

## Evaluation of the Oral Absorption of Heparin Conjugated with Sodium Deoxycholate as a Facilitating Agent in GI Tract

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**Abstract:** The oral delivery of heparin is the preferred therapy in the treatment of patients with a high risk of deep vein thrombosis and pulmonary embolism. New conjugates of heparin and sodium deoxycholate were synthesized in order to enhance the heparin absorption in the GI tract. After oral administration of DOC-heparin, the concentration in anti-FXa assay was increased with increasing amount of coupled DOC. The maximum concentration of DOC-heparin VIII conjugate was  $3.3 \pm 0.5$  IU/mL at an oral dose of 10 mg/kg, which was 3-fold higher than the baseline level. Finally, DOC coupled to heparin greatly enhanced the absorption of heparin in the GI tract, and this enhancing effect was not induced by changing the tissue structure of the GI wall.

**Keywords:** sodium deoxycholate, oral delivery, heparin, conjugate.

### Introduction

The development of oral drug delivery that lacks the intrinsic limitation of macromolecular drugs such as heparin, insulin and growth factor is of considerable interest in pharmaceutical and medical field.<sup>1,2</sup> One of candidate drugs is heparin which widely used to treat or prevent deep vein thrombosis (DVT) and pulmonary embolism (PE) in high-risk patients.<sup>3,4</sup> The drug has proved efficacious in the treatment of patients with venous or arterial thrombosis, when administered at high dosage which prevent coagulation of the blood. However, Heparin treatment is usually limited to hospitalized patients since the drug is given only by injection. Both hydrophilic and highly negative charged structures of heparin are major barriers in oral delivery.<sup>5,6</sup>

Many research groups have tried to develop new formulations or enhancers for oral heparin delivery. There have been several different dosage forms, such as liposomes, hydrophobic organic bases, spermine and lysine salts, or monoolein complex, in order to facilitate heparin absorption into

the gastrointestinal (GI) mucosa.<sup>6-8</sup> Formulations using enteric coating materials, intrapulmonary aerosol of sodium heparin, or heparin complex were also developed for oral delivery of heparin.<sup>9</sup> Other researchers attempted to evaluate the effects of EDTA, acidic buffer, or sulfated surfactants on the heparin absorption in the GI tract.<sup>10</sup> Recently, *n*-[8-(2-hydroxybenzoyl)amino] caprylate (SNAC) was developed as a potent promoter of heparin absorption from the GI tract.<sup>11,12</sup> Recently, our studies have reported that a conjugate of LMWH with DOCA (LMWH-DOCA) enhanced the absorption of LMWH in the GI tract. LMWH-DOCA significantly increased the bioavailability in rats up to 7.8%, while LMWH itself showed poor bioavailability (nearly 0%). This study found that the conjugated DOCA increased the permeability of LMWH-DOC in the intestine via LMWH hydrophobic properties, and enhanced the mucoadhesion of LMWH-DOCA to the intestinal membrane.<sup>13-15,17</sup> In this study, we developed new DOC-heparin conjugate by using the carboxylic group of heparin and hydroxyl group of DOC instead of carboxyl group of DOC which used classically to bind with LMWH in our group. For coupling of DOC and heparin, we designed DOC-heparin conjugates by modifica-

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tion of C3-hydroxyl group of DOC with 4-nitrophenyl chloroformate (4-NPC) and ethylenediamine (EDA)(Figure 1). Through the conjugation method, many DOC molecules were allowed to bind freely with one heparin structure. In addition, the conjugated heparin retained its ability to be absorbed in the GI tract. Finally, we evaluated absorption behaviors of DOC-heparin in the intestine.

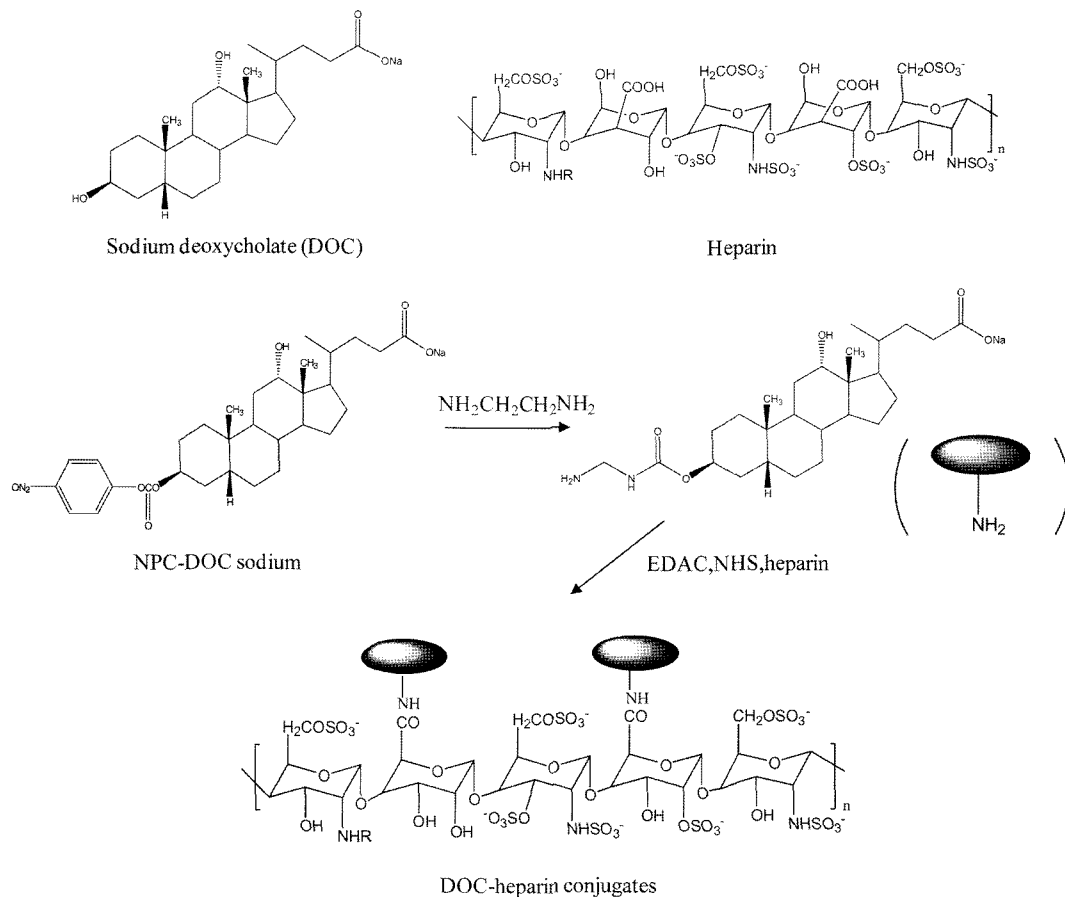
## Experimental

**Materials.** Low molecular weight heparin (Heparin; Fraxiparin<sup>®</sup>, 5,000 Dalton) was obtained from GlaxoSmith-Kline (Brentford, Middlesex, UK). Sodium deoxycholate (DOC), 4-nitrophenyl chloroformate, triethylamine, dicyclohexylcarbodiimide (DCC), hydroxysuccinimide (HOSu), 4-methylmorpholine, ethylene diamine, dimethyl sulfoxide (DMSO) and ethyl acetate were purchased from Sigma Chemical Co. (St. Louis, MO). Formamide was obtained from Merck (Darmstadt, Germany). Coatest Factor Xa assay kits were from Chromogenix (Milano, Italy). All reagents were of analytical grade and were used without further purification.

**Preparation of DOC-Heparin Conjugates.** The hydroxyl group ( $3\alpha$ ) of DOC (1.2 mmol) in 4.6 mL DMF solution

was derivatized by introducing a 4-nitrophenyl chloroformate (NPC, 6.0 mmol) and triethylamine (7.2 mmol) at 0 °C for 6 h. The resulting DOC-NPC intermediate solution was partitioned between ethyl acetate (25 mL) and 1 N HCl solution (25 mL). The aqueous layer was extracted with ethyl acetate (3×15 mL) and freeze-dried under vacuum for 2 days. To prepare aminated DOC, DOC-NPC intermediate (0.864 mmol) in DMF (5 mL) was reacted with 4-methylmorpholine (1.7 mmol) at 50 °C for 1 h and then added with ethylenediamine (0.086 mol) in 4.7 mL DMF solution for 16 h, forming deoxycholyethylamine. After reaction, the solution was concentrated and precipitated by adding acetonitrile solution (3×15 mL) to remove unreacted ethylenediamine, NPC and 4-methylmorpholine completely. After the product was dried for 2 days, the aminated DOC was obtained as a white powder.

For preparation of DOC-heparin conjugate, heparin (0.01 mmol) was dissolved in water and adjusted to pH 5.0 by adding 0.1 M HCl solution. The solution was mixed with EDAC (0.04 mmol), NHS (0.04 mmol) and aminated DOC (0.044 mmol). After 30 min, the mixture was dialysis (MWCO: 2000) against water to remove unreacted NHS, EDAC and aminated DOC. The final product, DOC-heparin was obtained and stored at 4 °C after freeze drying. The dried DOC-hep-



**Figure 1.** Composition and schematic diagram of DOC-heparin conjugate.

arin conjugate was analyzed by  $^1\text{H}$  NMR and FT-IR (Bruker, Germany). Values for  $^1\text{H}$  NMR of heparin( $\text{D}_2\text{O}$ ) were:  $\delta$  5.38 [H1 of Glucosamine residue(A)],  $\delta$  5.04 [H1 of iduronic acid residue(I)],  $\delta$  4.84 [I-5],  $\delta$  4.36-4.23 [A-6],  $\delta$  4.12-4.40 [I-3],  $\delta$  4.08 [I-4],  $\delta$  4.02 [A-5],  $\delta$  3.78 [I-2],  $\delta$  3.71 [A-4],  $\delta$  3.65-3.69 [A-3],  $\delta$  3.24 [A-2]. Values for  $^1\text{H}$  NMR of aminated DOC ( $\text{D}_2\text{O}$ ) were:  $\delta$  1.2-1.9 [m, five and six rings of DOC, 1H],  $\delta$  2.1-2.3 [m,  $\text{CH}_3$  of DOC, 1H],  $\delta$  3.15 [d, 12 $\alpha$ -OH of DOC, 2H],  $\delta$  8.0 [H of CONH]. Values of  $^1\text{H}$  NMR of DOC-heparin conjugates ( $\text{D}_2\text{O}$ ) were  $\delta$  1.2-1.9 [m, five and six rings of DOC, 1H],  $\delta$  3.24-5.38 [A or I of heparin],  $\delta$  8.0-8.2 [H of CONH of DOC-heparin].

**Ninhydrin Colorimetric Method.** For coupling ratio of DOC in DOC-heparin conjugate, ninhydrin colorimetric method was used as described previously.<sup>14</sup> In brief, 80  $\mu\text{L}$  of the ninhydrin solution was added to 300  $\mu\text{L}$  aminated DOC and cover the test tube with a piece of paraffin film to avoid the loss of solvent due to evaporation. With gentle stirring, the solution was heated for 5 min at 100  $^\circ\text{C}$ . After cooling to room temperature in a cold water bath, record the absorbance with spectrophotometer at 570 nm in wavelength. With the standard curves of aminated DOC, the coupling ratio of DOC-heparin conjugates by subtracting the OD values of remained aminated DOC.

**Anticoagulant Activity of DOC-Heparin Conjugates.** DOC-heparin conjugate (100  $\mu\text{L}$ ) was mixed with 100  $\mu\text{L}$  of antithrombin III (ATIII) solution for making DOC-heparin conjugate-ATIII complexes, where ATIII concentration was in excess of the DOC-heparin conjugate concentration. The solution was incubated at 37  $^\circ\text{C}$  for 3 min and 100  $\mu\text{L}$  of FXa was added to the solution. The resulting solution was then incubated for an additional 30 sec. The concentration of FXa was also in excess of the DOC-heparin conjugate concentration. The substrate (200  $\mu\text{L}$ , 0.8  $\mu\text{mol}/\text{mL}$ ) was then added and incubated at 37  $^\circ\text{C}$  for 3 min. The reaction was terminated by adding 300  $\mu\text{L}$  of 20% acetic acid. The bioactivity and the concentration of DOC-heparin conjugate were calculated from the absorbance at 405 nm.

**In Vivo Experiments.** Male ICR mice (25-30 g, Korean Animal Center, Seoul, Korea) were anesthetized with light diethyl ether and were administered heparin or DOC-heparin through an oral gavage that was carefully passed down the esophagus into the stomach. The gavage was made of

stainless steel with a blunt end to avoid causing lesions on the tissue surface. The heparin solution was prepared in sodium bicarbonate buffer (pH 7.4), and the dosage of heparin or DOC-heparin varied within the range of 3-10 mg/kg. The total volume of the administered HDL solution was 0.3 mL. Blood samples (450  $\mu\text{L}$ ) were collected from a capillary in the retroorbital plexus and directly mixed with 50  $\mu\text{L}$  of sodium citrate (3.8% solution), and were immediately centrifuged at 2,500 $\times$ g at 4  $^\circ\text{C}$  for 15 min. The concentration of heparin or DOC-heparin in blood was evaluated by anti-factor Xa assay.

**Statistics.** The cumulative data from animal experiments were expressed as mean  $\pm$  SD, and the paired *t*-test was to compare data before or after a treatment in the same animal and the ANOVA was also used for comparisons between groups. A value of  $P \leq 0.05$  was considered statistically significant.

## Results and Discussion

**Synthesis of DOC-Heparin Conjugates.** The conjugation of heparin and aminated DOC was confirmed by amide bonds formed by coupling carboxyl groups of heparin and the amine group of aminated DOC using FT-IR and  $^1\text{H}$  NMR. Briefly, the peaks at 1720 and 1585  $\text{cm}^{-1}$  in Fourier transform infrared spectrum indicated the presence of amide bonds in DOC-heparin. In the  $^1\text{H}$  NMR spectrum, the amide peak was also observed at 8.0 ppm, respectively. We synthesized DOC-heparin conjugates for cancer targeting and angiogenesis inhibition by linking polysaccharide heparin (about 5,000 Da) with sodium deoxycholate (DOC).<sup>16</sup> Conjugation between the carboxyl groups of polysaccharide heparin and the amine groups of DOC was confirmed by the presence of signals at  $\delta$  8.0-8.2 ppm in the  $^1\text{H}$  NMR spectrum. The selective modification of C3-OH was conducted by a suitable protected side chain of C12-OH group. It also took advantage of the known reactivity order of the two hydroxyl groups in DOC structure, C3-OH > C12-OH.<sup>18</sup> We also found a sharp peak at  $\delta$  2.2 ppm, indicating the presence of hydroxyl group at C12-OH position. The amount of DOC conjugated to polysaccharide heparin estimated by ninhydrin colorimetric method was maximized to 8.5 mol based on 1 mol of heparin (DOC-heparin VI) as shown in

**Table I. Reaction Condition and Coupled Ratio of DOC in DOC-Heparin Conjugates**

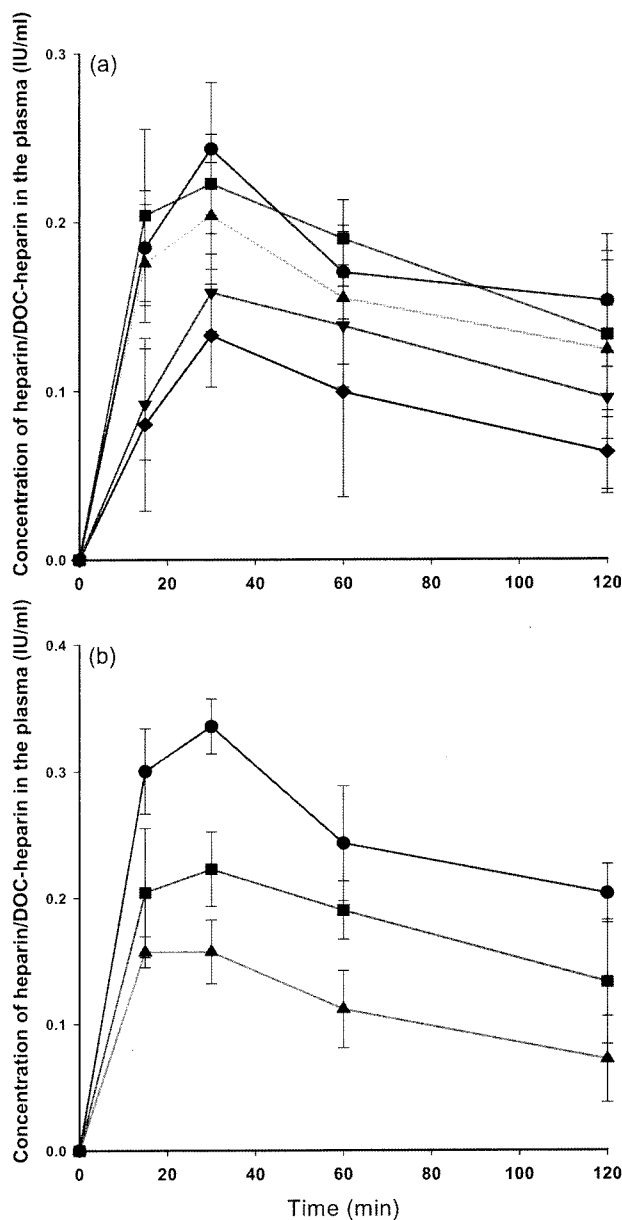
	DOC-Heparin I	DOC-Heparin II	DOC-Heparin II	DOC-Heparin IV	DOC-Heparin V	DOC-Heparin VI	DOC-Heparin VII	DOC-Heparin VIII
Fraxiparin( $\text{Ca}^{2+}$ , mol)	1	1	1	1	1	1	1	1
EDAC (mol)	1.2	2.2	2.4	3.2	3.6	4.8	6	12
HOSu (mol)	1.2	2.2	2.4	3.2	3.6	4.8	6	12
Aminated DOC (mol)	1.3	2.0	2.6	3.0	4	5.2	6.7	13.3
Coupling ratio	0.9	1.5	2.1	2.8	3.2	3.9	4.2	8.5

**Table II. Bioactivities of Heparin and DOC-Heparin Conjugates**

Sample	Absolute Bioactivity (IU/mg)	SD
Heparin (Ca <sup>2+</sup> )	101	-
DOC-Heparin I	106.7	1.5
DOC-Heparin II	110.0	1.5
DOC-Heparin III	109.8	2.1
DOC-Heparin IV	101.8	0.8
DOC-Heparin V	92.6	2.0
DOC-Heparin VI	86.5	5.7
DOC-Heparin VII	86.0	2.7
DOC-Heparin VIII	75.1	1.0

Table I. By changing feed mole ratio of heparin, EDAC, HOSu and aminated DOC, we controlled the coupling ratio between DOC and heparin. When dissolved in water, the DOC-heparin conjugates produced a clear solution at a concentration of 50 mg/mL. Bioactivities of DOC-heparin conjugates measured by Factor Xa chromogenic assay decreased to 75.1 IU/mg from bioactivity of heparin itself, 101 IU/mg. DOC-heparin showed lower bioactivity than unmodified heparin since there is 8-10 carboxyl groups in the active site of heparin (Table II). If other functional groups in heparin, such as amine group had been used for coupling reaction in order to increase the binding mole ratio of DOCA to heparin, the activity of heparin derivatives would have been completely preserved.<sup>14,15</sup> However, the number of amine group in the heparin was not enough to couple with DOC. Therefore, we controlled the binding ratio between DOC and heparin to have both high oral absorption and optimum bioactivities for oral absorption. Also, the steric hindrance of the coupled DOC might be low because DOC was linked with NPC and ethylenediamine.

**Oral Absorption of DOC-Heparin Conjugates.** Concentrations of heparin or DOC-heparin in the plasma could be determined using the anti-FXa assay. When 5 mg/kg of DOC-heparin VIII conjugates was administered to mice, the maximum anti-factor Xa activity was  $0.24 \pm 0.05$  IU/mL ( $P < 0.01$ ) after 30 h, which slowly decreased toward the baseline by 2 h after the single dose. The therapeutic window of heparin ranged from 0.1 to 0.2 IU/mL, indicating a 2 fold baseline in the therapeutic range as shown in Figure 2(a). The result of Figure 2(a) indicates that the absorption of DOC-heparin in the mice was significantly increased with the increase of coupled DOC in DOC-heparin. The absorption of DOC-heparin in the GI tract was determined according to the dose amount in the range of 3 to 10 mg/kg. In this experiment, the mole ratio of coupled DOC to heparin in heparin-DOC was 4.2. When raw heparin was administered orally to mice, the maximum concentration, measured by anti-factor Xa activity, was 0 IU/mL and it did not change with time. The average value of baseline was 0 IU/mL, indicating that raw heparin was never absorbed in the GI tract (data not shown). On the other hand, when 3 mg/kg of DOC-



**Figure 2.** The concentration curves of (a) DOC-heparin conjugates and (b) DOC-heparin VII conjugates; (●) DOC-heparin VIII, (■) DOC-heparin VII, (▲) DOC-heparin V, (▼) DOC-heparin III and (◆) DOC-heparin I (Figure 2(a)). (●) 10 mg/kg, (■) 5 mg/kg and (▲) 3 mg/kg (Figure 2(b)). The data are plotted as means  $\pm$  SD,  $n=5$ .

heparin was orally administered, concentration of DOC-heparin reached to the therapeutic window after 15 min as shown in Figure 2(b). Since the blood sampling was carried out at 15 min intervals and the maximum concentration was shown at the first 30 min, the real maximum concentration could not be determined. When heparin-DOC was dosed at 3, 5 and 10 mg/kg, the maximum concentration at 30 min were around 0.16, 0.24 and 0.33 IU/mL, respectively. Therefore, the coupled DOC to heparin greatly enhanced the absorp-

tion of heparin in the GI tract.

For toxicity of coupled DOC, we previously studied with DOCA that is free form of DOC.<sup>14</sup> From the result of H & E stain and TEM, the increased absorption of heparin derivatives was not due to tissue damage. The examination of the stomach and small intestine after 1, 2 and 3 h exposure to 100 mg/kg heparin-DOCA indicated no damage to the epithelium compare to the control. Some side effect with occasional epithelial cell shedding, some villi fusion, congestion of mucosal capillary with blood and focal trauma can be detected by H-E stain. But considerable side effects on GI-tract were not apparent. The data confirm that the increased absorption of heparin derivatives was not due to disruption of the intestinal epithelium. From transmission electron micrographs (TEM), no damage such as microvilli fusion, dissolution, disoriented cell layer with porosity and cytotoxic effect was detected. Therefore, we confirmed that DOCA or DOC in heparin derivatives didn't affect any damage on the surface of GI tract.

### Conclusions

In this study, we have prepared a new DOC-heparin conjugate for oral delivery. Heparin derivative conjugated via hydroxyl group of DOC were able to be absorbed in GI tract as was shown by different binding ratio of DOC. And concentration of DOC-heparin conjugate also is one of factors that increase the GI tract absorption. The absorption mechanism is remained to our further work. These findings may be a first step to overcome the obstacle of macromolecules and active large peptide drugs and be of great importance for the development of oral drug delivery, especially high molecular weight drugs.

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