Liphophilic complexation of heparin based on bile acid for oral delivery

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Abstract

Oral delivery of heparin will offer great advantages over injectable heparin therapy in the treatment of patients with deep vein thrombosis. Since heparin absorption in the intestine is restricted due to its physicochemical properties, we designed a bile acid derivative, cationic deoxycholylethylamine (DCEA), to be complexed with anionic low molecular weight heparin (LMWH). Complexation between LMWH and DCEA was saturated above 1:10 molar ratio and improved lipophilicity of LMWH. The LMWH/DCEA complex was completely solubilized in 80% propylene glycol solution. The oral absorption of LMWH in rats was proportional to the molar ratio of DCEA and the administered dose of complex. The Cmax values to the complex molar ratios of 1:0, 1:3, 1:5 and 1:10 were about 0.07, 0.27, 0.83, and 0.47 IU/ml, respectively, and the Cmax values to the doses of 10, 25, 50 mg/kg were 0.16, 0.44, and 0.83 IU/ml, respectively. The LMWH/DCEA complex was found to be absorbable through all regions of the small intestine of rats without causing tissue damage. This study demonstrates the feasibility of oral heparin delivery using the cationic DCEA for chronic administration in clinical trials as an effective therapy.

Keywords: Oral delivery; Heparin; Deoxycholylethylamine; Ionic complex; Lipophilicity

1. Introduction

Heparin, which is made up of heterogeneous polysaccharides, is widely used as an anticoagulant drug for the treatment and prevention of deep vein thrombosis (DVT) and pulmonary embolism (PE) [1,2]. Clinically, the treatment of DVT and PE is by hospitalizing patients and administering unfractionated heparin (UFH) intravenously. This heparin therapy is followed by the initiation of oral warfarin treatment after discharge from the hospital or by administering low molecular weight heparin (LMWH) subcutaneously twice-daily to the discharged patients, who must also complete a 5 to 7 day course with warfarin treatment started concomitantly at home [3,4]. However, warfarin has many unfavorable drug interactions, and careful patient monitoring is essential on account of its high protein binding properties [5]. In the view of the therapeutic problems associated with the current oral anticoagulant, heparin is believed to be the treatment of choice for preventing DVT and PE. Although the conventional injectable heparin therapy meets this goal, it is associated with several therapeutic disadvantages, such as pain and inconvenience. Among all the different alternative routes of heparin delivery, peroral administration is the most preferable, as it offers significant advantages in therapeutic and patient acceptability.

This heparin-based therapeutic approaches are limited because heparin is not absorbed in the intestine due to its high molecular weight, negatively charged structure and hydrophilic properties [6,7]. Therefore, many attempts have been made to find various alternatives to deliver heparin via non-invasive routes, including peroral, intranasal and pulmonary route [8–13]. Formulation strategies such as absorption enhancers, enteric coatings and liposomes have been investigated to circumvent the current obstacle to oral delivery of heparin [8–11]. Delivery agents, such as ethylenediaminetetraacetic acid, n-[8-(2-hydroxybenzoyl) amino] caprylate, 10-[N-(2-hydroxybenzoyl)amino] decanoate, sodium caprylate etc., have been developed to promote the absorption of heparin in the intestine. In addition, LMWH administered via the nasal or pulmonary routes has been reported
to increase the therapeutic anti-factor Xa levels using an enhancer or hydrophobic agents [12,13]. Recently, we developed a new form of orally active heparin derivative, a chemical conjugate of LMWH and deoxycholic acid (DOCA), which enhances heparin absorption in the intestine [14–16]. The chemically conjugated DOCA molecule constituent promotes intestinal absorption by enhancing the hydrophobic properties of LMWH and by increasing the interaction between heparin and the intestinal membrane without toxicity [17,18].

A more promising approach for enhancing oral absorption of heparin is the non-covalent approaches, in which the permeability of heparin is altered by using small organic molecules, termed carriers, that interact non-covalently with heparin to enable their oral absorption safely and effectively without changing chemical structure of heparin [10,19]. This approach has advanced the field of oral delivery of macromolecules further than any to date. Previously, we demonstrated oral absorption of insulin and ceftriaxone using non-covalent interaction with chemically synthesized derivatives of bile acid [20,21]. In this study we developed a new oral bile acid-based carrier, deoxycholylethylamine (DCEA), which could physically associate with LMWH by ion-pairing interaction without altering the structure of LMWH (Fig. 1). The aim of this study was to evaluate whether the LMWH/DCEA complex can be orally absorbed and to propose an oral formulation of heparin that is not affected by the intestinal conditions.

2. Materials and methods

2.1. Materials

Low molecular weight heparin (LMWH, Ardeparin; average molecular weight 4.1 kDa) was purchased from Celsus Laboratories Inc. (Cincinnati, OH) and used without further purification. Deoxycholic acid (DOCA), propylene glycol (PG), Azure A, hydrochloric acid, ethylenediamine, and ethylenediaminetetraacetate (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO). Fluorescen-5-isothiocyanate (FITC) was purchased from Fluka (Buchs, Switzerland). Deoxycholic acid (DOCA; 10 g, 23 mM) in 50 ml methanol was acidified with 1 ml concentrated hydrochloric acid. The mixture was stirred and heated to reflux for 1 h. The solution was cooled to 0 °C until recrystallization occurred. The product was filtered and washed with cold methanol twice and dried in vacuum to obtain methyl deoxycholate. Prepared methyl deoxycholate (3 g, 8.8 mM) was treated with an excess of ethylenediamine (15 ml) and the mixture was stirred and heated to reflux for 6 h. After the reaction, the mixture was poured to cold distilled water, and the obtained solid was filtered off, washed several times with ice cold distilled water and dried in vacuum. White solid with lumps were obtained, and this synthetic derivative was characterized by FT-IR spectrometer (SPECTRUM 2000, Perkin Elmer Co., UK) and 300 MHz NMR (JEOL JNM-LA 300WB FT-NMR, Tokyo, Japan) to prove the coupling between DOCA and ethylenediamine.

2.2. Preparation of deoxycholylethylamine (DCEA)

A solution of deoxycholic acid (DOCA; 10 g, 23 mM) in 50 ml methanol was acidified with 1 ml concentrated hydrochloric acid. The mixture was stirred and heated to reflux for 1 h. The solution was cooled to 0 °C until recrystallization occurred. The product was filtered and washed with cold methanol twice and dried in vacuum to obtain methyl deoxycholate. Prepared methyl deoxycholate (3 g, 8.8 mM) was treated with an excess of ethylenediamine (15 ml) and the mixture was stirred and heated to reflux for 6 h. After the reaction, the mixture was poured to cold distilled water, and the obtained solid was filtered off, washed several times with ice cold distilled water and dried in vacuum. White solid with lumps were obtained, and this synthetic derivative was characterized by FT-IR spectrometer (SPECTRUM 2000, Perkin Elmer Co., UK) and 300 MHz NMR (JEOL JNM-LA 300WB FT-NMR, Tokyo, Japan) to prove the coupling between DOCA and ethylenediamine.

2.3. Lipophilicity of LMWH/DCEA complex

The LMWH/DCEA complex was prepared by making a complex of LMWH with DCEA. Briefly, LMWH and DCEA were individually dissolved in distilled water. The DCEA solution was added in the LMWH solution while mixing by vortex. To determine the formation of complexes between LMWH and DCEA, the amount of unbound LMWH in the supernatant was analyzed after preparation of LMWH/DCEA complex followed by centrifugation. The drug complexes with various molar ratios between LMWH (10 mg/ml) and DCEA (0 to 26 mg/ml) were prepared (molar ratio of LMWH versus DCEA: 1:0, 1:1, 1:3, 1:5, 1:10, 1:15, 1:20, and 1:25), and Azure A assay was performed to estimate the amount of unbound LMWH in the supernatant. On the other hand, the partition coefficients of LMWH with or without DCEA in various molar ratios were determined between saturated water and n-octanol system. The LMWH complexes were prepared in water as described above and the same volume of n-octanol was added in the microcentrifugal tubes and vigorously vortexed. After mixing and occurrence of phase separation completely, Azure A assay was performed to estimate the amount of unbound LMWH in each phase. The partition coefficient (Log P_O/W) was calculated from \( P = \frac{C_{n-octanol}}{C_{water}} \), where \( C_{n-octanol} \) and \( C_{water} \) represent the concentrations of LMWH in the respective phase.

2.4. Formulation of LMWH/DCEA complex with propylene glycol (PG) solubilizer

To completely dissolve the LMWH/DCEA complex, it was dissolved at 40, 60, and 80% of propylene glycol (PG) in distilled water. Then, the transmittance of the complex solution was measured by UV–Vis spectrometry (CARY 1E, VARIAN, UK).
In animal experiments, 50 mg/kg of LMWH alone or LMWH/DCEA complex (molar ratio of LMWH versus DCEA = 1:5) in each concentration of PG solution was orally administered to male Sprague–Dawley (SD) rats weighing from 230 to 250 g (Samtako Bio Korea; Osan, Korea) by oral gavage. After oral administration of LMWH or LMWH/DCEA complex, blood samples (450 μl) were collected from the retro-orbital plexus at 0, 30, 60, 120, 180, 240, 300, and 360 min, directly mixed with 50 μl of sodium citrate (3.8% solution), centrifuged at 2500 ×g, and allowed to stand for 5 min at 4 °C. The LMWH concentration in plasma was measured by Coatest Factor Xa kit (Chromogenix, Milano, Italy). On the other hand, DOCA and ethylenediaminnetetraacetate (EDTA) were also known as an oral absorption enhancer. As a positive control, therefore, after physical complexation of LMWH with DOCA or EDTA (molar ratio of LMWH versus DOCA or EDTA = 1:5) in 80% PG solution, each complex was orally administered to rats by oral gavage. After oral administration of 50 mg/kg of LMWH/DOCA or LMWH/EDTA, the LMWH concentration in plasma was measured. All rats were kept in metabolic cages with free access to water only. All animal experimentation was reviewed and approved by our Institutional Animal Care and Use Committee, which is certified by the Institute of Laboratory Animal Resources at Seoul National University.

2.5. Oral absorption of LMWH/DCEA complex in rats

LMWH (50 mg/kg) was complexed with various molar ratios of DCEA ranging from 0 to 52 mg/kg (molar ratio of LMWH versus DCEA: 1:0, 1:3, 1:5, and 1:10) and reformulated in 80% PG solution. After fasting overnight, LMWH alone or different molar ratios of the LMWH/DCEA complexes were orally administered to male SD-rats through an oral gavage. The optimized molar ratio between LMWH and DCEA was determined, and we evaluated the dose-dependency of oral LMWH formulation ranging from 10 to 50 mg/kg. In order to compare oral LMWH efficiency with parenteral administration, 2 mg/kg of LMWH in saline was administered intravenously to the rats via cannulated jugular vein. After oral or intravenous administration of LMWH or LMWH/DCEA complex, blood samples were collected and the plasma concentration of LMWH was measured.

2.6. Histological evaluation and visualization of LMWH/DCEA complex absorption in the small intestine

To visualize the interaction of LMWH/DCEA in the small intestine, fluorescen-5-isothiocyanate (FITC) was conjugated with LMWH to form fluorescence-labeled LMWH. At 0 min, FITC-LMWH and FITC-LMWH/DCEA complex in 80% PG was orally administered to rats, respectively. At 30 and 60 min, each group of animals was sacrificed and sections of duodenum, jejunum, and ileum were removed. The tissues were fixed with 4% paraformaldehyde, dehydrated, and embedded in cryomatrix (frozen specimen embedding medium, Tissue-Tek®). The sections (10 μm) were analyzed by using a confocal laser scanning microscopy (CLSM; Leica DM IRB/E, Leica Co, Germany). In order to estimate the tissue damage to small intestine, the removed tissues of duodenum, jejunum and ileum were fixed in 4% paraformaldehyde buffer for 2 h. After dehydration, each tissue was freeze-dried and followed by coating with gold–palladium in a sputter coater. The tissues were analyzed by a scanning electron microscope (SEM) (JSM-5800, JEOL, Tokyo, Japan).

2.7. Data analysis

The pharmacokinetic parameters were calculated from the plasma anti-Factor Xa activities. The area under the time curves (AUC) was calculated by using SigmaPlot software (Windows version 9.01; Systat Software, Inc., San Jose, CA). Data are expressed as means±s.e. and analyzed using the Student’s t-test. P values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. Synthesis and characterization of DCEA

Deoxycholylethylamine (DCEA) was formed by an amide bond between methyl deoxycholate and ethylenediamine. The synthetic cationic delivery carrier, DCEA, was characterized for the characteristic amide bond peak by FT-IR and NMR. In the FT-IR spectrum, the amide bond was detected at 1700 cm⁻¹ and the N-terminal part of DCEA was detected at 3300 cm⁻¹. In addition, in the H-NMR spectrum, the amide bond and primary amine group were observed at 7.7 ppm and 3 ppm, respectively.

3.2. Lipophilicity of LMWH/DCEA complex

The partition coefficient of LMWH/DCEA was measured in the n-octanol/water system to evaluate the hydrophobic property (Fig. 2, open circle). LMWH itself exhibited a low partition coefficient (Log Po/w = −1.9±0.2) since it was hydrophilic. However, the complex of LMWH with DCEA showed substantial improvement of the partition coefficient up to 100 times in the

Fig. 2. Lipophilicity of LMWH/DCEA at different complexation molar ratios. After complexation of 10 mg/ml of LMWH with different molar ratio of DCEA (0 to 26 mg/ml), the amount of unbound LMWH in complex solution was measured by Azure A assay (●). Partition coefficient (Po/w) values of LMWH/DCEA complex were measured in n-octanol and water system (○).
molar ratio from 1:1 to 1:10, that is, Log\(P_{O/W}\) at 1:1, 1:3, 1:5, 1:10 were \(-0.05\pm0.01, 0.08\pm0.01, 0.48\pm0.09, \text{ and } 0.99\pm0.1\), respectively. Further improvement of its partition coefficient was not observed above the molar ratio 1:10. To confirm that the increment of partition coefficient of LMWH/DCEA was attributed to the binding of LMWH with DCEA, the amount of unbound LMWH in supernatant after precipitating the LMWH/DCEA complex by centrifugation was measured (Fig. 2, closed circle). About 10% of LMWH from the initial added amount was detected in supernatant above the 1:10 molar ratio and no further complexation was observed in the mole ratio above 1:10. This result indicated that complexation between LMWH and DCEA was saturated above the molar ratio of 1:10.

3.3. Formulation of LMWH/DCEA complex with propylene glycol (PG) solubilizer

The LMWH/DCEA complex was dissolved in different concentrations of propylene glycol (PG) in order to completely solubilize it for absorption in the intestine. For 40, 60, and 80% PG solution, the transmittance of the LMWH/DCEA complex was 1.4, 47.4, and 99.0%, respectively, that is, the complex in 80% PG was completely solubilized. Plasma concentration profiles following oral administration of 50 mg/kg of LMWH alone and LMWH/DCEA complex (in this case, molar ratio 1:5) in different concentrations of PG were measured in rats. The plasma concentration profiles changed distinctively depending on the concentration of PG (Fig. 3A). With increasing concentration of PG from 40 to 80%, the concentration of LMWH in plasma was significantly increased (1.8\(\pm\)0.5, 5.0\(\pm\)1.6, 11.8\(\pm\)4.2 fold, respectively, compared to LMWH alone in 80% PG). In the case of 40, 60 and 80% PG concentration, each \(C_{\text{max}}\) value was 0.11\(\pm\)0.01, 0.22\(\pm\)0.04, and 0.8\(\pm\)0.12 IU/ml, respectively. On the other hand, plasma concentration profiles following oral administration of 50 mg/kg of LMWH/DOCA or LMWH/EDTA in 80% PG solution were measured as a positive control (Fig. 3B). In each case of LMWH/DOCA or LMWH/EDTA, each \(C_{\text{max}}\) value was 0.34\(\pm\)0.06 and 0.40\(\pm\)0.07 IU/ml, respectively.

3.4. Pharmacokinetics of orally administered LMWH/DCEA complex in rats

When 50 mg/kg of LMWH in 80% PG was orally administered, LMWH was not appreciably absorbed (Fig. 4A).
On the other hand, when 50 mg/kg of a different molar ratio of LMWH/DCEA complex in 80% PG was orally administered in rats, the $C_{\text{max}}$ value to the molar ratios of 1:0, 1:3, 1:5, and 1:10 was 0.07, 0.27±0.05, 0.83±0.11, and 0.47±0.09 IU/ml, respectively. Fig. 4B showed the dose-dependent plasma concentration profiles of orally administered LMWH/DCEA that were complexed at fixed molar ratio of 1:5. In the case of 10, 25 and 50 mg/kg of LMWH/DCEA complex, the $C_{\text{max}}$ value was 0.16±0.03, 0.44±0.02, and 0.83±0.11 IU/ml, respectively. Table 1 showed the values of pharmacokinetic parameters following the oral administration of LMWH either alone or in complexation with DCEA. The absolute bioavailability of orally administered LMWH/DCEA at dose of 10, 25 and 50 mg/kg was 2.33, 3.23 and 3.08%, respectively. Whereas the bioavailability of orally administered LMWH at dose of 50 mg/kg was 0.12%, the absolute bioavailability of orally administered LMWH/DCEA increased about 25 times.

3.5. Histological analysis

To visualize that the LMWH/DCEA complex was absorbed in the small intestine, FITC-labeled LMWH was complexed with DCEA. At 30 or 60 min after the administration of 50 mg/kg of FITC-LMWH alone and FITC-LMWH/DCEA complex in 80% PG, the duodenum, jejunum, and ileum were removed from the rats and observed by using CLSM because the maximum plasma concentration of LMWH was observed at 1 h after its oral administration. No fluorescence was observed when the small intestine was exposed only to FITC-LMWH (Fig. 5). In the case of FITC-LMWH/DCEA complex, however, fluorescence was mainly observed in the duodenum and jejunum part of the small intestine at 30 min and in the jejunum and ileum part at 60 min. To confirm no tissue damage in the small intestine, the small intestinal tissue was removed at 6 h after the oral administration of 50 mg/kg of LMWH/DCEA complex in 80% PG and evaluated by SEM. Any tissue damage at any parts of small intestine was not observed and their morphologies were similar to those of the control (no treatment) (Fig. 6).

4. Discussion

Deoxycholic acid is an endogenous substance consisting of a facially amphiphilic steroid [22]. Synthesized from cholesterol in the liver, they are carried via bile duct to the small intestine, from where they return to the liver by reabsorption through the bile acid transporter. Our approach relies on the development of delivery agent of bile acid-based molecules that physically interacts with macromolecules to enable their oral absorption. This synthetic delivery carrier could maintain full biological activities of a therapeutic drug in a reduced dose as compared to conventional penetration enhancers because it could directly interact with drugs by electrostatic interaction.

DCEA is a positively charged deoxycholic acid derivative with the preserved properties of bile acid and can readily induce the formation of complexes by electrostatic interaction when mixed with LMWH in aqueous conditions. We hypothesized that physically associated DCEA could increase the lipophilicity of LMWH, which is known to be a key factor required for increasing drug permeability across cell membranes while maintaining the ability to be recognized by bile acid transporter in intestinal membranes which may allow drug absorption actively. DCEA improved lipophilicity of LMWH dependent on feed molar ratio (up to 1:10) between DCEA and LMWH. By adding DCEA, the lipophilicity of LMWH was enhanced.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Dose (mg/kg)</th>
<th>$C_{\text{max}}$ (IU/ml)</th>
<th>$T_{\text{max}}$ (min)</th>
<th>AUC (IU/ml/h)</th>
<th>$F$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMWH (iv)</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>2.40±0.17</td>
<td>–</td>
</tr>
<tr>
<td>LMWH (oral)</td>
<td>50</td>
<td>0.07±0.00</td>
<td>41.42±1.43</td>
<td>0.07±0.01</td>
<td>0.12</td>
</tr>
<tr>
<td>LMWH/DCEA</td>
<td>10</td>
<td>0.16±0.03</td>
<td>42.86±12.86</td>
<td>0.28±0.03</td>
<td>2.33</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.44±0.02</td>
<td>42.86±6.06</td>
<td>0.97±0.12</td>
<td>3.23</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.83±0.11</td>
<td>38.57±5.53</td>
<td>1.85±0.17</td>
<td>3.08</td>
</tr>
</tbody>
</table>

Table 1: Pharmacokinetic parameters following the administration of LMWH either alone or in complexation with DCEA.

Fig. 5. CLSM images of duodenum, jejunum, and ileum at 30 or 60 min after oral administration of 50 mg/kg of FITC-LMWH alone or FITC-LMWH/DCEA complex at the molar ratio of 1:5.

Fig. 6. SEM images of duodenum, jejunum, and ileum at 6 h after oral administration of 50 mg/kg of LMWH/DCEA complex at the molar ratio of 1:5. Control: no treatment. Sale bar: 20 μm.
However, the solubility was significantly reduced due to the larger sized particle. Improved absorption of poorly soluble drugs can be achieved by increasing the solubility of the drug by formulation. In this study, propylene glycol (PG), which is one of the most common water soluble organic solvent approved for oral formulation, was chosen as a vehicle for oral formulation of LMWH/DCEA complex. In comparison with none clearly dissolved drug, the absorption profile of clearly dissolved drug was significantly high.

The animal experiments indicated that DCEA significantly affected the oral absorption of the LMWH. When LMWH formed complex with DCEA at the molar ratio of 1:5, the oral absorption of LMWH was high. However, at the complex molar ratios of 1:3 and 1:10, they did not show significant effects even though the saturated complex molar ratio was 1:10. This was attributed to the fact that, at the complex molar ratio of 1:3, the complex was loosely formed while at the molar ratio of 1:10, the molecular weight of the complexes was higher than that of the molar ratio of 1:5. These kinds of factors hindered the drug from being absorbed in the small intestine. In this study, to compare with the absorption potency of DCEA, we also measured the plasma concentration profile of LMWH after oral administration of LMWH/DOCA or LMWH/EDTA complex. It has known that both DOCA and EDTA have an absorption enhancing effect. The oral absorption of LMWH/DCEA complex was higher than those of LMWH/DOCA and LMWH/EDTA complex, meaning that DCEA has higher potency as a new absorption enhancer. On the other hand, at 30 min after the administration of above 10 mg/kg of LMWH/DCEA complex, the bioactivity of LMWH in plasma reached the maximum peak and higher than 0.1 IU/ml of which concentration is the recommended minimum effective concentration for the prevention of venous thromboembolism [23,24].

The mechanism of improved LMWH absorption by DCEA is not clear. However, this is partly explained by the physico-chemical structure of LMWH/DCEA complexes. DCEA preserves the natural characteristic of bile acid and has the ability to form electrostatic interaction with LMWH. Once DCEA makes an ion-pairing interaction with LMWH based on opposite electrostatic charges, it may increase the lipophilicity and stability of the native drug by combining an amphiphilic steroid nucleus with the reactivity of the side groups of bile acid. Increased lipophilicity would improve permeability of drug against mucosal membrane and increased stability would prolong therapeutic activity of drug in gastrointestinal region. In the previous study, we experimentally showed the direct interaction between chemical conjugate of LMWH-DOCA and ileal brush border membrane surface by using surface plasmon resonance technique [25]. Hence, it is possible that our physically associated bile acid derivative would be recognized by the bile acid transporter, thus leading to the improvement of drug absorption; however, this mechanism will be verified in further studies.

In summary, a deoxycholic acid derivative was prepared with the aim of enhancing the oral bioavailability of LMWH. Physically associated LMWH/DCEA complexes significantly increased bioavailability and improved the pharmacokinetic effect in a dose-dependent fashion. It is anticipated that this new simple formulation strategy for peroral form may replace other injectable ionized drugs as well so as to improve patient acceptability and therapeutic effect.

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References


