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Formulation of self-microemulsifying drug delivery system (SMEDDS) by D-optimal mixture design to enhance the oral bioavailability of a new cathepsin K inhibitor (HL235)

Voradanu Visetvichaporn[a], Kyung-Hee Kim[b], Kyungjin Jung[b], Yun-Seok Cho[c], Dae-Duk Kim[a,*]

[a] College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 08826, Republic of Korea
[b] New Drug Development Center, Daegu-Gyeongbuk Medical Innovation Foundation, Daegu 41061, Republic of Korea
[c] R&D Center, Hanlim Pharm. Co., Ltd, Seoul 06634, Republic of Korea

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ABSTRACT

HL235 is a new cathepsin K inhibitor designed and synthesized to treat osteoporosis. Since HL235 has poor aqueous solubility, a self-microemulsifying drug delivery system (SMEDDS) was formulated to enhance its oral bioavailability. A solubility study of HL235 was performed to select a suitable oil, surfactant and cosurfactant. Pseudoternary phase diagrams were plotted to identify the microemulsion region and to determine the range of components in the isotropic mixture. D-optimal mixture design and a desirability function were introduced to optimize the SMEDDS formulation for the desired physicochemical characteristics, *i.e.*, high drug concentration at 15 min after dilution with simulated gastric fluid (SGF) and high solubilization capacity. The optimized HL235-loaded SMEDDS formulation consisted of 5.0% Capmul MCM EP (oil), 75.0% Tween 20 (surfactant) and 20.0% Carbitol (cosurfactant). The droplet size of the microemulsion formed by the optimized formulation was 10.7 ± 1.6 nm, and the droplets were spherical in shape. Pharmacokinetic studies in rats showed that the relative oral bioavailability of the SMEDDS formulation increased up to 3.22-fold compared to its solution in DMSO:PEG400 (8:92, v/v). Thus, the formulation of SMEDDS optimized by D-optimal mixture design could be a promising approach to improve the oral bioavailability of HL235.

1. Introduction

Bone is a living tissue that changes gradually throughout the lifetime. Osteoporosis is a bone disease that involves excessive loss of bone mass and density. This condition makes bone easy to break and leads to serious complications, such as bone fractures, especially in elderly patients. Bone loss is caused by an imbalance between bone formation and resorption (Stoch and Wagner, 2008). Thus, osteoporosis could be prevented or treated by increasing bone formation and/or inhibiting bone resorption. Cathepsin K is a lysosomal cysteine protease highly expressed in osteoclasts, which plays an important role in the degradation of bone components by osteoclastic bone resorption (Boonen et al., 2012; Stoch and Wagner, 2008). Thus, the direct inhibition of cathepsin K has been studied as a novel target to treat osteoporosis (Rachner et al., 2011; Rodan and Duong, 2008). Cathepsin K inhibitors have been shown to increase bone mineral density and reduce the biomarkers of bone resorption in clinical studies (Eisman et al., 2011; Stoch et al., 2009). However, many of them were discontinued due to safety issues, including dermatological or cardiovascular adverse events (Lu et al., 2018; Runger et al., 2012). The selectivity of cathepsin K inhibitors against other highly homologous cathepsins (i.e., B, L, and S) is the main property associated with the side effects. Compounds with basic and lipophilic properties tend to accumulate at high levels in the acidic lysosome (lysosomotropism), resulting in interaction with other cathepsins and less selectivity for cathepsin K. (Black and Percival, 2006; Falgueyret et al., 2005). In addition, the cardiotoxicity-related adverse events caused by off-target interactions with human ether-a-go-go-related gene (hERG) were also major concerns for new drug development since several drugs in the market were withdrawn because of hERG blocking activity (Kalyaanamoorthy and Barakat, 2018). Therefore, HL235 (Fig. 1) was designed and synthesized as a new cathepsin K inhibitor with neutral property, but high potency (IC_{50} = 2.1 nM) against cathepsin K. It thus has high selectivity (more than 5000-fold) against cathepsin B, L and S. Moreover, it has good *in vitro* metabolic stability without drug-induced blockade of hERG (unpublished data). However, HL235 has poor solubility in water, resulting in low

* Corresponding author.
E-mail address: ddkim@snu.ac.kr (D.-D. Kim).

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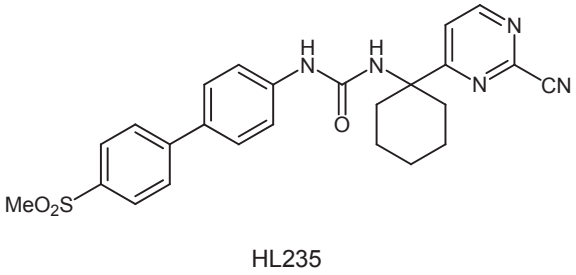

Fig. 1. Chemical structure of HL235.

bioavailability after oral administration.

Numerous pharmaceutical approaches, including lipid-based formulations, have been developed to improve the dissolution rate and the absorption of poorly water-soluble drugs in the gastrointestinal (GI) tract by enhancing their solubility in vehicles (Kalepu and Nekkanti, 2015; Yeom et al., 2015). Among them, the self-emulsifying drug delivery system (SMEDDS) has been successfully developed and made available in the market (Kamboj and Rana, 2016; Yeom et al., 2015; Zhang et al., 2008), including Neoral® (cyclosporine A), Fortovase® (saquinavir) and Agenerase® (amprenavir) (Porter et al., 2008). SMEDDS is an isotropic mixture of oil, surfactant and cosurfactant, that can continuously form fine oil-in-water (o/w) emulsions upon exposure to the GI fluid with gentle agitation from GI tract motility as demonstrated in Fig. 2. Many studies have demonstrated that SMEDDS is useful for improving the oral bioavailability of poorly water-soluble drugs (Wu et al., 2015; Yeom et al., 2015; Zhang et al., 2008).

However, the development of SMEDDS formulations based on the conventional "trial-and-error" process is time-consuming and labor-intensive. Thus, many statistical tools based on response surface methodology (RSM) and experimental designs have been used for the optimization of SMEDDS, including Box-Behnken design, factorial design and D-optimal mixture design (Cho et al., 2013; Holm et al., 2006; Kamboj and Rana, 2016; Shaji and Lodha, 2008; Yeom et al., 2015). Statistical optimization can simultaneously estimate both the main effects and the interaction of all variables of a SMEDDS formulation. Among many statistical tools, D-optimal mixture design is known to be suitable for the optimization of SMEDDS formulations since it considers the total of SMEDDS as 100% (Yeom et al., 2015). Thus, the objective of this study was to develop and optimize HL235-loaded SMEDDS formulation by using D-optimal mixture design to enhance its oral bioavailability. After characterization of the optimized SMEDDS formulation, the *in vivo* oral bioavailability was compared with that of HL235 solution in rats.

2. Materials and methods

2.1. Materials

HL235 was synthesized and supplied by Hanlim Pharmaceutical Co., Ltd. (Seoul, South Korea). Etodolac was purchased from Tokyo Chemical Industry (Tokyo, Japan). Capmul MCM EP (glyceryl caprylate/caprate) was received as a sample from Abitec Corp. (Wisconsin, United States). Capryol 90 (propylene glycol monocaprylate), Labrafil M1944 CS (oleoyl polyoxyl-6 glycerides), Labrafil M2125 CS (linoleoyl polyosyl-6 glycerides), Labrafil M2130 CS (lauroyl polyoxyl-6 glycerides), and Labrasol (caprylocaproyl polyoxyl-8 glycerides) were purchased from Gattefossé (Saint Priest, France). Cotton seed oil, soybean oil, sunflower seed oil, Tween 20, Tween 80, polyethylene glycol 400 (PEG400), Solutol HS 15 (poly-oxyethylene esters of 12-hydroxystearic acid), Carbitol (diethylene glycol ethyl ether) and Span 80 (sorbitan monooleate) were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade.

2.2. Solubility studies

The solubility of HL235 was determined in various natural oils (cotton seed oil, sunflower seed oil, soybean oil), synthetic/semi synthetic oils (Capmul MCM EP, Labrafil 1944 CS, Labrail M2125 CS, Labrafil M2130 CS, ethyl oleate), and surfactants/cosurfactants (Tween 20, Tween 80, PEG 400, Labrasol, Solutol HS 15, Carbitol, Span 80). Excess amounts of HL235 were added to 1 mL of each oil/surfactant and vortexed until completely dispersed. Then, the mixtures were kept in a shaking water bath (Lab Companion, BS-21, Jeiotech Co., Ltd., Republic of Korea) at 50 rpm and 37 ± 0.5 °C for 24 h, followed by centrifugation (Centrifuge 5415 R, Eppendorf, Germany) at 16,168*g* for 5 min. The supernatants were collected and filtered through a 0.45 μm syringe filter (Minisart®-RC, Sartorius, Germany). The filtrates were analyzed by high-performance liquid chromatography (HPLC) with a fluorescence detector after appropriate dilution with a mixture of dimethyl sulfoxide (DMSO) and acetonitrile (ACN) (50:50, v/v).

2.3. Construction of the pseudoternary phase diagram

Based on the solubility (Table 1) and hydrophilic-lipophilic balance (HLB) values, Capmul MCM EP, Tween 20 and Carbitol were chosen as the oil, surfactant and cosurfactant, respectively. To determine the microemulsion area, pseudoternary phase diagrams were constructed employing the water titration method at 37 °C. Mixtures of surfactant and cosurfactant (S-mix) were prepared in different volume ratios (3:1,

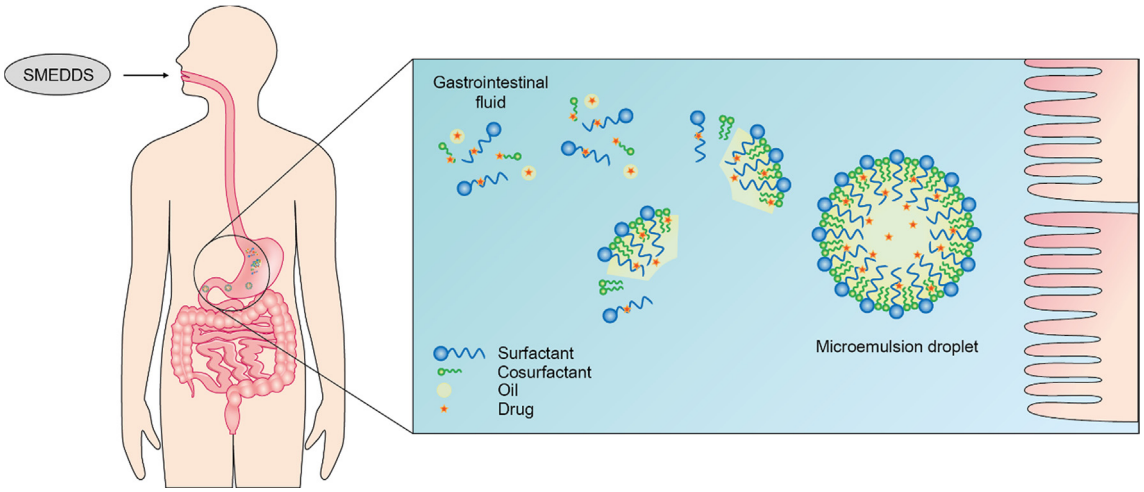

Fig. 2. Schematic illustration of forming microemulsions after oral administration of SMEDDS.

Table 1
Solubility of HL235 in various excipients.

Excipient	Solubility (mg/mL)
Oil	
Capmul MCM EP	0.239 ± 0.035
Capryol 90	0.191 ± 0.009
Labrafil M2130 CS	0.136 ± 0.003
Labrafil M2125 CS	0.097 ± 0.002
Labrafil M1944 CS	0.054 ± 0.003
Cotton seed oil	0.004 ± 0.000
Soybean oil	0.004 ± 0.000
Sunflower seed oil	0.002 ± 0.000
Ethyl oleate	0.002 ± 0.000
Surfactant	
Tween 20	7.461 ± 0.759
PEG400	5.022 ± 0.200
Labrasol	3.306 ± 0.198
Tween 80	2.284 ± 0.135
Solutol HS 15	1.860 ± 0.980
Cosurfactant	
Carbitol	6.563 ± 0.109
Span 80	0.015 ± 0.007

Values are presented as the mean ± SD (n = 3).

2:1, 1:1, 1:2, 1:3). Each ratio of S-mix was combined with oil in different ratios of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1 (total volume of 1 mL). The prepared mixtures were vortexed and titrated with water dropwise under gentle agitation by a magnetic stirrer (75 rpm) up to 100 mL (1:100 dilution). After each addition, the emulsion was observed visually (turbid or clear). Each experimental component of blank SMEDDS was marked with an open circle (microemulsion; clear) or a closed circle (macroemulsion; turbid). The microemulsion region was identified by constructing a pseudoternary phase diagram using Sigma Plot® software (Sigma Plot, USA).

2.4. Optimization of HL235-loaded SMEDDS formulations using D-optimal mixture design

The composition of the SMEDDS formulation was optimized by using D-optimal mixture design. Based on the solubility study (Table 1) and the pseudoternary phase diagram of microemulsion (Fig. 3), the amounts of oil, surfactant and cosurfactant were chosen as the independent variables. The range of Capmul MCM EP (oil; X_1), Tween 20 (surfactant; X_2), and Carbitol (cosurfactant; X_3) were set to 5–15%, 55–75%, and 15–35%, respectively (Table 2). The total of X_1, X_2 and X_3 in each formulation summed to 100%. The drug concentration at 15 min after dilution with simulated gastric fluid (SGF, pH 1.2) (1:250 dilution) (DIL; Y_1) and the solubilization capacity (SC; Y_2) were determined as dependent variables, to find the optimal SMEDDS formulation. The experimental design in this study was developed and evaluated by Design Expert® Software version 7 (Stat-Ease Inc., Minneapolis, MN, USA). Sixteen formulations were obtained from the program to fit the statistical models (Table 3). DIL and SC were fitted in the polynomial model (linear, quadratic, cubic and special cubic model), and the equations were generated by the software. The most suitable mathematical fitting model was selected based on the comparison of various statistical parameters provided by the analysis of variance (ANOVA) such as sequential p-value, lack of fit p-value, r-squared and adequate precision. The selected models for each response were used to predict the desirable results of the optimized independent factors by using the desirability function.

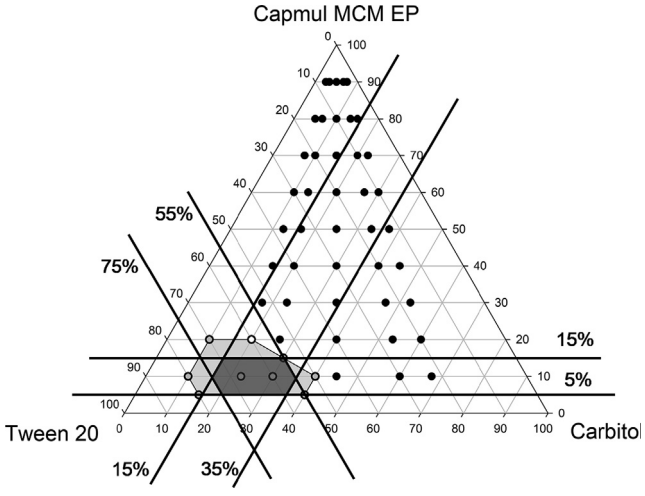

Fig. 3. Pseudoternary phase diagram of Capmul MCM EP (oil), Tween 20 (surfactant), and Carbitol (cosurfactant) and design space for D-optimal mixture design.

Table 2
Variables used in the D-optimal mixture design.

Independent variables	Range (%)	
	Minimum	Maximum
X_1: Oil (Capmul MCM EP)	5	15
X_2: Surfactant (Tween 20)	55	75
X_3: Cosurfactant (Carbitol)	15	35
Dependent variables		Goal
Y_1: Drug concentration at 15 min after dilution with SGF (1:250 dilution) (μg/mL; DIL)		Maximize
Y_2: Solubilization capacity (mg/mL; SC)		Maximize

2.5. In vitro evaluation and optimization of SMEDDS formulation

2.5.1. Drug concentration at 15 min after dilution with SGF (1:250 dilution) (DIL)

The blank SMEDDS formulation without HL235 was first prepared by gently mixing oil, surfactant, and cosurfactant. Then, HL235 was loaded into the blank SMEDDS to make 2.5 mg/mL HL235-loaded SMEDDS. The mixture was vortexed until completely dispersed and

Table 3
Composition and observed responses from randomized runs in the D-optimal mixture design.

Mixture Number	Independent variables			Dependent variables (Responses)	
	Capmul MCM EP (%; X_1)	Tween 20 (%; X_2)	Carbitol (%; X_3)	DIL (μg/mL; Y_1)	SC (mg/mL; Y_2)
1	5	65	30	1.80 ± 0.01	6.76 ± 0.04
2	5	70	25	1.89 ± 0.07	6.43 ± 0.07
3	5	75	20	2.14 ± 0.10	6.23 ± 0.05
4	5	75	20	2.20 ± 0.11	6.67 ± 0.06
5	5	60	35	1.58 ± 0.05	6.91 ± 0.10
6	5	60	35	1.62 ± 0.02	6.67 ± 0.11
7	10	75	15	2.15 ± 0.11	5.65 ± 0.04
8	10	75	15	2.05 ± 0.14	5.53 ± 0.11
9	10	65	25	1.61 ± 0.08	5.74 ± 0.07
10	10	60	30	1.53 ± 0.01	5.89 ± 0.10
11	15	55	30	1.40 ± 0.10	5.04 ± 0.04
12	15	55	30	1.42 ± 0.10	5.06 ± 0.06
13	15	60	25	1.61 ± 0.06	4.98 ± 0.02
14	15	65	20	1.60 ± 0.07	5.02 ± 0.05
15	15	70	15	1.82 ± 0.06	4.75 ± 0.01
16	15	70	15	1.81 ± 0.08	4.87 ± 0.02

DIL, Drug concentration at 15 min after dilution with SGF (1:250 dilution); SC, Solubilization capacity.

then kept in a shaking water bath at 50 rpm and 37 ± 0.5 °C for 3 h to obtain a clear homogenous solution. Then, an aliquot (100 μL) of the HL235-loaded SMEDDS formulation was added to 25 mL of SGF at 37 °C and gently stirred at 75 rpm with a magnetic stirrer for 15 min. Samples (1 mL) were collected and filtered through a 0.2 μm membrane. The filtrate was centrifuged at 16,168*g* for 2 min. The supernatant was collected and diluted with a mixture of DMSO and ACN (50:50, v/v), after which 100 μL of the sample was analyzed by HPLC. SGF (pH 1.2) in this study was prepared by dissolving 2.0 g of sodium chloride in 7.0 mL of hydrochloric acid and adjusting the volume to 1000 mL with water according to the method in United States Pharmacopoeia (USP) 39th edition, without the addition of purified pepsin.

2.5.2. Solubilization capacity (SC)

An excess amount of HL235 was added to 1 mL of blank SMEDDS of each formulation and vortexed until the drug was completely dispersed. The samples were kept in a shaking water bath at 50 rpm and 37 ± 0.5 °C for 24 h. Then, the samples were centrifuged at 16,168*g* for 10 min. The supernatants were collected and filtered through a 0.45 μm syringe filter (Minisart®-RC, Sartorius, Germany). The filtrates were analyzed by HPLC after appropriate dilution with a mixture of DMSO and ACN (50:50, v/v).

2.5.3. Droplet size (DS) and polydispersity index (PDI)

The optimized SMEDDS formulation (20 μL) containing 2.5 mg/mL of HL235 was diluted with 1000 μL of double-distilled water (DDW). After gentle shaking, the microemulsion was transferred into an optical polystyrene cuvette, and the DS and PDI were measured using zeta-potential & particle size analyzer (Photal, ELSZ, Otsuka Electronic, Japan).

2.5.4. Morphology of microemulsion

The morphology of the microemulsion droplets formed from the optimized SMEDDS formulation containing 2.5 mg/mL of HL235 was observed using an energy-filtering transmission electron microscope (TEM; LIBRA 120; Carl Zeiss, Jena, Germany) at 80 kV. SMEDDS formulation (20 μL) was added to 1000 μL of DDW (1:50 dilution), and the sample drop was placed on a copper grid. The sample was subsequently stained with uranyl acetate solution for 10 s. It was washed with water for 1 s twice, and the excess was removed with a filter paper.

2.6. In vivo pharmacokinetic studies

The pharmacokinetics of HL235 after intravenous and oral administration were studied in rats. The study protocol was in accordance with the National Institutes of Health guidelines on the principles of laboratory animal care, and was approved by the Institutional Animal Care and Use Committee of the College of Pharmacy, Seoul National University (Seoul, Republic of Korea). Male Sprague-Dawley rats (250–300 g) were obtained from Orient Bio (Kyunggi-Do, Korea), and were fasted with free access to water for approximately 12 h before experiments. A solution of HL235 (2.5 mg/mL) in a mixture of DMSO and PEG400 (8:92, v/v) was prepared for intravenous and oral administration by dissolving HL235 in DMSO and then adding PEG400. The solution was vortexed and stirred at room temperature for 1 h, after which it was intravenously injected through the femoral vein at a dose of 5 mg/kg. For oral administration, HL235 solution or HL235-loaded SMEDDS formulation containing 2.5 mg/mL of HL235 was administered at a dose of 5 mg/kg. Rats in "with water" groups were promptly given 1 mL of water after oral administration. Blood samples (approximately 300 μL) were collected from the cannulated femoral artery at predetermined time intervals into a polyethylene micro test tube, after which they were immediately centrifuged at 16,168*g* for 3 min at 4 °C. Supernatant plasma samples were collected and stored at −20 °C until further analysis.

Plasma samples (100 μL) were mixed with 1 mL of ACN containing 400 ng/mL internal standard (*i.e.*, etodolac). Each sample was shaken for 5 min, followed by centrifugation at 16,168*g* for 5 min at 4 °C. The supernatant was collected and evaporated to remove ACN under nitrogen gas on a pressured gas blowing concentrator (EYELA, MGS-2200, Tokyo Rikakikai, Japan). The residue was reconstituted with 100 μL of DMSO:ACN (10:90, v/v) and vortexed for 5 min. The concentration of HL235 was analyzed by HPLC described below.

The pharmacokinetic parameters of HL235 were calculated by a noncompartmental model using WinNonlin (version 5.0.1, Pharsight, CA, USA). The area under the plasma concentration versus time curve from zero to 5 h (AUC_{last}) was calculated using the trapezoidal method. The maximum plasma concentration (C_{max}), the time to reach C_{max} (T_{max}) and half-life ($t_{1/2}$) were obtained from the plasma data. The significance differences observed for the mean pharmacokinetic parameters were determined using analysis of variance (ANOVA) at a significance level of $P < 0.05$. Tukey's multiple comparison was used as a subsequent analysis by Graphpad PRISM® Software (Version 5.01).

2.7. HPLC analysis of HL235

The HL235 concentrations in the samples acquired from solubility and *in vivo* pharmacokinetic studies were determined by using an isocratic HPLC system equipped with a pump (We2695; Waters Corporation, Milford, MA, USA), fluorescence detector (W2475; Waters Corporation, Milford, MA, USA) and chromatographic XBridge Shield RP18 column (4.6 × 250 mm, 5 μm; Waters Corporation, Milford, MA, USA). The flow rate was set to 1.0 mL/minute at 25 °C. The mobile phase consisted of ACN and water (65:35, v/v) for solubility studies or of ACN:10 mM phosphate buffer (pH 2.5) (57:43, v/v) for plasma samples. The excitation and emission wavelengths of fluorescence detection were set to 295 and 395 nm, respectively, for HL235, while those of etodolac were set to 235 and 345 nm, respectively. The injection volume was 20 μL. The chromatograms were evaluated with Empower 2 Software (Waters Corporation, Milford, MA, USA). The retention times of etodolac and HL235 were 7.2 min and 8.3 min, respectively, under these conditions. The lower limit of quantification (LLOQ) of HL235 was 40 ng/mL with acceptable accuracy and precision.

3. Results and discussion

3.1. Selection of oil, surfactant and cosurfactant

The solubility of HL235 in various excipients is shown in Table 1. Among the oils tested, Capmul MCM EP showed the highest solubility (0.239 ± 0.035 mg/mL), while Tween 20 (7.461 ± 0.759 mg/mL) and Carbitol (6.563 ± 0.109 mg/mL) showed the highest solubility among surfactants and cosurfactants, respectively. The HLB value of surfactants should also be considered when selecting surfactants and cosurfactants for the SMEDDS formulation. Water-soluble surfactants with HLB values higher than 12 are generally recommended for SMEDDS formulation due to their high micelle-forming ability (Dokania and Joshi, 2015; Pouton and Porter, 2008). However, surfactants with low HLB values can help to reduce the interfacial tension of the film formed by emulsion droplets and ensure the flexibility of the film. Thus, the combination of low HLB (HLB < 10) and high HLB (HLB greater than 10) surfactants can prolong the stability of the formulation. Based on the solubility study, Capmul MCM EP was selected as an oil phase to form an oil-in-water (o/w) emulsion, while Tween 20 (HLB = 16.7) and Carbitol (HLB = 4.2) were selected as the surfactant and cosurfactant, respectively. Then, the composition was further evaluated using a pseudoternary phase diagram.

3.2. Construction of the pseudoternary phase diagram

Fig. 3 shows the pseudoternary phase diagrams plotted for Capmul

MCM EP (oil), Tween 20 (surfactant), and Carbitol (cosurfactant), with each composition marked as 100% at the apex of the diagram. Only three combinations among various ratios of S-mix and oil showed the formation of microemulsions (marked as open circles) when diluted 100 times with water. Six additional ratios near these three points were further confirmed to form microemulsions after dilution 100 times. However, the other combinations of oil and S-mixture showed turbid emulsion after dilution with water no more than 20 times (marked as closed circle). The microemulsion region was thus shown in the light gray region as in Fig. 3.

3.3. Statistical analysis using the D-optimal mixture design of HL235-loaded SMEDDS

A D-optimal mixture design was applied to optimize the SMEDDS formulation. It was reported that the amount of oil, surfactant and cosurfactant were major factors influencing the *in vitro* dispersion of SMEDDS formulations (Kamboj and Rana, 2016; Yeom et al., 2015). Thus, these factors were set as the input variables, and their design space was selected from the pseudoternary phase diagram shown as a dark gray region where the robustness of microemulsion formation is ensured (Fig. 3), *i.e.*, oil (X_1, 5–15%), surfactant (X_2, 55–75%), and cosurfactant (X_3, 15–35%). DIL (Y_1) and SC (Y_2) were chosen as response variables since they are critical properties of SMEDDS to enhance the oral absorption of poorly water-soluble drugs. Table 2 summarizes the range of independent variables and the goals of dependent variables used in this D-optimal mixture design.

Droplet size (DS) and size distribution (*i.e.*, PDI) are also important characteristics affecting the *in vitro* dissolution and *in vivo* absorption of emulsions (Liu et al., 2009). However, in our preliminary study, the average microemulsion droplet size and polydispersity index after diluting HL235-loaded SMEDDS with water (1:50 dilution) of the experimental formulations were small (DS = 10.3–13.9 nm) and homogeneous (PDI = 0.010–0.161) as shown in Table S1. Thus, these two parameters were not included in this design since they were not significantly different among the 16 formulations.

Table 3 shows the sixteen formulations of SMEDDS obtained from Design Expert® software, together with the dependent variables obtained from each formulation. These data were statistically fitted to different models and the polynomial equations of the responses were generated. As shown in Table 4, the quadratic and linear mathematical models were suggested to fit Y_1 and Y_2, respectively. A sequential p-value of < 0.05 indicates that the model terms are significant. Additionally, a lack of fit p-value value of greater than 0.1 indicates adequacy of the model fit. Multiple regression and analyses of the regression for each model were expressed by R^2, adjusted R^2 and adequate precision. The R^2 values for the responses Y_1 and Y_2, which imply the total variation explained by the model, were higher than 97%. Moreover, the adjusted R^2 values, which reflect the influence of increasing and decreasing the number of model terms, were also desirably similar to R^2, indicating that the fit was sufficient.

3.4. Influence of independent variables on DIL (Y_1)

Drug precipitation during dissolution and/or digestion in the GI tract is a major concern regarding lipid-based formulations since it would result in decreased drug absorption and eventually low bioavailability (Khan et al., 2016; Pouton, 2000). Thus, the SMEDDS formulations was optimized to have a maximized DIL (Y_1) value. As shown in Table 3, formulation 4 and 11 showed the highest (2.20 ± 0.11 μg/mL) and the lowest (1.40 ± 0.10 μg/mL) values, respectively. The data were statistically fitted well to the quadratic model (Table 4), and the following polynomial equation was obtained from the program based on the results of analysis of variance to validate the relationship between the independent variables and DIL (Y_1) (Table 5).

$$\text{DIL } (Y_1) = +1.80X_1 + 2.47X_2 + 1.52X_3 - 1.57X_1X_2 - 0.84X_1X_3 - 0.66X_2X_3 \quad (1)$$

The magnitude of the coefficient, which indicates the influence of the response, was in the order $X_2 > X_1 > X_3$. The significantly highest magnitude and the positive coefficient of X_2 imply that the amount of Tween 20 (X_2) was a critical factor and had a positive effect on Y_1. In other words, the risk of precipitation can be decreased by increasing the Tween 20 content, which was also demonstrated in the contour and three-dimensional response surface plots in Fig. 4(a). The plots show the relationship between the independent variables and DIL by changing color from blue to red as DIL increases. However, the negative coefficients of combined independent variables (X_1X_2, X_1X_3, and X_2X_3) imply an inverse relationship between X_2 and other parameters on Y_1.

3.5. Influence of independent variables on SC (Y_2)

SC is related to the ability to maintain the solubilized form of HL235 in the SMEDDS formulation. Because high SC would prevent drug precipitation and result in high absorption, the SMEDDS formulation was optimized to have a maximized SC (Y_2) value. As shown in Table 3, it is notable that SC increased up to 6.91 mg/mL in the SMEDDS formulation. The linear model was suggested as a statistical fit to the data (Table 4), and the following equation was obtained from the program based on the results of analysis of variance to validate the relationship between the independent variables and SC (Y_2) (Table 5).

$$\text{SC } (Y_2) = +2.47X_1 + 6.36X_2 + 6.86X_3 \quad (2)$$

The magnitude of the coefficient was in the order $X_3 > X_2 > X_1$. The positive value of all coefficients indicated that SC would increase as the oil, surfactant and cosurfactant contents increased. The contour and three-dimensional response surface plots of SC in Fig. 4(b) were also consistent with these results. Moreover, the coefficient values demonstrate that Tween 20 (X_2) and Carbitol (X_3) were more significantly responsible for the SC of the SMEDDS than oil (X_1), which can also be expected from the result of the solubility study in Table 1.

3.6. Optimization of SMEDDS formulation by desirability function

The desirability function of the Design Expert® program was used for optimization of all the responses, where Y_1 and Y_2 were set to be maximized (Table 2). After calculation by the combination of all the polynomial equations mentioned above, the program suggested the independent variables of 5.0% Capmul MCM EP (X_1, oil), 75.0% Tween 20 (X_2, surfactant) and 20.0% Carbitol (X_3, cosurfactant) as the optimized formulation with a desirability value of 0.878 (Fig. 5). The contour and three-dimensional response surface plot of the optimized formulation are shown in Fig. 5. The SMEDDS of the optimized formulation was prepared to validate the D-optimal design model, and the experimentally measured response values were compared with the predicted values (Table 6). The percentage prediction errors of Y_1 and

Table 4
Summary of statistical analyses and model equations for the measured responses.

Response	Model	Sequential p-value	Lack of fit p-value	SD	%CV	PRESS	R^2	Adjusted R^2	Predicted R^2	Adequate precision
DIL (Y_1)	Quadratic	0.0487	0.1596	0.06	3.10	0.069	0.9708	0.9561	0.9327	22.120
SC (Y_2)	Linear	< 0.0001	0.9660	0.12	2.11	0.30	0.9779	0.9745	0.9652	37.055

Table 5
Analysis of variance of measured responses.

Response	Source	Sum of squares	df	Mean square	F-value	P-value prob. > F	Remark
DIL (Y_1)	Model	0.99	5	0.20	66.39	< 0.0001	Significant
	Linear mixture	0.96	2	0.48	160.36	< 0.0001	
	X_1X_2	0.010	1	0.010	3.40	0.0952	
	X_1X_3	2.88×10^{-3}	1	2.88×10^{-3}	0.97	0.3490	
	X_2X_3	0.015	1	0.015	4.90	0.0513	
	Residual	0.030	10	2.982×10^{-3}			
	Lack of Fit	0.022	5	4.304×10^{-3}	2.59	0.1596	Not significant
	Pure Error	8.302×10^{-3}	5	1.660×10^{-3}			
	Corrected total	1.02	15				
SC (Y_2)	Model	8.51	2	4.25	287.41	< 0.0001	Significant
	Linear Mixture	8.51	2	4.25	287.41	< 0.0001	
	Residual	0.19	13	0.015			
	Lack of Fit	0.052	8	6.530×10^{-3}	0.23	0.9660	Not significant
	Pure Error	0.14	5	0.028			
	Corrected total	8.70	15				

Note: df, degrees of freedom.

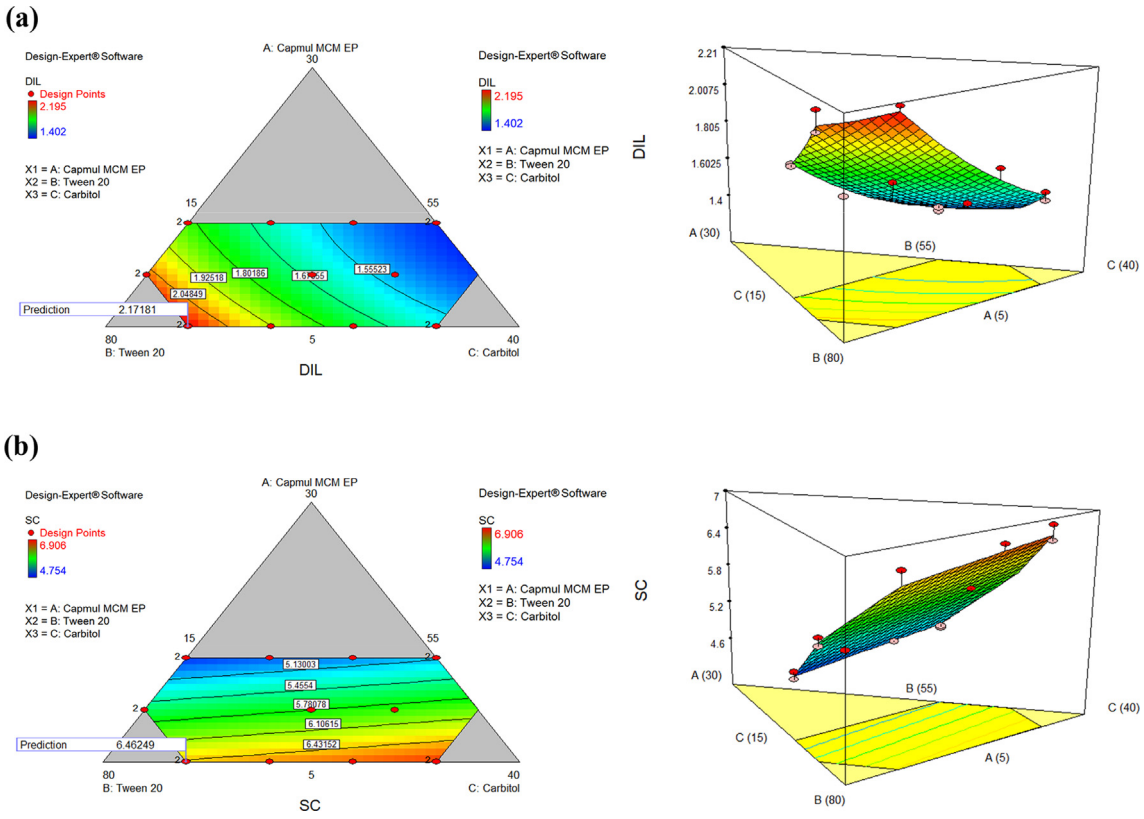

Fig. 4. Contour and three-dimensional response surface plots of (a) DIL and (b) SC.

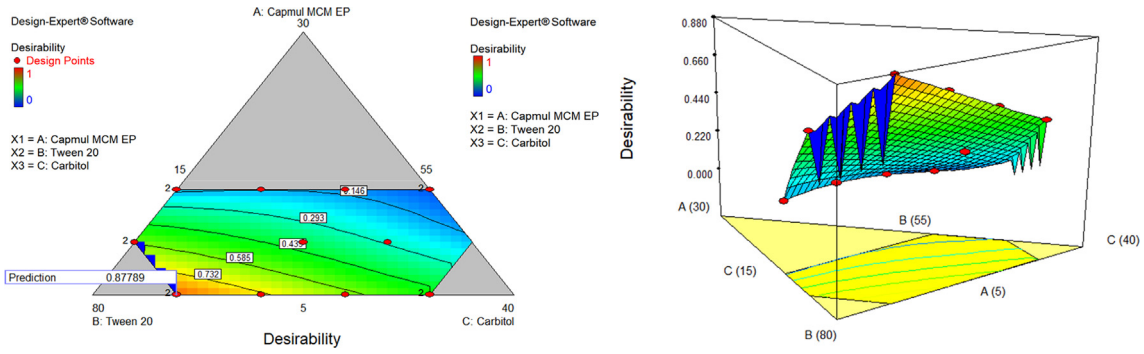

Fig. 5. Contour and three-dimensional response surface plots of optimized HL235-loaded SMEDDS formulation using desirability approach.

Table 6
Predicted and experimental results of optimized HL235-loaded SMEDDS formulation.

Response	Predicted value	Experimental value	Prediction error (%)
DIL (μg/mL; Y_1)	2.17	2.34 ± 0.21	−7.83
SC (mg/mL; Y_2)	6.462	6.164 ± 0.06	4.61

Note: Prediction error (%) was calculated using the formula ([predicted value – experimental value]/predicted value) × 100; values are presented as the mean ± SD (n = 3).

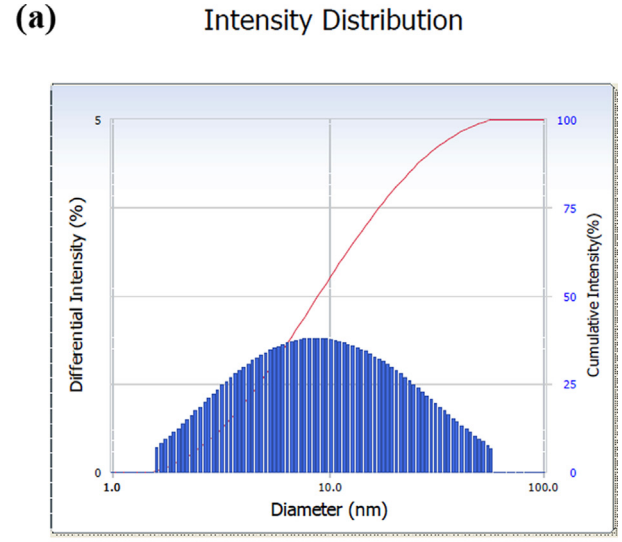

Fig. 6. (a) Droplet size distribution and (b) TEM image of the microemulsions from the optimized HL235-loaded SMEDDS formulation after 50 times dilution with double-distilled water (1:50 dilution). The scale bar represents 200 nm.

Y_2 were −7.83 and 4.61, respectively, suggesting that the D-optimal design successfully optimized the SMEDDS formulation of HL235. Moreover, the dilution stability of optimized SMEDDS was also observed in simulated intestinal fluid (SIF, pH 6.8) (Table S2), indicating

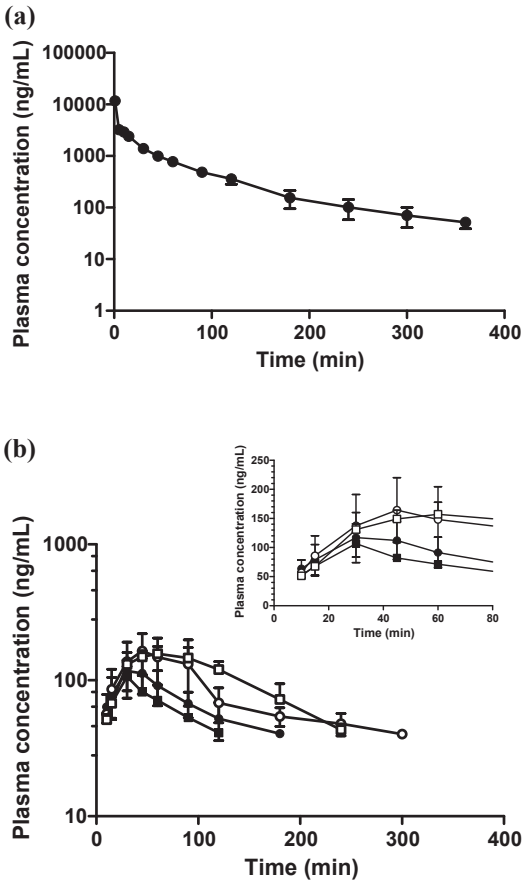

Fig. 7. Plasma concentration profiles of HL235 after (a) intravenous injection of HL235 solution and (b) oral administration of HL235 solution without water (●) and with water (■) or HL235-loaded SMEDDS without water (○) and with water (□) in rats at a dose of 5 mg/kg. For solution, HL235 was dissolved in a mixture of DMSO and PEG400 (8:92, v/v) at 2.5 mg/mL for intravenous injection and oral administration.

that HL235 in the optimized formulation will be stabilized upon dilution throughout the gastrointestinal tract.

The droplet size of the optimized formulation containing 2.5 mg/mL of HL235 was 10.7 (± 1.6) nm with a PDI value of 0.015 (± 0.0), indicating a homogenous size distribution as shown in Fig. 6(a). The morphology of the microemulsions formed from the optimized SMEDDS formulation was also observed by TEM. The image in Fig. 6(b) shows the spherical shape of the emulsion droplets without aggregation. Moreover, the droplet size of the microemulsions in the TEM image (approximately 10 nm) was similar to that measured by the electrophoretic light-scattering spectrophotometer in Fig. 6(a).

The SMEDDS components are common pharmaceutical excipients for oral use. Capmul MCM EP is listed in Generally Recognized as Safe (GRAS), according to Code of Federal Regulations Title 21 (21 CFR184.1505) (FDA, 2018). Also, Tween 20 and Carbitol are safe food additives. Although the content of a surfactant and a cosurfactant is high in the optimized formulation, the amount of Tween 20 (~1.5 mg/kg body weight) is within the acceptable daily intake (ADI) as a food additive for human (0–25 mg/kg body weight), based on World Health Organization (WHO) guideline (Joint et al., 1974; Rowe et al., 2009). In addition, the amount of Carbitol used in this study (~0.4 mg/kg body weight) is within estimated oral permissible daily exposure (PDE) level of 10 mg/kg/day (Sullivan Jr. et al., 2014).

3.7. *In vivo pharmacokinetic studies in rats*

Plasma concentration-time profiles and the pharmacokinetic

Table 7
Pharmacokinetic parameters after intravenous or oral administration of HL235 (5 mg/kg) in rats.

Parameters	Intravenous Injection[1]	Oral administration			
		Solution (without water)[1]	Solution (with water)[1,2]	SMEDDS (without water)	SMEDDS (with water)[2]
C_{max} (ng/mL)	–	122.20 ± 38.80	108.13 ± 18.53	164.27 ± 55.87	162.99 ± 14.00
T_{max} (min)	–	33.75 ± 7.50	33.75 ± 7.50	45.00 ± 0.00	63.75 ± 18.87$^{**,\#\#}$
AUC_{last} (μg·min/mL)	226.71 ± 24.60	11.04 ± 1.77	5.85 ± 2.45	18.87 ± 7.94$^{\#\#}$	22.64 ± 3.67$^{*,\#\#}$
AUC_{inf} (μg·min/mL)	233.45 ± 28.43	15.83 ± 2.36	9.04 ± 2.65	25.78 ± 9.61$^{\#\#}$	29.09 ± 4.95$^{*,\#\#}$
$t_{1/2}$ (min)	93.59 ± 44.86	79.80 ± 45.50	43.42 ± 12.56	110.76 ± 64.75	86.33 ± 19.21
Bioavailability (%)	100	6.78	3.87	11.04	12.46

The data are presented as mean ± standard deviation (n = 4).
[1] HL235 was dissolved in the mixture of DMSO and PEG400 (8:92, v/v) at 2.5 mg/mL.
[2] Rats were promptly given 1 mL of water after oral administration of solution or SMEDDS formulation.
* $P < 0.05$.
** $P < 0.01$ when compared with the "solution without water" group.
$P < 0.01$ when compared with the "solution with water" group.

parameters of HL235 after intravenous and oral administration (5 mg/kg) in rats are shown in Fig. 7 and Table 7, respectively. To mimic the actual clinical situation in which patients take medication with water, rats were immediately given 1 mL of water after oral administration in the "with water" group. Since the plasma concentration of HL235 after oral administration of the suspension (2.5 mg/mL in water) was below the detection limit of HPLC analysis (data not shown), HL235 was dissolved in a mixture of DMSO and PEG400 (8:92, v/v). The $t_{1/2}$ of HL235 was not significantly different among groups in ANOVA test. However, it is interesting to note that the AUC value of the "solution with water" group was lower than that of the "solution without water" group, resulting in 1.75-fold lower bioavailability. This could be due to the precipitation of HL235 in the GI tract, indicating that the dissolution will decrease with water intake by patients. In contrast, pharmacokinetic parameters were not significantly different between "SMEDDS without water" and "SMEDDS with water", indicating that the SMEDDS easily formed a microemulsion in the GI fluid. Additionally, dilution with water intake for medication showed no significant effect on the absorption of HL235. The phenomena could be explained by the different interaction with GI fluid after administration of the solution and the SMEDDS. For the solution, the solubilized drug in the cosolvent system rapidly begun to diffuse into aqueous phase, leading to drug precipitation. On the other hand, the micelles were formed in the initial phase of the dilution of SMEDDS in the GI tract due to the high concentration of surfactants, which is an advantage of SMEDDS to prevent the precipitation of drug. After further dilution progress, the reorientation of the surfactant molecules occurred and oil was covered inside the surfactant layer to form an oil-in-water emulsion (Hauss, 2007). Although the absorption of drug from oral SMEDDS is still unclear, it was reported that the drug entrapped in the oil-in-water emulsions formed by self-emulsifying formulation might release in an unstirred layer and directly penetrate into an intestinal membrane without involving of the bile salt-mixed-micelle transport system (Araya et al., 2006). The most notable result was that the AUC values of SMEDDS were significantly higher than those of the solution groups, which could result from the improvement in the solubility and/or dissolution rate of HL235 by the optimized SMEDDS formulation. Thus, the oral bioavailability of HL235 from the optimized "SMEDDS without water" increased 1.63-fold and 2.85-fold compared to that of the "solution without water" and the "solution with water", respectively. Moreover, the "SMEDDS with water" group showed 1.84-fold and 3.22-fold higher oral bioavailability of HL235 compared to that in the "solution without water" and the "solution with water" groups, respectively. Moreover, hematoxylin and eosin (H&E) staining of rat intestinal epithelia after oral administration of the optimized SMEDDS with and without HL235 were not different from those of the control (double distilled water) group (Fig. S1). Thus, oral intake of HL235 and pharmaceutical excipients including surfactant/cosurfactant in SMEDDS did not cause the irritation in the GI tract and showed no evidence of pathological sign(s). Therefore, the SMEDDS formulation optimized by D-optimal mixture design successfully enhanced the oral absorption of HL235 by improving its solubility and/or dissolution without irritation on epithelium.

4. Conclusions

The SMEDDS formulation of HL235 was successfully optimized by using the D-optimal mixture design. The optimized SMEDDS formulation significantly enhanced the oral bioavailability of HL235 in a pharmacokinetic study in rats. Thus, statistical experimental design is a useful tool to optimize SMEDDS formulation. Additionally, SMEDDS is a promising approach to enhance the oral bioavailability of a poorly water-soluble cathepsin K inhibitor, HL235.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2019.118772.

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