiles of LMWH and LHD1.5 with or without Poloxamer 407. The maximum anticoagulant activities of LHD1.5 and LHD1.5−Poloxamer 407 were 0.20 ± 0.06 and 0.44 ± 0.11 IU/mL, respectively. The effective AUCs for LHD1.5 and LHD1.5−Poloxamer 407 were 0.59 ± 0.19 and 1.46 ± 0.35 IU·h/mL, respectively. By comparing the mean AUC value obtained after intravenous administration of LHD1.5 with the AUC obtained after oral administration, the oral bioavailabilities of LHD1.5 and LHD1.5−Poloxamer 407 were calculated as 9.3 and 22.9%, respectively. The oral absorption of LHD1.5 with Poloxamer 407 was enhanced by 2.46 times compared to LHD1.5. On the other hand, LMWH and LMWH with Poloxamer 407 were rarely absorbed via oral route.

In Vivo **Experiments in Monkeys.** To assess the absorption of LHD1.5 and its solid oral formulation in monkeys, we measured the concentration of LHD1.5 in the plasma at each sampling time after a single oral dose (Figure 4). When 5 and 10 mg/kg of LHD1.5 formulated with Poloxamer 407 were administered orally to monkeys, the concentration of LHD1.5 in the plasma remained above the minimum effective concentration (MEC) for 10 h and the maximum anti-FXa activities were 0.26 ± 0.04 and 0.35 ± 0.03 IU/mL, respectively. On the other hand, plasma anti-FXa activities of LMWH and LHD1.5 were around 0.09 ± 0.13 and 0.15 ± 0.02 IU/mL, respectively. The bioavailability of LHD1.5 was significantly enhanced by Poloxamer 407 with 17.4% oral bioavailability for the 5 mg/kg dose (Table 3).

Pharmacological Effect of Orally Administered LMWH−DOCA. To evaluate the pharmacological effect, an animal model for DVT was prepared and thrombus formation was evaluated by measuring the weight of the wet thrombus after subcutaneous injection of LMWH (enox-

(26) Kim, S. K.; Lee, D. Y.; Lee, E. H.; Lee, Y. K.; Kim, C. Y.; Moon, H. T.; Byun, Y. Absorption study of deoxycholic acid-heparin conjugate as a new form of oral anticoagulant. *J. Controlled Release* **2007**, *120*, 4–10.

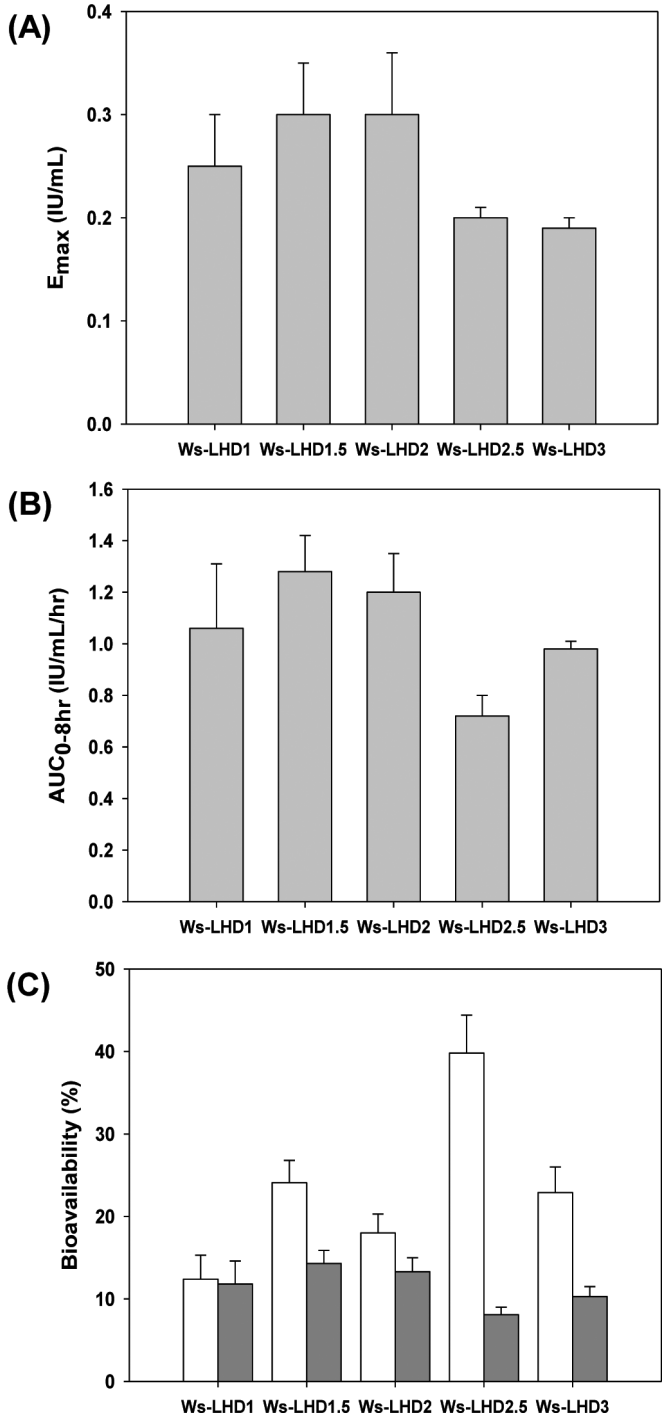

Figure 1. Pharmacokinetic values for water-soluble LMWH−DOCA conjugates in rats: (A) E_{max}, maximal anti-FXa activity. (B) AUC_{0-8h}, area under the concentration−time curve from zero to 8 h. (C) Bioavailability, absolute oral bioavailability calculated by area under the concentration−time curve from zero to 8 h after oral and intravenous administration of each LMWH−DOCA conjugate (open bars). Relative oral bioavailability calculated by area under the concentration−time curve from zero to 8 h after oral administration of each LMWH−DOCA conjugate and area under the concentration−time curve from zero to 8 h after intravenous administration of LMWH (gray bars). The data are plotted as mean ± SEM ($n = 4$).

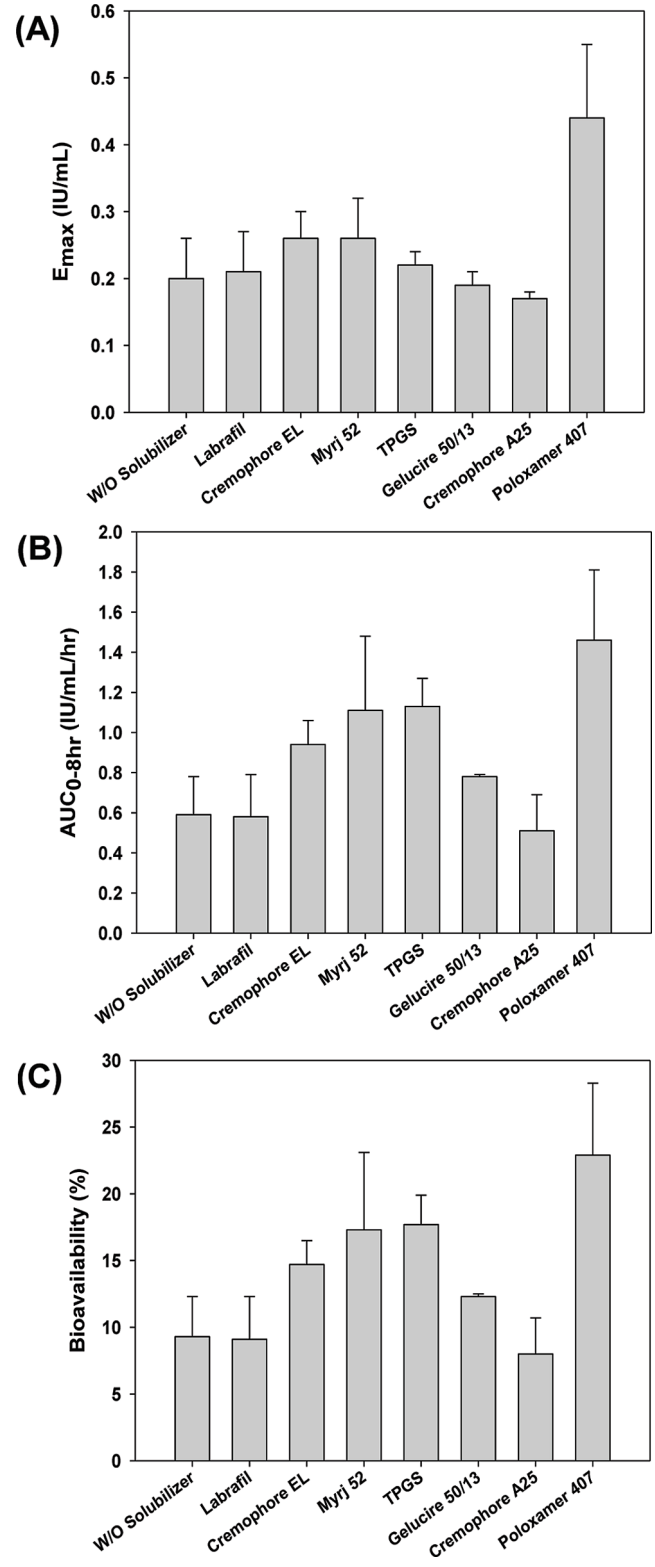

Figure 2. Pharmacokinetic values for LHD1.5 and LHD1.5 with solubilizers in rats: (A) E_{max}, maximal anti-FXa activity. (B) AUC_{0-8h}, area under the concentration−time curve from zero to 8 h. (C) Bioavailability, absolute oral bioavailability calculated by area under the concentration−time curve from zero to 8 h after oral of LHD1.5 with or without solubilizer and intravenous administration of LHD1.5. The data are plotted as mean ± SEM ($n = 4$).

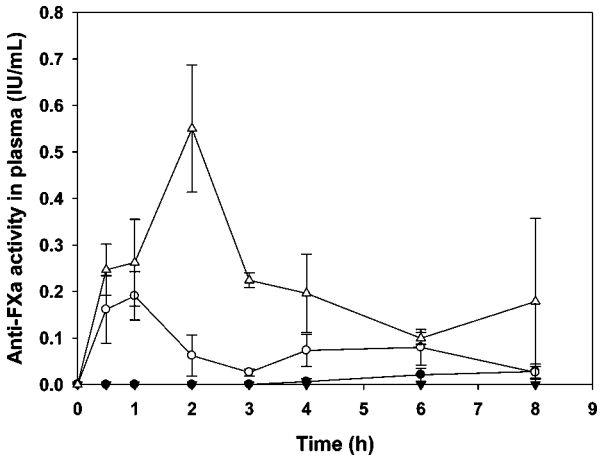

Figure 3. Anti-FXa activity of orally administered 10 mg/kg of LMWH and LHD1.5 with or without 1 mg/kg of Poloxamer 407 in rats; LMWH (●), LHD1.5 (○), LMWH−Poloxamer 407 (▼), LHD1.5−Poloxamer 407 (△). The data are plotted as mean ± SEM ($n = 4$).

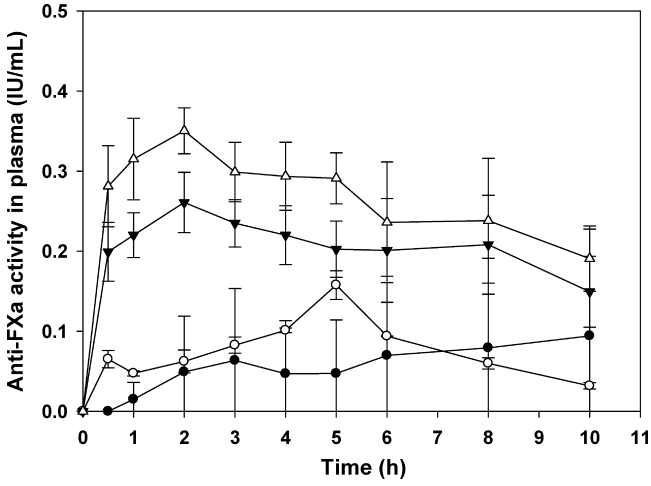

Figure 4. Anti-FXa activity of orally administered LMWH and LHD1.5 with or without Poloxamer 407 in monkeys; LMWH (100 mg/kg, (●)), LHD1.5 (10 mg/kg, (○)), LHD1.5−Poloxamer 407 (5 mg/kg, (▼)), LHD1.5−Poloxamer 407 (10 mg/kg, (△)). The data are plotted as mean ± SEM ($n = 6$).

aparin) or oral administration of LHD1.5 with Poloxamer 407. With the control phosphate buffer treatment, thrombi weighing 20.7 ± 6.4 mg were formed. Subcutaneous administration of enoxaparin (100 IU/kg) reduced thrombus formation by 49.4 ± 17.8%. In contrast, 3 mg/kg (255 IU/kg), 5 mg/kg (425 IU/kg), and 10 mg/kg (850 IU/kg) of LHD1.5 with Poloxamer 407, each administered orally, reduced thrombus formation by 49.5 ± 26.7%, 63.9 ± 16.6%, and 76.4 ± 17.3%, respectively (Figure 5).

Discussion

In this study, we synthesized chemical conjugates of LMWH with DOCA at various DOCA conjugation ratios to maximize oral absorption. The conjugation of hydrophobic DOCA molecule increases the hydrophobicity of heparin and enhances its intestinal membrane permeability. According to our previous absorption mechanism study, the conjugated DOCA interacts with bile acid transporters, which mainly exist on the ileum, thereby increasing its interaction and concentration gradient through the intestinal membrane.[23] LMWH−DOCA has been proven to permeate through the intestinal membrane via transcellular pathway owing to its amphiphilicity.[26] However, although the permeability of LMWH by the conjugated DOCA molecules was increased, their anticoagulant activities decreased as the number of conjugation ratio increased because the conjugated DOCA molecules protect the active sites of LMWH.[27]

On the other hand, LMWH−DOCA formed self-assembled particles in aqueous conditions because of the amphiphilic properties of LMWH and the presence of hydrophobic DOCA. The nanoparticle structure of LMWH−DOCA could not enhance its permeation in the intestine because the conjugated DOCA, located inside of the particles, cannot interact with bile acid transporters and the intestinal wall. In the previous study, we introduced DMSO as a solubilizer to prevent particle formation.[28] DMSO is widely used as a solubilizer to dissolve highly hydrophobic materials during the screening process of new drugs. This solvent can form hydrogen bonding via water and hydrophobic interaction with hydrophobic drug. In the case of DOCA conjugated materials, the bound DMSO caused hydrophobic interaction with DOCA and hydrogen bonding to water molecules through its oxygen atom, so particle formation induced by conjugated DOCA could be prevented.[29] In this study, we used DMSO as a model solubilizer to evaluate the effect of DOCA conjugation ratio on oral absorption and to find the optimum conjugation ratio for maximum oral bioavailability. The Ws-LHD series prepared by freeze-drying LMWH−DOCA with 10% DMSO solution produced the maximum oral bioavailability at the DOCA conjugation ratios of 1.5. Although greater DOCA conjugation can induce more interaction with bile acid transporters and enhance absorption, water-soluble LHD still formed particles in aqueous solution. Additionally, higher DOCA conjugation decreased the anticoagulant activity of LHD; therefore, the relative oral bioavailability, which was calculated based on anticoagulant activity of LMWH, reached maximum at LHD1.5. Based on the oral absorption and biological activity, LHD1.5, which has 1.5 molecules of DOCA to one LMWH molecule, was selected as the best heparin derivative for an oral anticoagulant drug.

Although the bound DMSO is a well-known absorption enhancer and good solubilizer for LMWH−DOCA, DMSO has not been approved by the FDA for oral dosage and controlling

(27) Linhardt, R. J. Heparin: structure and activity. *J. Med. Chem.* **2003**, *46*, 2551–2564.
(28) Kim, S. K.; Lee, D. Y.; Kim, C. Y.; Hyun, T. M.; Byun, Y. Prevention effect of orally active heparin derivative on deep vein thrombosis. *Thromb. Haemostasis* **2006**, *96*, 149–153.
(29) Kim, S. K.; Lee, D. Y.; Kim, C. Y.; Nam, J. H.; Moon, H. T.; Byun, Y. A newly developed oral heparin derivative for deep vein thrombosis: non-human primate study. *J. Controlled Release* **2007**, *123*, 155–163.

Table 3. Pharmacokinetic Values for LMWH, LHD1.5 and LHD1.5 with Poloxamer 407 in Monkeys

	dose (mg/kg)	E_{max}[a,b] (IU/mL)	T_{max} (h)	$AUC_{0-600min}$[b,c] (IU·min/mL)	F[d] (%)
		Iv Administration			
LMWH	1			1814.6 ± 313.9	
LHD1.5	1			1603.7 ± 410.2	
		Oral Administration			
LHD1.5− Poloxamer 407	5 1.08	0.26 ± 0.04	2	1394.3 ± 199.3	17.4
LHD1.5− Poloxamer 407	10 2.16	0.35 ± 0.03	2	1823.6 ± 239.6	11.4
LHD1.5	10	0.15 ± 0.02	5	458.6 ± 25.5	2.9
LMWH	100	0.09 ± 0.13	10	345.0 ± 212	0.2

[a] Maximal effect. [b] Mean ± SEM ($n = 6$). [c] Area under the concentration−time curve from zero to 600 min. [d] Absolute bioavailability.

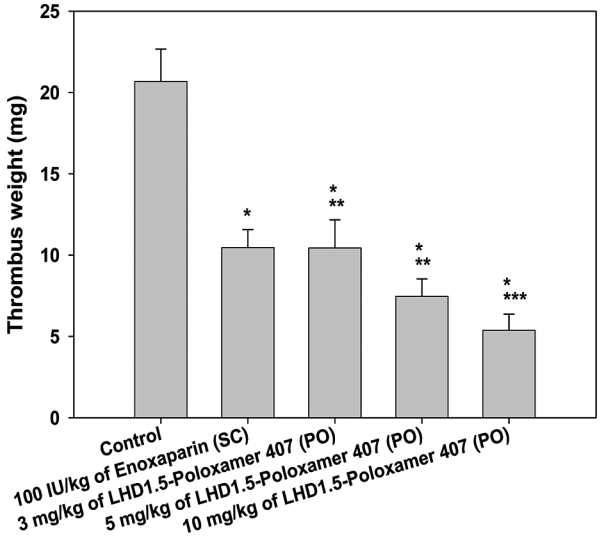

Figure 5. Inhibition effect of thrombus formation by LMWH (Enoxaparin) and LHD1.5−Poloxamer 407 in rat venous thrombus model. Enoxaparin (100 IU/kg, sc) or LHD1.5−Poloxamer 407 (3, 5, and 10 mg/kg, PO) was measured for their ability to inhibit venous thrombosis and compared with a control (saline). Clot formation was measured the weight of the resulting thrombus. Eight animals were used in each group, mean ± SEM. * $p = < 0.005$ vs control. ** $p = > 0.05$ vs Enoxaparin. *** $p = 0.007$ vs Enoxaparin.

the exact weight of bound DMSO is also difficult. Of different solubilizers tested in the oral absorption study of LHD1.5, Poloxamer 407 provided the maximum oral bioavailability and presented similar oral absorption capability to the water-soluble form. Poloxamer is a polyethylene polyoxypropylene block copolymer and is widely used as an oral solubilizer. Its hydrophilic−lipophilic balance (HLB) is about 23 and is higher than that of other solubilizers. A hydrophilic solubilizer is more suitable to solubilize LMWH−DOCA because hydrophobic parts of the solubilizer can prevent the conjugated hydrophobic DOCA molecules from gathering inside against the aqueous conditions and the hydrophilic portions of the solubilizer can more effectively expose them to aqueous conditions by enhancing the attraction of water molecules. In this way, a conjugated DOCA can readily interact with bile acid transporters. Compared to LHD1.5, the oral bioavailability of LHD1.5 formulated with Poloxamer 407 was increased by 2.46-fold. On the other hand, the oral absorption of LMWH with Poloxamer 407 was negligible. Therefore, Poloxamer itself did not enhance the oral absorption of LMWH.

After oral administration of 5 and 10 mg/kg of LHD1.5 with Poloxamer 407 to monkeys, the plasma concentration of LHD1.5 remained higher than 0.1 IU/mL, which is the minimum therapeutic concentration level for treating DVT and PE, for 10 h. However, there was no dose linearity in their absorption. In this study, the oral availability of LHD1.5 was significantly enhanced by introducing Poloxamer as a solubilizer and produced similar results to those of the water-soluble form. Also, the final dosage form could be obtained as a tablet or capsule.

The oral administration of 3, 5, and 10 mg/kg of LHD1.5 with Poloxamer 407 reduced thrombus formation more significantly than the subcutaneous administration of 100 IU/kg of enoxaparin in a rat DVT model. Based on the oral bioavailability in monkeys and the antithrombogenic effects, it is safe to conclude that 5 mg/kg of LHD1.5 with Poloxamer 407 prevents DVT and PE, and that 200−300 mg b.i.d. should be the optimum dose for clinical trials to establish the final formulation in a capsule or tablet form containing Poloxamer as a solubilizer. Considering oral dose and bioavailability, our results showed higher oral absorption efficacy than any other attempts at oral administration of heparin.

In conclusion, we propose a novel solid oral formulation of the chemical conjugate of LMWH and DOCA containing Poloxamer as a solubilizer for the treatment of DVT and PE. Considering the oral absorption efficacy and the therapeutic effect, the optimum DOCA conjugation ratio was about 1.5 molecules of DOCA per molecule of heparin. Furthermore, the oral absorption of LHD1.5 was enhanced by formulation with Poloxamer 407 and provided about 22.9% and 17.4% oral bioavailability in rats and monkeys. Therefore, we assert that LHD1.5 with Poloxamer formulation will open a new vista in controlling and preventing thrombotic events empirically, a venture more effective than conventional injectable heparin.

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