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ORIGINAL ARTICLE

Pre-Formulation Studies of Lipid-Based Formulation Approach for a Poorly Water-Soluble Biopharmaceutics Classification System Class II Model Drug: Bosentan

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ABSTRACT

Objectives: This study aimed to perform pre-formulation studies, formulation development, and formulation optimization for self-nanoemulsifying drug delivery systems (SNEDDS), a lipid-based formulation approach to improve the low solubility of bosentan monohydrate (BOS).

Materials and Methods: Pseudo-ternary phase diagrams were created for pre-formulation studies and formulation design for SNEDDS. The SNEDDS was optimized with BBD. The optimized BOS-loaded SNEDDS formulation was characterized by droplet size (DS), polydispersity index (PDI), dispersibility, an efficiency test of self-nanoemulsification, % transmittance, turbidity, robustness, and the effects of pH, viscosity, and thermodynamic and long-term stability studies. The *in vitro* dissolution studies were performed in distilled water containing 1% sodium lauryl sulfate, which is a Food and Drug Administration-recommended medium, and in biorelevant media. *Ex vivo* studies were conducted in biorelevant media.

Results: The optimum BOS-loaded SNEDDS had a DS of 16.76 nm and PDI of 0.200. The characterization studies satisfied SNEDDS requirements (does not deteriorate when diluted at different pHs; resistant to thermodynamic changes; self-emulsifying within 1 minute; Grade A; and transparent) for both blank and BOS-loaded SNEDDS. In long-term stability studies, it was found to be stable for six months. When *in vitro* dissolution was compared to the performance of the commercial product (Tracleer®), the BOS-loaded SNEDDS showed 2.88, 7.63, 3.83, and 4.23 increases in the percentages of cumulative dissolution in fasted state simulated intestinal fluid (FaSSIF), fed state simulated intestinal fluid (FeSSIF), FaSSIF-V2, and FeSSIF-V2, respectively. The *ex vivo* permeation study showed 12.2-, 19.1-, 20.3-, and 13.1-fold increases in drug permeation in FaSSIF, FeSSIF, FaSSIF-V2, and FeSSIF-V2 for the SNEDDS formulation, as compared to the commercial product, respectively.

Conclusion: Pre-formulation and formulation studies were carried out successfully, and lipid-based optimum BOS-loaded SNEDDS were obtained. The present study confirms the potential of optimum BOS-loaded SNEDDS, which was found to be stable over the long term, to increase the drug's solubility, *in vitro* dissolution, and *ex vivo* permeability. This formulation approach has been promising for further *in vivo* studies, to improve the oral bioavailability of BOS.

Keywords: Bosentan, experimental design, lipid-based formulation, self-nanoemulsifying drug delivery system, SNEDDS, pre-formulation studies

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INTRODUCTION

Many known and newly discovered drug candidates show limited solubility in water, which affects variable bioavailability and poor oral absorption. Advances in oral drug delivery systems can provide formulation options to resolve the solubility limitations of such compounds, thereby enabling the drug to be administered in a form that overcomes poor solubility problems by changing the formulation rather than the molecular structure. Different conventional techniques are used to increase solubility.[1] However, lipid formulations have emerged as a successful new approach to increasing the solubility of poorly soluble drugs belonging to the Biopharmaceutics Classification System (BCS) class II.

Self-nanoemulsifying drug delivery systems (SNEDDS) are one of the most common and commercially applicable lipid-based approaches for poorly water-soluble drugs. This system consists of oil, surfactant, cosurfactant, and active substance, and the agitation of this mixture forms *o/w* nanoemulsions in the gastrointestinal tract. As a result of the small droplet size (DS), the nanoemulsified drug can be effectively taken along the lymphatic path without undergoing hepatic first-pass effects.[2,3]

Bosentan monohydrate (BOS) is a BCS class II drug (solubility water: 1 mg/100 mL, pKa: 5.8, log P: 4.94) for the treatment of pulmonary arterial hypertension (PAH).[4-6] The bioavailability of BOS is approximately 50%. Despite the potential benefit of PAH treatment, the use of BOS is controversial due to its considerable cost.[7] Therefore, it would be crucial for both pharmacoeconomics and treatment to find a dosage form that produces the same or greater therapeutic efficacy with a lower dose.

The design of experiments has recently become common practice in both industry and academia. This systematic approach provides an understanding of the interactions among the variables.[8] Box-Behnken design (BBD) is a 3-factor, 3-level statistical approach used for this purpose. The interaction effects of the amounts of components of the optimum SNEDDS formulation, developed in our study, on the response variables were evaluated using BBD.[9]

Existing micro and nanoemulsion studies in the literature report that formulations formed of varied lipids increased the % cumulative dissolved of BOS because of the enhancement of its solubility.[10,11] This study focused on identifying, formulating, and optimizing different formulation components for oral delivery of BOS *via* SNEDDS, a lipid-based formulation approach, to increase solubility, dissolution, and permeability compared to the commercial product (Tracleer®). For this purpose, a solubility-based screening was conducted for groups of formulation components. Then, pseudo-ternary phase diagrams were created, and formulation combinations were obtained. Researchers continued the studies by selecting the most suitable formulation by analyzing their pseudo-ternary phase diagrams and fundamental formulation characteristics of SNEDDS. The selected formulation was optimized with BBD and examined in terms of physicochemical properties, as well as *in vitro* and *ex vivo* characterization, and stability. It was evaluated whether it could be a candidate for *in vivo* studies.[2,12] This study includes pre-formulation part of the previous studies. After this detailed pre-formulation study, further *in vivo* studies were conducted successfully.

MATERIALS AND METHODS

Materials

Methanol, acetonitrile, triethylamine, and sodium lauryl sulfate were purchased from Merck (Germany). Methanol and phosphoric acid were purchased from Sigma-Aldrich (France). Cremophor® RH 40 was purchased from BASF (Germany). Black seed oil, flaxseed oil, sesame oil, cotton oil, olive oil, and grape seed oil were purchased from ZadeVital® (Türkiye). Corn oil, oleic acid, and sunflower oil were purchased from the Turkish drug market. Imwitor® 988, Imwitor® 948, Miglyol® 812, Miglyol® 818, and Miglyol® 840 were provided as samples by Oleochemicals (Germany). Captex® 355 and Capmul® MCM C8 were provided as samples by Abitec (USA). Propylene glycol dicaprolate/dicaprate Labrafac®, Labrasol®, Capryol® 90, Maisine®, and Peceol® were provided as samples by Gattefossé (France). BOS was provided by Abdi İbrahim (Türkiye). Biorelevant media were purchased from biorelevant.com (UK). All chemicals and reagents were of analytical grade.

Bosentan monohdyrate quantification

BOS was quantified using a ultraviolet (UV)-spectrophotometer (Cary 60 UV-visible, Agilent, Germany) for solubility studies, and an high performance liquid chromatography (HPLC) system from Agilent (1020 Series, Germany) for *in vitro* and *ex vivo* quantification analysis.

Detailed information on UV and HPLC analysis methods has been given in the previous study.[12] The chromatographic separation was performed using the XSelect® HSS C 18, 250x4.6 mm, 5 µm (Waters, Ireland) at 220 nm. The separation was achieved using the mobile phase composed of a buffer solution, prepared by adding 1 mL of triethylamine to 1 L of distilled water and adjusting with phosphoric acid to a pH of 2.5, acetonitrile in a 45:55 *(v/v)* ratio. The flow rate was 1.5 mL/min, and the injection volume was 100 µL.

Solubility studies

Various oils, surfactants, and cosurfactants such as medium-chain triglycerides (Captex® 355, Labrafac®, Miglyol® 812, Miglyol® 818, and Miglyol® 840), medium-chain mono and diglycerides (Capmul® MCM C8, Imwitor® 988, and Imwitor® 948), edible oils (corn oil, sunflower oil, black seed oil, flaxseed oil, sesame oil, cotton oil, olive oil, oleic acid, and grape seed oil), long-chain mono and diglycerides (Maisine® and Peceol®), and propylene glycol ester (Capryol® 90) were investigated and screened for BOS solubility and lipid-based SNEDDS formulation. Solubility studies were performed as described in our previous study.[12] Components of BOS with the highest solubility were selected for pseudo-ternary phase diagram studies (Table 1).

Table 1. Evaluation of different formulation

	Self-emulsifying mixture composition (*w/w* %)				HLB	PDI*	Zeta potential*	Z-average (d.nm)*	Self-emulsification time (sec.)	Dispersibility	Self-emulsion area
	S_{mix}	Oil	Surfactant	Cosurfactant							
F1	1:4	Maisine® 39.0	Labrasol® 12.2	Tween® 20 48.8	10.3	0.628±0.144	-8.44±0.12	434±27	288	Grade D	615.3
F2	1:4	Maisine® 42.2	Labrasol® 11.6	Tween® 80 46.2	9.0	0.751±0.034	-7.16±0.26	764±52	352	Grade D	639.7
F3	1:4	Maisine® 40.1	Solutol® HS 15 12.0	Tween® 20 47.9	10.2	0.713±0.148	-5.88±0.17	468±23	136	Grade D	504.3
F4	1:4	Maisine® 42.0	Solutol® HS 15 11.6	Tween® 80 46.4	9.1	0.972±0.006	-6.56±0.80	571±3	523	Grade D	434.3
F5	4:1	Capryol® 90 33.3	Tween® 20 53.3	Transcutol® HP 13.3	11.1	0.499±0.005	-5.15±0.19	165±2	70	Grade A	684.4
F6	4:1	Capryol® 90 22.2	Labrasol® 62.2	Tween® 20 15.6	12.5	0.524±0.009	-1.53±0.24	95±4	36	Grade C	550.2
F7	4:1	Capryol® 90 22.2	Labrasol® 62.2	Tween® 80 15.6	12.2	0.624±0.207	-0.47±0.05	156±53	28	Grade C	437.7
F8	4:1	Capryol® 90 22.2	Solutol® HS 15 62.2	Tween® 20 15.6	13.1	0.134±0.004	-3.05±0.15	12±0.3	177	Grade A	473.9
F9	9:1	Peceol® 10.1	Cremophor® RH 40 80.9	Labrasol® 9.0	13.5	0.149±0.008	-6.86±	14±0.2	60	Grade A	788.5

*n=3, HLB: Hydrophilic-lipophilic balance, PDI: Polydispersity index, S_{mix}: Surfactant: cosurfactant ratio, Z-average: Droplet size diameter, sec: Second, d.nm: Diameter values in nanometers

Construction of pseudo-ternary phase diagram and selection of SNEDDS formulation

The oils (Maisine®, Peceol®, and Capryol® 90), a mixture of surfactants (Solutol® HS 15, Tween® 20, Labrasol®, and Cremophor® RH 40) with cosurfactants (Tween® 20, Tween® 80, Labrasol®, and Transcutol® HP) at different ratios, were mixed at weight ratios of 9:1-1:9, according to the solubility study results. The optimum surfactant: cosurfactant ratio (S_{mix}) was determined. The pseudo-ternary phase diagram was constructed with a homogeneous mixture of SNEDDS components and water. The endpoint of the transparency-to-turbidity transition was noted and determined by identifying the nanoemulsifying area.[13] The candidate system was examined for specific SNEDDS formulation properties such as hydrophilic-lipophilic balance (HLB), polydispersity index (PDI), zeta potential, DS, self-emulsification time, and dispersibility, based on the center of the obtained pseudo-ternary phase diagrams. According to the lipid formulation classification system (LFCS), a candidate formulation was selected by considering the properties that SNEDD system formulations should have, such as Type IIIB formulations containing that contain relatively limited amounts of glyceride lipid (<20%) and larger quantities (20-50%) of hydrophilic components (HLB>12). These formulations do not undergo significant phase changes or potential loss of solvent capacity upon aqueous dilution and have a narrow DS (50-100 nm).[1] After the candidate formulation was optimized with BBD, characterization studies were carried out for the resulting formulation.

Experimental design

Design Expert® V10 (Stat-Ease Inc., MN) was used to evaluate design parameters with a 3-factor, 3-level BBD. Formulation components, their main effects, and interaction effects were investigated. As determined by BBD, 15 experimental studies (containing three central points) were studied to define the precision of the design, assess experimental error, and optimize the performance of the SNEDDS. The program determined the low, middle, and high levels of the independent variables low, middle, and high levels. The levels (3-levels) coded as -1 (low), 0 (middle), and +1 (high) for each independent variable (3-factors) were chosen from the self-nanoemulsification area in the pseudo-ternary phase diagram. The amounts of oil (Peceol®) (X1), surfactant (Cremophor® RH 40) (X2), and cosurfactant (Labrasol®)

(X3) were chosen as independent variables, while particle size (Y1) and PDI (Y2) were the dependent variables. The response surface methodology was used to determine the ideal formulation composition. The models were validated by the multiple correlation coefficients (R^2), the lack of fit tests, and the analysis of variance (ANOVA) tests.

Preparation of lipid-based formulations

BOS was dissolved in the optimum SNEDDS formulation (Peceol®, Cremophor® RH 40, and Labrasol® were selected as oil, surfactant, and co-surfactant, respectively) obtained with BBD. The components were mixed at 37 ˚C until a homogeneous preparation was obtained.

Characterization of SNEDDS

PDI and DS

The PDI and DS were examined after diluting 1 mL formulation with 250 mL of distilled water at 25±0.5 ˚C. All the PDI and DS measurements were carried out by Malvern (Nano ZS, Malvern Instruments, UK).

Efficiency test of self-nanoemulsification

Self-emulsification time and formulation dispersibility are measured to characterize the spontaneous formation of SNEDDS.[14] For dispersibility, SNEDDS formulations were added to 500 mL of water at 37±0.5 ˚C, at 50 rpm. For self-emulsification time, SNEDDS formulations were added to 250 mL of water under the same conditions. The formulations' final appearance and time of emulsification are evaluated by a grading system.[15]

% transmittance and turbidity

The transmittance studies of the optimum SNEDDS formulations were conducted at 638 nm using a UV spectrophotometer.[16] The transparency of the formulations is verified if the % transmittance is greater than 99%.

For the optimum SNEDDS formulation and self-nanoemulsion, turbidity measurements were carried out at 25±0.5 ˚C, with readings from 0 to 200 NTU.

Viscosity

The viscosity was measured and evaluated as described in our previous study.[12] The SNEDDS formulation (0.5 mL) was placed in a Brookfield viscometer (DV III+Rheometer, USA). The viscosity studies were carried out in triplicate.

Robustness and pH effect

The effect of volume and pH change on the DS of the aqueous medium used to dilute the optimum SNEDDS was examined. The optimum SNEDDS formulation was diluted in 1:100, 1:250, and 1:500 ratios in aqueous media without enzyme at pH 1.2 and 6.8.

Morphological imaging

Transmission electron microscopy (TEM) was used to image the droplet's morphology. Distilled water was used to dilute the formulation. It was then stirred with a magnetic stirrer, and a drop of the sample was placed on a copper grid with a phosphotungstic acid solution (1% *w/v*) for 5 min at room temperature (FEI, USA).[17]

Thermodynamic and long-term stability studies

The studies were carried out for heating-cooling cycles (3 cycles, 4˚C - 45˚C), centrifugation (at 3500 rpm for 30 min), and freeze-thaw (3 cycles, -21 ˚C - 25 ˚C). Each cycle should be at least 48 hours for each of the heating-cooling and freezing-thawing cycles. After the successful thermodynamic studies, the optimum formulation was evaluated with PDI and DS.

The physical and chemical stability of the optimum formulation was evaluated under different storage conditions, namely 4˚C, 25±2˚C/60±5% relative humidity (RH), and 40±2 ˚C/75±5% RH. The optimum formulation was evaluated at 0, 1, 3, and 6 months for physical appearance, PDI, and DS.

Dissolution study

The *in vitro* dissolution study was conducted in United States Pharmacopeia apparatus II (Variant, USA) at 37±0.5 ˚C and 50 rpm in 900 mL of dissolution medium. The 1% SLS in distilled water, which is Food and Drug Administration (FDA)-recommended media (before the updated 09/15/2023 revision in the FDA dissolution database), and biorelevant media were used as media. Based on satueferation solubility, the relative sink condition was calculated for the dissolution study. This evaluation was conducted for the FDA-recommended medium only. To prepare a supersaturated solution, an excess dose of BOS and 10 mL of dissolution medium were added to vials (at 37 ˚C, n=3). Samples were taken from the vials at the end of 24 hours and analyzed with a UV spectrophotometer. The relative sink condition values of the FDA medium, 1% SLS containing distilled water, were evaluated at the 24th hour under the temperature conditions of the dissolution studies 37 ˚C. The ratio of saturation solubility to drug concentration (CS/CD) was calculated by dividing the dose by 900 mL of dissolution medium to represent the sink condition for commercial products and SNEDDS formulation. One g of SNEDDS formulation was placed in a Capsugel® capsule (00el, Belgium), which was placed in a sinker that would prevent it from floating, then left in the dissolution medium. The samples (5 mL) were withdrawn at 0.25, 0.5, 0.75, 1, and 1.5 h. They were filtered and analyzed at 220 nm by HPLC (1220 LC Agilent, Germany).[12] The dissolution profiles of commercial products (125 mg) and optimum SNEDDS formulation (28 mg) were compared and evaluated by the DDSolver® (with similarity factor) (f2).

Ex vivo permeability studies

Ex vivo permeability studies used a Franz diffusion cell (diffusional area: 1 cm^2, cell chamber capacity 2.5 mL). Biorelevant media and goat intestine membranes were used for the study. One mL of optimum SNEDDS and commercial product suspension were used, and the study was carried out at 37 ˚C for 10 h. The samples were analyzed using HPLC at 220 nm.[12] The steady-state section of the permeation profile and the ratio of concentration to flux in the donor chamber were used to calculate the permeability coefficient (*P*) and flux values, respectively.

Flux (J, µg. cm-2. h-1) is the amount of active substance penetrating a unit area per unit of time. Equation 1 was used to determine the slope of the linear portion in the graph showing the quantity of BOS that permeated the acceptor compartment vs. time.

$$J=dQ/A\,dt \qquad \text{(Equation 1)}$$

Q is the amount of penetrating drug, A is the area of the tissue/membrane, and t is time.

Statistical analysis

Student *t-tests* and one-way ANOVA were used for statistical analysis. The significance of the difference, which is at a 0.05 probability level, was assessed using IBM® SPSS® 22.

RESULTS

Solubility studies

The oil with a higher solubilization capacity of the drug has a higher drug loading potential.[18,19] In Figure 1, BOSs solubility was significantly higher in synthetic oils than in other edible oils, showing high solubility in medium-chain mono/diglyceride and long-chain mono/diglyceride groups.

Construction of pseudo-ternary phase diagram and selection of SNEDDS formulation

The red areas in the pseudo-ternary phase diagram represent the areas where a system without any active substance forms a self-nanoemulsion, as defined by water titration (Figure 2). The most suitable combinations for each SNEDD system were determined by trying ratios from 9:1 to 1:9.

The HLB values are a critical factor in the formulation of SNEDDS. Based on the HLB values, suitable components for the *o/w*, (required HLB values: 8-18) emulsion were selected, and the total HLB values for SNEDD systems were calculated (Table 1). PDI, zeta potential, DS, self-emulsification time, and dispersibility characterizations were made for nine different SNEDDS combinations based on the centers of the obtained areas (Table 1).

Experimental design

The BBD program presented 15 formulations based on the experimental design. The influence of independent variables on dependent variables was examined (Table 2). The best-fit models were found for the SNEDDS PDI and DS dependent variables. The correlation coefficients of the equations were evaluated using experimental values and were found to fit the data well, yielding the R^2 values present in Table 3. The 'Prob>*f*' values must be less than 0.05 for the model terms to be significant ($p<0.05$ in all cases) (Table 4).

Tables 3 and 4 contain the model that fitted the experimental data and the ANOVA results for DS and PDI of SNEDDS. The DS responses for SNEDDS formulation were evaluated, and the 2-factor interaction (2 FI) model was selected by Design Expert® as the appropriate model. In the model, the terms A, B, C, AB, and AC were significantly effective ($p<0.0001$). For the PDI responses in the Peceol formulation, the terms A, B, and AB were found to be significant for SNEDDS. The contour and surface graphs of the independent factors statistically impacted the DS responses (Figures 3a and 3b for A, B, and AB and Figures 3c and 3d for A, C, and AC).

For the optimum SNEDDS from the F9 SNEDDS formulation, the PDI was found to be 0.149±0.008. The DS was 14.44±0.21 nm (Figure 4). Twenty-eight milligrams of BOS could be dissolved in 1 gram of the SNEDDS formulation. Other characterization

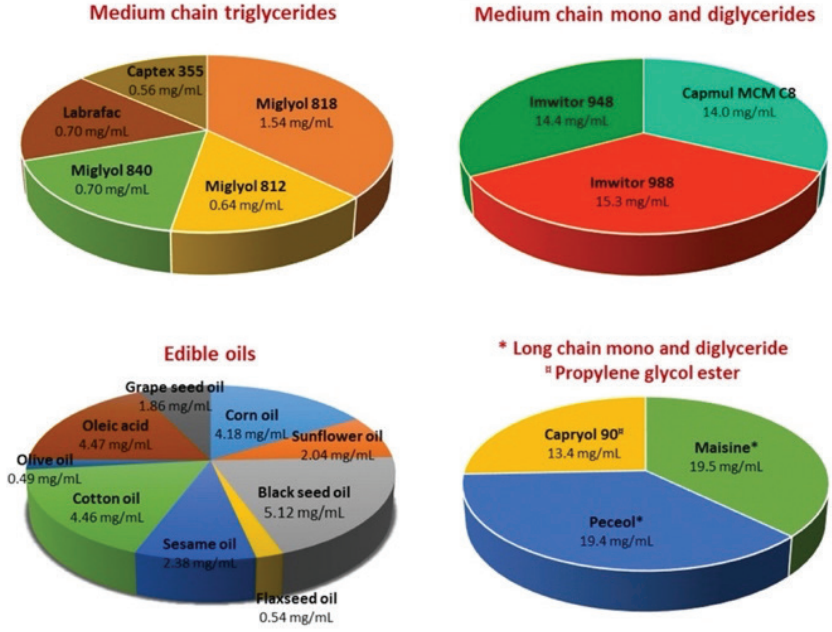

Figure 1. Solubility of BOS in different oil groups

BOS: Bosentan monohydrate

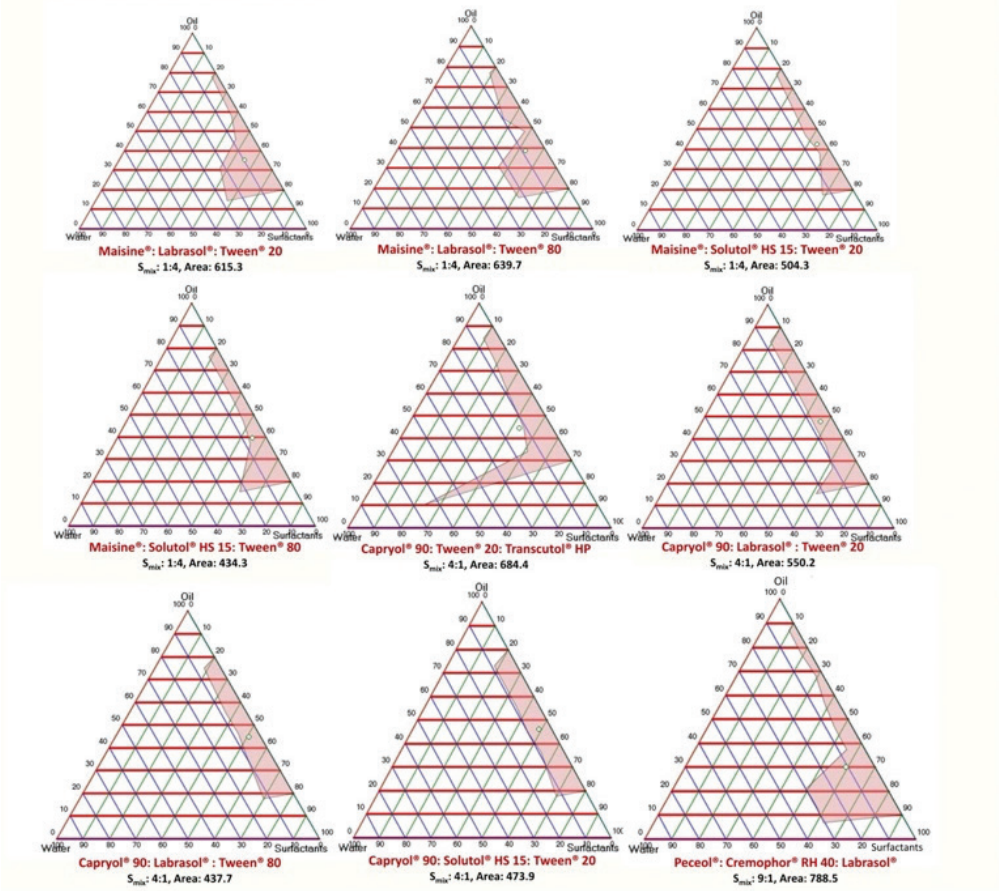

Figure 2. The pseudo-ternary phase diagrams of different oils, surfactants, and cosurfactants

Table 2. Variables used in the Box-Behnken design for optimum SNEDDS formulation

	Levels, actual (coded)		
	Low (-1)	**Medium (0)**	**High (+1)**
Independent variables			
X_1: Amount of Peceol added (g)	0.9	1.45	2
X_2: Amount of Cremophor RH 40 (g)	4.5	5.85	7.2
X_3: Amount of Labrasol (g)	0.5	0.65	0.8
Dependent variables			
Y_1: Droplet size (nm)		<100 nm	
Y_2: PDI		<0.2	

PDI: Polydispersity index, SNEDDS: Self-nanoemulsifying drug delivery systems

Table 3. Model fitting for DS and PDI of optimum SNEDDS formulation

	BFM	**Lack of fit**	**R^2**	**Adjusted R^2**	**Predicted R^2**
DS model	2FI	0.1424	0.9985	0.9973	0.9928
Equation	Y_1=87.26+62.28*A-41.04*B+9.23*C 27.46*AB+9.10*AC				
	BFM	**Lack of fit**	**R^2**	**Adjusted R^2**	**Predicted R^2**
PDI model	Quadratic	0.7479	0.9878	0.9659	0.9059
Equation	Y_2=0.95+0.28*A-0.11*B+0.17*AB				

Pred R^2: Predictive R^2, Adj R^2: Adjusted R^2, BFM: Best fitted model, DS: Droplet size, PDI: Polydispersity index

Table 4. Statistical analysis of the fitted model for DS and PDI of optimum SNEDDS formulation

Dependent variables	DS (Y_1)			PDI (Y_2)		
	Independent variables	*f* value	*p* value	Independent variables	*f* value	*p* value
	A	3312	<0.0001	A	204	<0.0001
	B	1438	<0.0001	B	29.4	0.0029
	C	72.7	<0.0001	C	2.53	0.1723
	AB	322	<0.0001	AB	39.0	0.0015
	AC	35.3	0.0003	AC	0.19	0.6798
	BC	4.99	0.0560	BC	2.73	0.1593
				A2	104	0.0002
				B2	0.85	0.3999
				C2	27.3	0.0034
Model ANOVA						
	f value:	864		*f* value:	45.1	
	R^2:	0.9985		R^2:	0.9878	
	p:	<0.0001		p:	0.0003	

DS: Droplet size, PDI: Polydispersity index, SNEDDS: Self-nanoemulsifying drug delivery systems, ANOVA: Analysis of variance

studies were carried out on the BOS-loaded optimum SNEDDS formulation.

Characterization of SNEDDS

PDI and DS

All formulations developed according to BBD were confirmed to be in the nanometer sizes, with DS ranging from 15.9 to 215 nm and PDI ranging from 0.121 to 1.000. The BOS-loaded SNEDDS formulation was diluted 250-fold with distilled water, and the PDI and mean DS were found. The PDI for the BOS-loaded SNEDDS formulation prepared with the optimum formulation was 0.200±0.025, and the DS was 16.76±1.78 nm (Figure 4).

Efficiency test of self-nanoemulsification

The formulation created a clear and fine system with an emulsification time of less than 1 minute. The results are shown in Table 5. The BOS-loaded SNEDDS formulation passed this test in Grade A, and it is hypothesized that it will form nanoemulsions when dispersed in GI fluid.

% transmittance and turbidity

Evaluated SNEDDS formulation showed a transmittance of over 99%, confirming the nanoemulsification efficiency of the SNEDDS.[20] Turbidity results are given in Table 5.

Viscosity

The SNEDDS formulation result is shown in Table 5.

Robustness and pH effect

Diluting the SNEDDS formulation 100, 250, and 500 times with distilled water and pH 1.2 and pH 6.8 phosphate buffer, which simulate the gastrointestinal system, revealed the effect of robustness and dilution media (Table 5).[21] Precipitation was not seen in either alkaline or acidic environments, indicating that the formulation is stable in dispersion.

Morphological imaging

The spherical shapes ranging from 10 to 100 nm were visualized using TEM pictures (Figure 5).

Thermodynamic and long-term stability studies

The SNEDDS formulation passed the heating-cooling cycle, centrifugation study, and freeze-thaw test. The DS and PDI results for the SNEDDS formulation are shown in Table 6. The study indicated that the SNEDDS formulation showed no precipitation or phase separation.[22]

The SNEDDS formulation was found to be physically stable for six months at 4 °C, 25±2 °C/60±5% RH, and 40±2 °C/75±5% RH (Table 6).

Dissolution studies

The dissolution profiles of SNEDDS formulation (28 mg BOS) and commercial products (Tracleer® 125 mg film tablet) were compared under conditions of 1% SLS in distilled water and biorelevant media (Figure 6).

The relative sink condition values (CS/CD) for the distilled water medium containing 1% SLS, which is the dissolution medium recommended by the FDA, were found to be greater than 3 for both the commercial product (10.3) and Peceol® SNEDDS formulation (47.6). The C_S/C_D values greater than 3 are considered to provide sink conditions.[23]

In the distilled water with 1% SLS, more than 80% of the BOS was released from the formulation and commercial product after 15 min, and 100% release was obtained from both within 30 min. In addition, for the SNEDDS formulation, more than

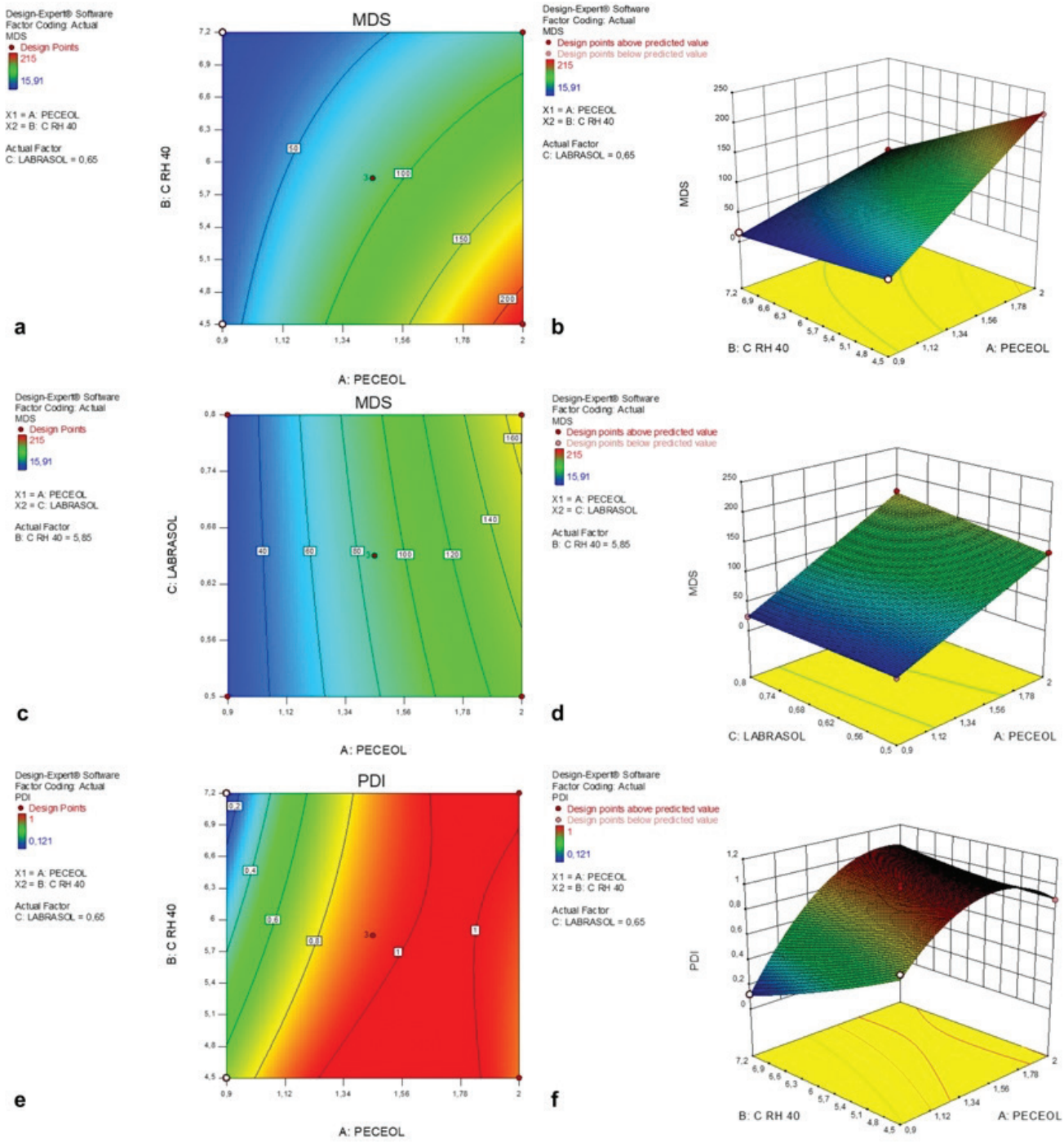

Figure 3. a) Surface and b) contour graphs of Peceol® and Cremophor® RH 40 for droplet size, c) Surface and d) contour graphs of Peceol® and Labrasol® for droplet size, e) Surface and f) contour graphs of Peceol®, Cremophor® RH 40 and Peceol®-Cremophor® RH 40 for PDI

MDS: Mean diffusional size, PDI: Polydispersity index, RH: Relative hydrophobicity

80% of the releases were obtained in 30 minutes for FaSSIF, FeSSIF, and FeSSIF-V2, while the release in FaSSIF-V2 was 77% in 30 minutes and exceeded 80% at the end of 90 minutes. However, within 90 minutes, the commercial products were able to release almost 32%, 11%, 22%, and 2% in FaSSIF, FeSSIF, FaSSIF-V2, and FeSSIF-V2, respectively.

SNEDDS formulation and commercial product dissolution profiles did not give similar dissolution curves with f_2=14 for FaSSIF, f_2=9 for FeSSIF, f_2=14 for FaSSIF-V2, and f_2=9 for FeSSIF-V2.

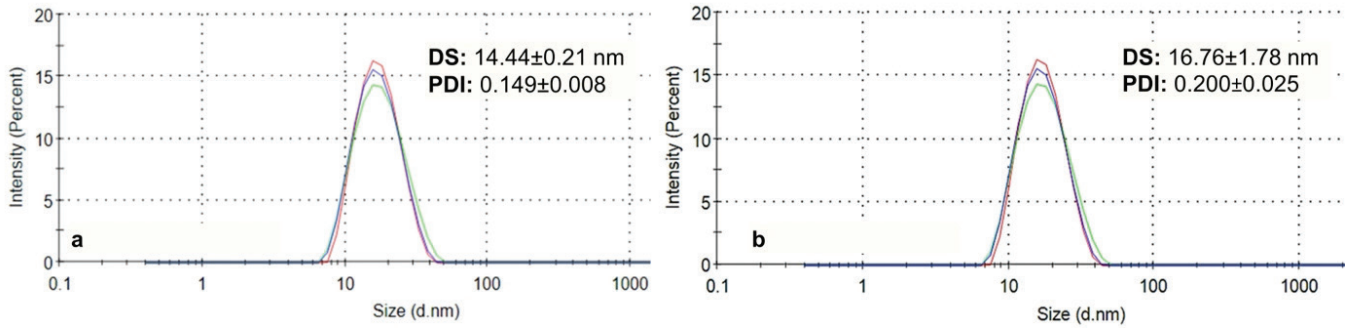

Figure 4. Droplet size and droplet size distributions (PDI) of optimum SNEDDS formulation and BOS-loaded SNEDDS formulation
BOS: Bosentan monohydrate, PDI: Polydispersity index, SNEDDS: Self-nanoemulsifying drug delivery systems

Table 5. Characterization of BOS-loaded SNEDDS formulation

Dispersibility	Self-emulsification time (sec.)	Transmittance %	Turbidity (NTU)	Viscosity (cP)
Grade A	54±6	No dilution 99.9±0.1	No dilution 12.4±0.2	474
		250-time dilution 99.6±0.0	250-time dilution 10.9±0.3	

Robustness and effect of pH		DS (nm)	PDI
Distilled water	1:100	15.4±1.2	0.173±0.030
	1:250	16.8±1.8	0.200±0.025
	1:500	25.2±10.9	0.178±0.051
pH 1.2	1:100	18.0±1.4	0.205±0.026
	1:250	27.0±15.4	0.201±0.057
	1:500	34.5±20.9	0.225±0.035
pH 6.8	1:100	16.0±0.8	0.128±0.045
	1:250	17.0±1.3	0.165±0.032
	1:500	21.0±1.2	0.230±0.017

*Mean ± standard deviation, BOS: Bosentan monohydrate, SNEDDS: Self-nanoemulsifying drug delivery systems, NTU: Nepholometric turbidity units, cP: Centipoise, DS: Droplet size, PDI: Polydispersity index

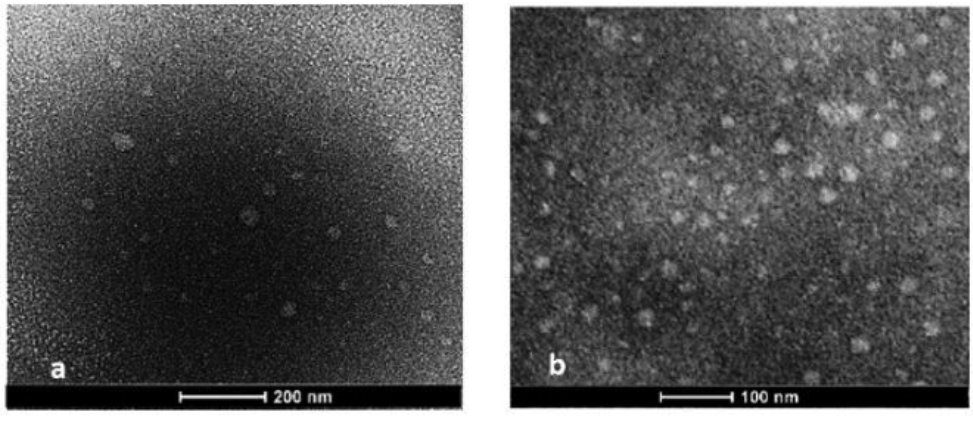

Figure 5. Morphology of a) optimum SNEDDS formulation and b) BOS-loaded SNEDDS formulation
BOS: Bosentan monohydrate, SNEDDS: Self-nanoemulsifying drug delivery systems

Table 6. Thermodynamic and long-term stability studies of BOS-loaded SNEDDS formulation

	Thermodynamic stability			Long term stability			
	DS (nm)*	PDI*		Time (month)	PA	DS (nm)*	PDI*
CS	15.4±0.7	0.136±0.027	4 °C	0	Clear	14.4±0.2	0.149±0.008
				1	Clear	23.2±6.9	0.186±0.043
				3	Clear	17.2±2.8	0.202±0.044
				6	Clear	17.0±1.2	0.176±0.063
Heating cooling	16.7±1.9	0.175±0.080	25±2 °C 60±5% RH	0	Clear	14.4±0.2	0.149±0.008
				1	Clear	24.3±2.3	0.190±0.072
				3	Clear	15.4±0.2	0.154±0.043
				6	Clear	18.3±1.9	0.208±0.058
Freeze thaw	16.6±1.8	0.229±0.038	40±2 °C 75±5% RH	0	Clear	14.4±0.2	0.149±0.008
				1	Clear	18.0±3.3	0.201±0.031
				3	Clear	15.2±0.6	0.128±0.039
				6	Clear	16.0±1.1	0.141±0.065

*Mean ± standard deviation, DS: Droplet size, PDI: Polydispersity index, CS: Centrifugation study, PA: Physical appearance, BOS: Bosentan monohydrate, SNEDDS: Self-nanoemulsifying drug delivery systems, RH: Relative hydrophobicity

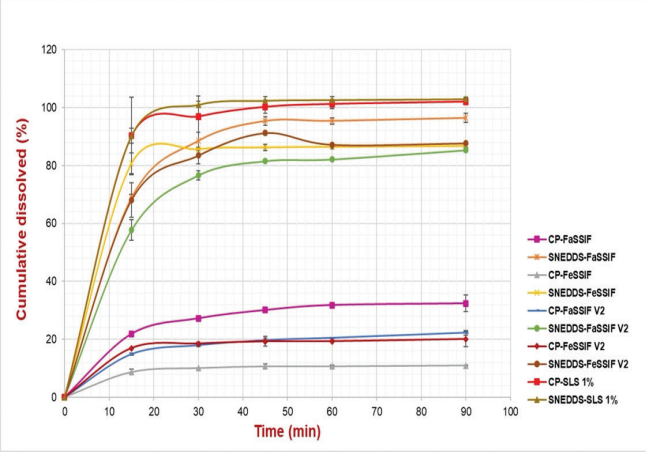

Figure 6. *In vitro* dissolution profiles of commercial product and BOS-loaded SNEDDS for 1% SLS in distilled water, FaSSIF, FeSSIF, FaSSIF-V2, and FeSSIF-V2 (n=3, mean ± SD)

CP: Commercial product, SD: Standard deviation, BOS: Bosentan monohydrate, SLS: Sodium lauryl sulfate, FaSSIF: Fasted state simulated intestinal fluid, FeSSIF: Fed state simulated intestinal fluid

Ex vivo permeability studies

The results of the SNEDDS formulation and the commercial product are shown in Figure 7. Compared to the commercial product, the SNEDDS formulation increased drug permeability by 12.2, 19.1, 20.3, and 13.1-fold in FaSSIF, FeSSIF, FaSSIF-V2, and FeSSIF-V2, respectively.

DISCUSSION

BOS is a weakly acidic drug, and pH is very important for such substances.[24] While BOS is less soluble in water: low pH aqueous solutions, its solubility increases specifically as pH increases (pH 1.1 and 4.0: 0.001 mg/mL, pH 5.0: 0.002 mg/mL, and pH 7.5: 0.43 mg/mL).[25] For solubility studies, the high solubility of the drug in the synthetic oil phase allowed less use of surfactants and cosurfactants, which are other components required for the system. The use of fewer surfactants and cosurfactants reduced the toxicity associated with these substances.

According to the solubility results, the construction of the pseudo-ternary phase diagram was carried out to identify the self-nanoemulsifying area for the SNEDDS.[26] Transparent isotropic areas are regions where nanoemulsions spontaneously form. S_{mix} ratios must be high to maintain droplet stability and to obtain smaller droplets, thus increasing the surface area.[27] The elevated S_{mix} ratios enhance water uptake capacity and maximize the nanoemulsion area for SNEDDS. Also, the choice of constituents in an SNEDDS is based on both HLB values and solubility. It is known that surfactants and cosurfactants with an HLB of 12-15 generally show better self-nanoemulsification.[28] For all these, the most suitable candidate SNEDDS formulation, which meets the requirements of being an SNEDDS according to LFCS (DS<100 nm; 0.200<PDI; dispersibility: Grade A) and also considering the size of the self-emulsification areas, was determined to be the optimum formulation,

recognized as the F9 SNEDD formulation (Table 1).[1,29,30] Optimization studies with BBD experimental design were continued with the F9 formulation. F9 formulation consists of Peceol® (HLB=1), Cremophor® RH 40 (HLB=15), and Labrasol® (HLB=14), serving as the oil, surfactant, and cosurfactant, respectively.

The experimental design study aimed to minimize DS (<100 nm) and PDI (<0.2) for F9 SNEDDS formulation, to obtain optimal SNEDDS. BBD has been proposed to study the relationship

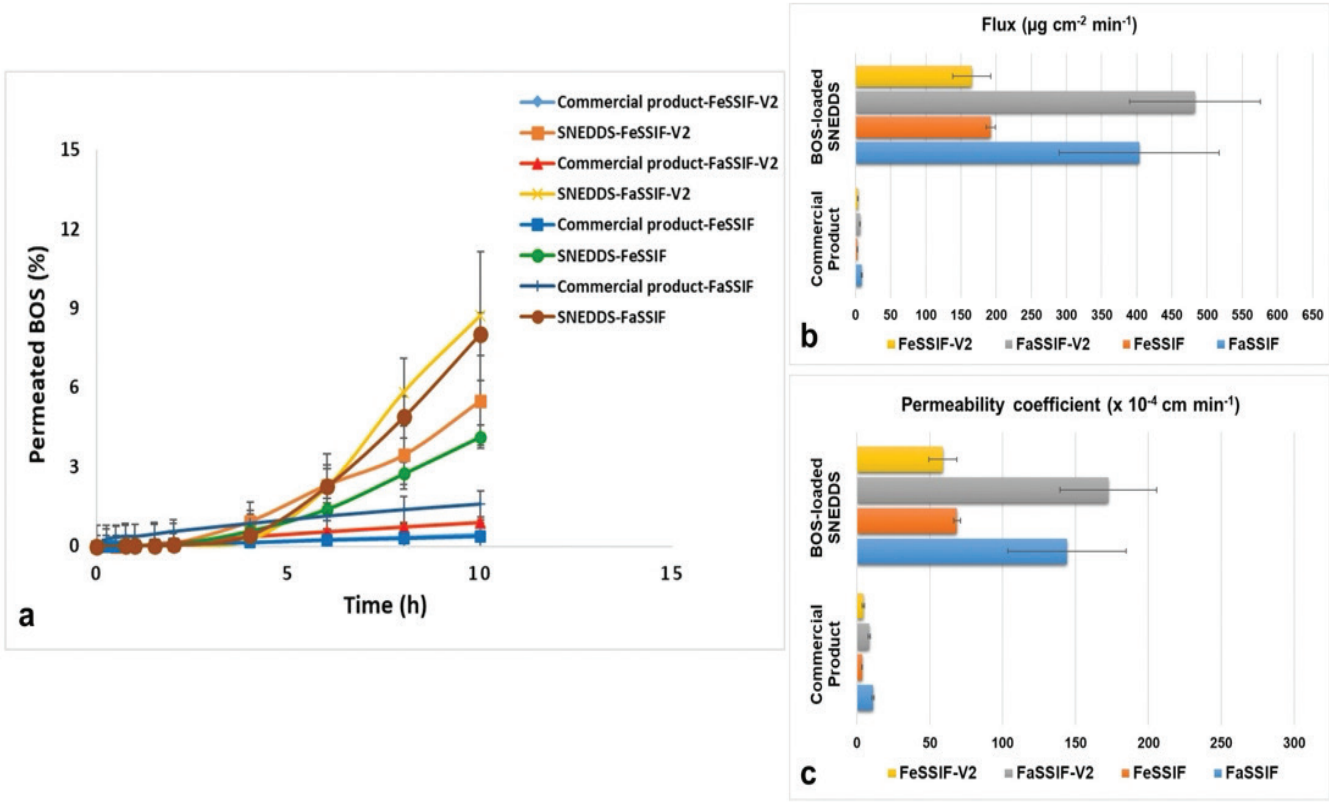

Figure 7. a) *Ex vivo* permeation profiles of commercial product and SNEDDS, b) flux values calculated from the permeation-time profiles of BOS across goat intestine membrane, c) permeability coefficients calculated from the permeation-time profiles of BOS across goat intestine membrane in FaSSIF, FeSSIF, FaSSIF-V2, and FeSSIF-V2 (n=3)

BOS: Bosentan monohydrate, FaSSIF: Fasted state simulated intestinal fluid, FeSSIF: Fed state simulated intestinal fluid, SNEDDS: Self-nanoemulsifying drug delivery systems

between dependent and independent factors, along with experimental parameters and response variables, and has been used successfully in many studies.[29] Another advantage of BBD is that it includes combinations in which all variables are simultaneously examined at their highest or lowest values.

In SNEDDS formulations, the DS should be smaller than 100 nm. In the evaluation of the graphs in Figure 3a and 3b, with the quantity of Labrasol, the cosurfactant, kept constant, the effect of Peceol®-Cremophor® RH 40 interaction on the DS was examined. It was observed that DS decreased when the amount of Peceol® selected as oil was decreased and the amount of Cremophor® RH 40 used as a surfactant was increased. The effect of the interaction of the quantity of Peceol®-Labrasol® on the DS was investigated by keeping the surfactant Cremophor® RH 40 constant (Figures 3c and 3d). It was observed that smaller DSs were obtained by decreasing the amount of Peceol® selected as oil while increasing the amount of Labrasol®, which has a relatively minor effect on the formulation, used as cosurfactant. Figures 3e and 3f, corresponding to the variables A, B, and AB, show the surface and contour graphs of the independent variables that have a statistically significant effect on the PDI responses in the model equation proposed by the software as a result of the design. When the impact of the interaction of Peceol®-Cremophor® RH 40 levels on PDI was evaluated, it was observed that as the quantity of Peceol® decreased, the DS reduced, while the quantity of Cremophor® RH 40, used as a surfactant enhancer, increased. The intended characterization features for developing the SNEDDS formulation were DS<100 nm and PDI<0.2. The test results were reported in a 95 percent confidence interval based on the best results from the formulation's characterization properties, the model results, and the experimental design.

The emulsion's PDI and DS are significant determinants in self-emulsification since they specify the rate and extent of *in vivo* drug release. As the DS of the nanoemulsion gets smaller, the surface area will increase, and the increased surface area will enhance the bioavailability of the drug, which has poor water solubility.[31] However, the volume of liquid in the stomach and its pH vary between individuals. It is essential to ensure that homogeneous nanoemulsions are formed at different dilution conditions, and the SNEDDS should disperse quickly and completely without precipitation when subjected to aqueous dilution under the agitation of GIT due to peristaltic activity.[30] Furthermore, there was a strong relationship between the SNEDDS' visual appearance and the formulation's turbidity. With smaller DSs, transparent systems exhibit a higher transmittance

percentage. Furthermore, the lower viscosity of the system indicates that SNEDDS has a propensity to create an oil-in-water *(o/w)* type nanoemulsion.The SNEDDS characterization studies have confirmed that transparent systems with suitable viscosity, small size, and homogeneous distribution, which are resistant to different dilution conditions, were obtained.

According to thermodynamic stability studies, stable emulsion formulations may withstand centrifugal stress and a wide range of temperature fluctuations without leading to drug precipitation or phase separation.[32] No change in the physical appearance of SNEDDS formulation was observed during the stability studies. The SNEDDS formulation remained transparent with no turbidity or Precipitation. The PDI and DS were similar in all the storage conditions for the SNEDDS formulation. It can be concluded that the SNEDDS formulation would stay stable at 25±2 ˚C/60±5% RH for long-term stability conditions, and at 4 ˚C and at 40±2 ˚C/75±5% RH for accelerated conditions, for six months.

In drug development, the quality of the drug product is utilized to evaluate how the formulation parameters would affect bioavailability, with *in vitro* dissolution tests being the most important measurement of performance.[33] When employed to examine solubility and dissolution, the classic dissolution media in the pharmacopeia provides useful information. However, these media are inadequate in reflecting physiological conditions, especially with BCS Class II poorly water-soluble drugs.[23]

Aqueous solutions of poorly water-soluble drugs such as BOS do not reach sufficient solubility in the physiological pH 1.2-6.8 range. BOS is a BCS Class IIa active substance with pH-dependent solubility and acidic properties.[34] A weak acid's saturation solubility, and consequently the rate and amount of dissolution, can be significantly influenced by the rate at which it ionizes. Weakly acidic drugs have low solubility in the stomach, and their solubility in the intestine increases with increasing pH. Because of their composition, biorelevant media produce more consistent results for simulating *in vivo* conditions. BCS Class II drugs have better results in *in vitro* dissolution compared to compendial media.[35]

The reason for the lower release of BOS, an active substance of BCS Class II, in the FeSSIF and FeSSIF-V2 media is that the active substance exhibits poor acidic properties. The pH values of FeSSIF (pH 5.0) and FeSSIF-V2 (pH 5.8) are less than FaSSIF (pH 6.5), and FaSSIF-V2 (pH 6.5).[36] The SNEDDS formulation, in comparison to the commercial product (Tracleer®), enhanced the percent cumulative dissolution by 2.98, 7.88, 3.84, and 4.37-fold in FaSSIF, FeSSIF, FaSSIF-V2, and FeSSIF-V2, respectively (Figure 6). The results of the commercial product and SNEDDS were analyzed, and the increases obtained with the formulation were significant ($p<0.05$). The enhanced dissolution rate of BOS from the developed SNEDDS could be because the SNEDDS formulation resulted in the spontaneous formation of a nanoemulsion with a much faster and smaller DS compared to the BOS commercial product. These results show that biorelevant media could better forecast the *in vivo* performance for BOS-loaded SNEDDS formulation. Hence, this greater availability of dissolved BOS from the formulation could enhance oral bioavailability.

In an *ex vivo* permeability studies study, biorelevant media were used as buffers to better simulate *in vivo* conditions. Furthermore, because it includes a significant level of surfactant and does not reflect *in vivo* conditions, the medium with 1% SLS was not included in the *ex vivo* studies. Although BCS Class II drugs do not inherently have a permeability problem, increased solubility may relatively enhance their permeability. BOS is a weakly acidic active compound, and its solubility is pH-dependent. The increase in the pH of the medium and the SNEDDS formulation strategy enhances the solubility and permeability of BOS. In comparison to FeSSIF (pH 5.0) and FeSSIF-V2 (pH 5.8), which are used for formulation and commercial product testing, BOS showed higher permeability in FaSSIF and FaSSIF-V2 (pH 6.5) (Figure 7). The results of the commercial product and SNEDDSs were analyzed, and the increases obtained with the formulation were significant ($p<0.05$).

The formulation was developed with the data obtained from our pre-formulation studies mentioned in this publication.[2] The main purpose of the studies was to develop the SNEDDS formulation, in which BOS is the most soluble, and then to develop the solid SNEDDS. Based on F9, Maisine®, a long-chain mono and diglyceride group oil with high BOS solubility, was preferred while the other components were kept constant. It was found that a formulation with a DS of 17 nm and a PDI of 0.180, consisting of 10.11% Maisine®, 80.90% Cremophor® RH 40, and 8.99% Labrasol®, and 30 mg BOS in 1 g SNEDDS, exhibited dispersibility of: Grade A, a self-emulsification time of 47 seconds, transmittance greater than 99%, and a viscosity of 571 cP. Similar results to those of the Maisine® SNEDDS formulation were obtained, as shown in Table 1 and Table 5. The dissolution study results did not show statistical significance ($p>0.05$). However, the dissolution of BOS per gram in Maisine® SNEDDS formulation (30 mg) was found to be relatively greater than that of the F9 (28 mg) formulation. Therefore, further studies were conducted using the Maisine® formulation.

CONCLUSION

With this study, the BOS-loaded SNEDDS formulation was effectively developed, characterized, and evaluated. The SNEDDS was optimized using the BBD. The *in vitro* evaluation of SNEDDS indicated characteristics of a self-nanoemulsifying system, including transparency, robustness to dilution, thermodynamic stability, and rapid dissolution. The formulation was found stable in long-term stability studies. An *in vitro* dissolution study was carried out to assess the biorelevant media performance of BOS. According to the findings, solubility *in vitro* was significantly improved for the formulation compared to the commercial product. Due to its increased solubility, SNEDDS improved the permeability of BOS. It can be concluded that the SNEDDS of BOS is a promising lipid-based drug delivery system to increase bioavailability. Further, *in vivo* studies are needed to test the performance of BOS-loaded SNEDDS formulation.

Ethics

Ethics Committee Approval: Not required.

Informed Consent: Not required.

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Footnotes

Authorship Contributions

Concept: D.Y.U., Z.Ş.T., Design: D.Y.U., Z.Ş.T., Data Collection or Processing: D.Y.U., Z.Ş.T., Analysis or Interpretation: D.Y.U., Z.Ş.T., Literature Search: D.Y.U., Z.Ş.T., Writing: D.Y.U., Z.Ş.T.

Conflict of Interest: The authors declare no conflicts of interest.

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