Design and evaluation of self-nanoemulsifying drug delivery systems (SNEDDSs) for senicapoc

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Abstract**:** Senicapoc (SEN), a potent antisickling agent, shows poor water solubility and poor oral bioavailability. To improve the solubility and cell permeation of SEN, self-nanoemulsifying drug delivery systems (SNEDDSs) were developed. Capryol PGMC®, which showed the highest solubilization capacity, was selected as the oil. The self-emulsification ability of two surfactants, viz., Cremophor-EL® and Tween® 80, was compared. Based on a solubility study and ternary phase diagrams, three optimized nanoemulsions with droplet sizes less than 200 nm were prepared. An *in vitro* dissolution study demonstrated the superior performance of the SNEDDS over the free drug. During *in vitro* lipolysis, 80% of SEN loaded in the SNEDDS remained solubilized. An *in vitro* cytotoxicity study using the Caco-2 cell line indicated the safety of the formulations at 1 mg/mL. The transport of SEN-SNEDDSs across Caco-2 monolayers was enhanced 115-fold ($p < 0.01$) compared to that of the free drug. According to these results, SNEDDS formulations could be promising tools for the oral delivery of SEN.

Keywords: Senicapoc; Self-nanoemulsifying drug delivery systems; drug solubility; oral bioavailability

1. Introduction

Senicapoc (SEN), also identified as ICA-17043, is an ion-channel blocker that selectively blocks potassium efflux through the Gardos channel in red blood cells (RBCs) (Ataga et al., 2011). Preclinical studies and studies in transgenic models of sickle cell disease have shown that SEN increases hemoglobin levels and decreases the density of cells and hemolysis. SEN is well tolerated when administered at a dose of 10 mg to sickle cell disease patients, producing a dose-dependent increase in hemoglobin and a decrease in markers of hemolysis (Ataga et al., 2009). Additionally, SEN has demonstrated pharmacological activity against malaria (Tubman et al., 2016), chronic asthma (Van Der Velden et al., 2013), liver disease (Paka et al., 2017) and cancer (Mohr et al., 2019).

Despite these promising pharmacological activities, SEN has some limitations. SEN is a very hydrophobic drug (logP 3.59) with poor aqueous solubility (975 µg/mL) and moderate oral bioavailability (51%). It has been reported that SEN has a half-life of 1 h in rats, with a maximum concentration attained after 4 h when administered orally (McNaughton-Smith et al., 2008).

In recent years, much attention has been focused on lipid-based formulations to increase the solubility and oral bioavailability of poorly water-soluble compounds (Qian et al., 2017). One of the most popular approaches is the incorporation of drug compounds into inert lipid vehicles such as surfactant dispersions (Park et al., 2006), solid lipid nanoparticles (Kushwaha et al., 2013), nanoemulsions (Piazzini et al., 2017), microemulsions (Yin et al., 2009), self-nanoemulsifying drug delivery systems (Dou et al., 2018) and liposomes (Pereira et al., 2016). To enhance the ocular bioavailability of SEN, Phua and coworkers prepared novel topical nanoliposomes that improved the residence time by up to 12-fold that of the free drug (Phua et al., 2018). However, when applied to oral delivery, liposomes are characterized by several limitations, such as high cost, limited drug loading, poor scaling up and the use of organic solvents (Cardona et al., 2019; Zaichik et al., 2019).

To overcome these drawbacks, self-nanoemulsifying drug delivery systems (SNEDDSs) appear to be an effective and suitable alternative. SNEDDSs are isotropic mixtures of oils, surfactants and cosurfactants that form oil-in-water nanoemulsions upon mild agitation in aqueous media, such as gastro-intestinal (GI) fluid (Mou et al., 2007). Due to their anhydrous nature, SNEDDSs can be orally administered in soft or hard gelatin capsules. They can produce nanoemulsions with droplet sizes between 20 and 200 nm upon dilution (Date et al., 2010). SNEDDSs have generated tremendous interest owing to their capability to increase drug solubilization and the oral bioavailability of poorly water-soluble compounds (Cardona et al., 2019). Many poorly water-soluble drugs such as docetaxel, resveratrol, quercetin, and amphotericin B have been encapsulated into SNEDDSs, leading to improved oral bioavailability (Rani et al., 2019).

SNEDDSs seem to be a plausible drug delivery system for the oral delivery of SEN. However, to the best of our knowledge, the use of a SNEDDS for SEN oral delivery has not yet been exploited.

Therefore, the aim of this study was to develop self-nanoemulsifying drug delivery systems of SEN to enhance its aqueous solubility and oral bioavailability. The developed SEN-SNEDDS formulations were characterized in terms of emulsification time, percentage of transmittance, particle size, and zeta potential. Afterward, the *in vitro* dissolution, *in vitro* lipolysis, cytotoxicity and intestinal permeability of the optimized SEN-SNEDDS were assessed.

2. Materials and Methods

2.1 Materials

Senicapoc with a purity greater than 98% was purchased from Ark Pharma, Inc. (Arlington Hts, USA). Cremophor- EL® (polyoxyl -35 castor oil) and polyethylene glycol 400 (PEG 400) were kindly provided by BASF (Ludwigshafen, Germany). Labrafil M® 1944 CS (oleoyl polyoxyl-6-glycerides), Labrafil M® 2125 CS (linoleoyl polyoxyl-6-glycerides), Labrasol AFL® (caprylocaproyl polyoxyl-8-glycerides), Transcutol HP® (diethylene glycol monoethyl), Capryol®90 (propylene glycol monocaprylate type II)**,** Capryol PGMC® (propylene glycol monocaprylate type I), Labrafac lipophile WL® 1349 (triglycerides medium-chain), Lauroglycol®90 (propylene glycol monolaurate) and Maisine® 35-1 (glycerol monolinoleate) were kind gifts from Gattefossé (Saint-Priest, France). Tween® 80 (polysorbate 80), Tween®20 (polysorbate 20), sodium taurodeoxycholate (NaTDC), L-α-phosphatidylcholine (TLC), 4bromophenylboronic acid, Triton X-100, thiazolyl blue tetrazolium bromide (MTT) and porcine pancreatin extract (P7545, 8x USP specification activity) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Propylene glycol and oleic acid were purchased from Fagron (Colombes, France). HPLC grade solvents such as acetonitrile (Thermo Fisher Scientific, MA, USA), formic acid (VWR chemicals, Leuven, Belgium), and dimethylsulfoxide (Carl Roth, Karlsruhe, Germany) were used for chromatography studies.

2.2 Development of SNEDDS formulations

2.2.1 Screening of formulation components based on saturation solubility studies

An excess amount of SEN was added to 1 g of each vehicle, followed by mixing (100 rpm) in a shaking incubator (Infors AG, Bottmingen, Switzerland) at 37 °C for 48 h. Afterward, the equilibrated samples were centrifuged (Eppendorf centrifuge 5804 R, Hamburg, Germany) at 4000 x g for 30 min (37°C) to remove the insoluble drug. The concentration of SEN in supernatants was measured by HPLC-UV (Shimadzu C 204353, Kyoto, Japan) after dilution with acetonitrile.

2.2.2 Screening of surfactants and cosurfactants for emulsifying ability

The emulsification ability of various surfactants was screened as described by Date et al. (2007) with minor modifications. In brief, selected oils and surfactants were mixed 1:1 (w/w), heated at 40-45 °C and mixed to homogenize the components. The mixture (500 mg) was accurately weighed and dispersed into 10 mL of deionized water under gentle stirring. Visual evaluation was used to assess the relative turbidity. The resulting dispersions were allowed to stand for 2 h, and their transmittance values were measured at a wavelength of 550 nm using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific) against deionized water as a control.

Various cosurfactants were screened by mixing the surfactant with each selected cosurfactant in a 2:1 (w/w) ratio. The oily phase was added to this mixture in a 1:3 ratio and homogenized with the aid of gentle stirring and heat (40-45°C). The resulting dispersions were accessed for different parameters as mentioned for the surfactant screening.

2.2.3 Ternary phase diagrams

Different surfactants (Cremophor-EL® and Tween® 80) and cosurfactants (Transcutol HP® and PEG 400) were mixed in various weight ratios (1:1, 2:1, 3:1, 3:2) to form Smix. Oil and Smix were mixed

thoroughly in different weight ratios from 0:10 to 10:0 (0:10, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1 and 10:0) for the four Smix ratios. To determine the feasibility of the self-(nano)emulsification, 1 g of the mixture (oil + Smix) was slowly titrated with deionized water (100 mL, 37°C), gently stirred and visually examined for transparency. The droplet size and its distribution (polydispersity index, PDI) were determined at 37°C by photon correlation spectroscopy using a Nano ZS system (Malvern Instruments Ltd, UK). Pseudoternary plots were then constructed using Chemix School vers. 3.60 software (Arne Standnes, Norway).

2.2.4 Preparation of SEN-loaded SNEDDSs and maximum drug content determination

The blank SNEDDSs were prepared by mixing the appropriate quantities of oil, surfactant and cosurfactant under agitation (100 rpm, 35 min). Then, 10 mg of SEN was added to 600 mg of each blank SNEDDS and mixed under agitation (100 rpm, 35 min) for dissolution until a transparent preparation was obtained. To determine the maximum loading content of SEN in each formulation, an excess amount of SEN was added to 1 g of each blank SNEDDS formulation by mixing (100 rpm) in a shaking incubator at 37°C for 48 h. The equilibrated samples were centrifuged at 4000 x g for 30 min to remove the excess SEN, and the concentration of SEN in the supernatant was determined by HPLCUV after appropriate dilution with acetonitrile.

2.3 Characterization of optimized formulations

2.3.1 Transmittance percentage

The percentage transmittance was evaluated as described by Shakeel et al. (2013). Briefly, the SNEDDS formulations (1 g) were nanoemulsified in 100 mL of deionized water and allowed to stabilize for an hour. The transmittance percentage of samples was measured at 550 nm wavelength using a Nanodrop UV spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) against deionized water as a control.

2.3.2 Viscosity measurement

Viscosities of formulations were determined with the aid of a modular compact rheometer (MCR 102, Anton Paar Instruments Ltd, Graz, Australia) equipped with a temperature control system. A parallel plate (50 mm) was used for the measurements. The gap size was set at 500 µm, and 4 µL of each preconcentrated SNEDDS was used. The shear stress was measured at varying rates from 0.1 to 100 s^{-1} for 5 min. All rheological measurements were made at 25°C, and data were analyzed with Rheocompass software (version 1.13.44-release, Anton Paar Instruments Ltd, Graz, Australia).

2.3.3 Emulsification time

One gram of each formulation was added to 500 mL of 0.1 HCl and maintained at 37 ± 0.5 °C under gentle agitation (100 rpm). The time required in seconds to obtain a clear dispersion was recorded as the emulsification time (Basalious et al., 2010).

2.3.4 Determination of size and zeta potential

The average globule size and polydispersity index (PDI) of formulations were determined by dynamic light scattering (DLS) at 37°C using a Nano ZS system (Malvern Instruments Ltd, UK). To prepare

samples, 600 mg of each formulation was dispersed in 200 mL of deionized water, PBS pH 6.8, FaSSGF pH 1.6 and FaSSIF pH 6.8. The droplet size and PDI of the resulting emulsions were directly measured.

Zeta potential was measured by electrophoretic mobility (PCS) using a Zetasizer Nano ZS system (Malvern Instruments Ltd, UK). After diluting the SNEDDS formulation (600 mg) with 200 mL of deionized water, the samples were directly measured.

2.3.5 Thermodynamic stability studies

The formulations were subjected to heating-cooling cycles. Six cycles between 4°C and 40°C were applied with storage at each temperature for not less than 48h. Those formulations, which were stable (no phase separation) were subjected to freeze-thaw cycles involved three cycles between -21°and 20°C with storage at each temperature for not less than 48h. Further, centrifugation was performed at 4000 x g for 30 min to observe phase separation.

2.4 HPLC analysis of senicapoc

Reversed-phase HPLC-UV was used for the analysis of SEN (Phua et al., 2018). The HPLC (Shimadzu C 204353, Kyoto, Japan) consisted of an LC-20A pump equipped with an SPD-20A intelligent UV/VIS detector and a SIL-20A autosampler. Chromatographic separation was performed on a CC 250-4.6 Nucleosil 100-5, C18 HD HPLC column (Macherey-Nagel, Germany). A mixture of 80% (acetonitrile + 0.1% formic acid) and 20% (water + 0.1% formic acid) was used as the mobile phase. All samples were analyzed under isocratic elution at a flow rate of 1.0 mL/min, and the effluent was monitored at 261 nm. A 10 µL sample was injected into the Rheodyne and analyzed at 25°C. The method was linear (r^2=0.99) in the concentration range of 0.7-100 µg/mL, and the retention time of SEN was approximately 4.31 ± 0.71 min. The limit of detection (LOD) and limit of quantification (LOQ) were 1.3 µg/mL and 4 µg/mL, respectively.

2.5 *In vitro* dissolution profile

Dissolution studies were performed using a drug dissolution tester (Sotax AT7, CH-4008 Basel, Switzerland) according to US Apparatus II (paddle method). Pure SEN and SEN-SNEDDS formulations (600 mg) equivalent to 10 mg filled in size "0'' hard gelatin capsules (Capsugel Inc., Morristown, NJ, USA) were placed in 500 mL of USP buffer (pH 1.2) used as dissolution media. A paddle rotation speed of 100 rpm and a temperature of 37 ± 0.5 °C were used. At predefined time intervals (5, 10, 20, 30, 40, 50 and 60 min), a 2 mL aliquot was withdrawn and replenished with a similar volume of fresh blank media. The withdrawal samples were filtered through 0.22 µm Rotilabo® syringe filters (Carl Roth, Karlsruhe, Germany) and transferred into glass vials. Then, 10 µL of the resulting filtrate was quantified by HPLC-UV to measure the concentration of SEN.

2.6 *In vitro* lipolysis

Lipolysis experiments were carried out according to the procedure described by Crum et al. (2016) with minor adjustments. The experimental setup consisted of a T5 Mettler Toledo pH-stat titration unit (Greifensee, Switzerland) comprising a combined pH Ag/AgCl electrode (DGI 115-SC) and coupled to a 30 mL DV 1020 Mettler Toledo autoburette (Greifensee, Switzerland), an IKA C-MAG HS7 thermostat-jacketed glass reaction vessel (Staufen, Germany) and a compact stirrer (Mettler Toledo).

The SEN-SNEDDS formulations were gently dispersed into 40 mL of digestion buffer (comprising 1.4 mM $CaCl_2.2H_2O$, 2 mM Tris-maleate, 150 mM NaCl, 3 mM NaTDC, and 0.75 mM TLC). After 15 min, the pH was automatically adjusted to 6.5 ± 0.05 with 0.5 M NaOH. The *in vitro* lipolysis was initiated by the addition of 4 mL of pancreatin extract containing lipase (lipase activity equivalent to 8X USP specifications) and other pancreatic enzymes (amylase, protease and ribonuclease). The pancreatin extract was freshly prepared before each experiment by mixing 1 g of pancreatic powder with 5 mL of digestion buffer and 20 µL of 0.5 M NaOH solution to reach the target pH 6.5. The resulting enzyme suspension was centrifuged (4000 x g, 4°C, Eppendorf centrifuge 5804 R, Hamburg, Germany) for 15 min.

During the experiment, the released fatty acids were automatically titrated with 0.5 M NaOH to maintain pH 6.5. Two milliliters of digestion medium was withdrawn in 5 min intervals up to 60 min. The lipase activity was inhibited by the addition of 10 µL of 1.0 M 4-bromophenylboronic acid (in methanol). The samples were vortexed and centrifuged (6700 x g, 4°C MiniSpin, Eppendorf AG, Hamburg, Germany) for 15 min, resulting in the separation of the digestion content in a clear supernatant and off-white pellet. The drug content in the supernatant was quantified by HPLC-UV following appropriate dilution with acetonitrile. Lipolysis was also performed with blank digestion medium containing no SNEDDS.

2.7 Transport of senicapoc through the Caco-2 cell monolayer

2.7.1 Cytotoxicity assessment of the SNEDDS formulations

The viability of Caco-2 cells against blank SNEDDS formulations was evaluated as described by Memvanga et al. (2013). Briefly, Caco-2 cells were seeded on 96-well plates (2x 10^4 cells/well; 100 µL per well) in culture medium consisting of Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (HyClone®, Thermo Scientific, UK), 1% (v/v) L-glutamine, 1% (v/v) nonessential amino acids and penicillin/streptomycin solution (10 units/ 10 µg/mL) and were incubated at 37 °C in a 10% CO_2 humidified incubator for 24 h. Once the cells were confluent, they were washed with phosphate-buffered saline (PBS) at 37 °C and treated with 100 µL of free SEN, unloaded-SNEDDS or SEN-SNEDDS dispersed in Hank's salt balanced solution (HBSS). Samples were prepared at concentrations varying from 0.3 mg/mL to 6 mg/mL. HBSS served as a negative control, and 1% (v/v) Triton X-100 served as a positive control. After 2 h of incubation, cells were washed with HBSS at 37°C, treated with 100 µl of MTT solution (0.5 mg/mL in DMEM) and further incubated for 3 h. Next, 200 µL of DMSO was added to solubilize the formazan crystals formed during the incubation, and the product of the reaction was measured at 545 nm using a Multiskan Spectrum microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). Cell viability rates of the samples were calculated according to equation (1): Cell viability (%) = A_s/A_c x 100

where A_s is the sample absorbance and A_c is the absorbance measured after treatment of cells with HBSS.

2.7.2 Cell culture for transport studies

The *in vitro* transport studies were carried out as described by Memvanga et al. (2013). Caco-2 cells (5x10^5 cells/well) were seeded on 12-well cell culture inserts with a 1 µm pore diameter and 0.9 cm^2 surface area (Corning Costar®, NY, USA) and were grown in culture medium at 37 °C in an atmosphere of 10% CO_2. Cell culture medium was added to the apical (0.5 mL) and basolateral (1.2 mL) sides, and

the medium was replaced every 2 days. After 21 days of incubation, only Caco-2 cell monolayers with initial transepithelial electrical resistance (TEER) values between 300 and 600 ohm/cm^2 were selected.

Before the transport study, the culture medium was replaced with preheated (37°C) HBSS. After the cell monolayer was equilibrated for 30 min at 37°C, TEER values of monolayers were determined in triplicate. The apical to basolateral transport experiments across Caco-2 cell monolayers were conducted by adding 0.5 mL of SEN free drug (1 mg/mL SEN in HBSS) or 0.5 mL of dispersed formulations in HBSS (1 mg/mL SEN-SNEDDS, i.e., 16.6 µg/mL SEN) on the apical side of the inserts and 1.2 mL of HBSS on the basolateral side. After 2 h, samples from the basolateral compartment were withdrawn to determine the permeation of free SEN or SEN loaded in the SNEDDSs. The amount of SEN that crossed the Caco-2 cell monolayers was determined by HPLC-UV. The apparent permeability coefficient (P_{app}) was determined using the following equation (2): $P_{app} = \frac{dQ}{dt} \times \frac{1}{CoA}$

where dQ/dt (transport rate) is the amount of SEN (µg) appearing per time unit (s) in the receiver compartment, C_o is the initial concentration in the donor compartment (µg/mL) and A is the surface area of the monolayer (A = 0.9 cm^2).

2.8 Statistical analysis

Student's t-test and analysis of variance (ANOVA) were used to compare different groups. A P-value less than 0.05 was considered statistically significant. GraphPad Prism version 8 (San Diego, CA, USA) was used for statistical analysis of the data. Unless otherwise stated, the data are the mean ± SD for n=3.

3. Results and discussion

3.1 Development of SNEDDS formulations

3.1.1 Selection of components

The selection of an appropriate oil is crucial for the preparation of SNEDDSs. Two important factors to be considered are the ability of an oil to solubilize the drug and its ease of emulsification. The oil with the maximum drug solubilizing capacity is typically selected due to its key influence in achieving optimal drug loading (Elsheikhet al., 2012; Qian et al., 2017). This property in turn is important in avoiding drug precipitation during the emulsification process. The results of solubility studies in oily phases are depicted in Fig. 1. Among all oils screened, the maximum solubilization capacity was exhibited by Lauroglycol®90 (14.8 ± 0.3 mg/mL) and Capryol PGMC® (14.5 ± 1.6 mg/mL); hence, they were selected as the oily phases for further studies.

The solubility of SEN in various surfactants and cosurfactants is graphically represented in Fig. 1. Labrasol AFL® showed the highest solubility (37.4 ± 2.8 mg/mL), followed by Cremophor-EL® (27.2 ± 0.7 mg/mL) and Tween®80 (22.1 ± 1.5 mg/mL). These three nonionic surfactants are known to be less toxic than ionic surfactants (Rani et al., 2019). Among the tested cosurfactants, Transcutol HP® yielded the highest solubility for SEN (16.5 ± 2.2 mg/mL), followed by PEG 400 (14.2 ± 1.2 mg/mL).

Surfactants screening was based on their emulsification abilities toward the selected oils. Lauroglycol® 90 and Capryol PGMC® were screened against Labrasol ALF®, Cremophor-EL® and Tween®-80. The

percentage transmittance values of various dispersions were measured (table 1) and clearly distinguished the ability of various surfactants to emulsify Lauroglycol®90 and Capryol PGMC®. It can be observed that Cremophor-EL® (HLB =13) had a very good ability to emulsify Capryol PGMC®, followed by Tween® 80 (HLB= 15), whereas Labrasol ALF® (HLB = 14) was a poor emulsifier for Capryol PGMC®. The emulsification ability of a surfactant is typically influenced by its structure and HLB. The HLB values of selected surfactants were above 12, and the considerable differences in their ability to emulsify Capryol PGMC® could be explained by their structural differences (Basalious et al., 2010).

None of the selected surfactants could effectively emulsify Lauroglycol®90. Chemically, Lauroglycol®90 and Capryol PGMC® are propylene glycol monolaurate (C12) and propylene glycol monocaprylate (C8), respectively. Borhade and coworkers reported that the emulsification of oil and its amount incorporated into the nanoemulsion are affected by its molecular volume (Borhade et al., 2011). The increase in the number and length of hydrophobic alkyl chains increases the molecular volume, which renders emulsification of the oil difficult. This fact could explain the poor emulsification of Lauroglycol® 90 (C12) compared to Capryol PGMC® (C8). Therefore, Capryol PGMC® was selected as the oily phase for further studies due to its ease of emulsification.

In SNEDDS development, the role of the surfactant is to lower the interfacial tension, which ultimately eases the dispersion process during nanoemulsion formation. Reducing the interfacial tension to zero results in a negative free energy that makes the system stable and favors spontaneous emulsification (Patki et al., 2019). However, surfactant alone may not be able to sufficiently lower the interfacial tension to form nanoemulsions (Rajesh et al., 2018). Hence, the use of cosurfactants or cosolvents is important in the preparation of SNEDDSs. The cosolvent is used to cooperate with the surfactant in reducing the interfacial tension, increasing the drug solubility and enhancing the dispersibility of surfactant in the oily phase, thus promoting formulation homogeneity and stability (Li et al., 2019). All the hydrophilic cosolvents improved the emulsifying ability of Cremophor-EL®, whereas some of them (PEG 400 and propylene glycol) were less effective as a cosolvent for Tween® 80 (Supplementary table S1). Lipophilic cosurfactants were less effective because they could not improve the emulsification of the selected surfactants. In general, cosurfactants increase the interfacial fluidity by penetrating the surfactant film, creating void spaces between the surfactant molecules. Their performance is affected by their structure and chain length (Malcolmson et al., 1998; Warisnoicharoen et al., 2000), which might explain the lower effectiveness of lipophilic cosurfactants compared to hydrophilic cosurfactants. Paradoxically, Labrafil M® 1944 CS did not follow this behavior. Labrafil M® 1944 CS, which has an oleic acid backbone, showed a good ability to improve the emulsification of selected surfactants, probably owing to its better hydrophilicity and surfactant-like properties than those of other cosurfactants. These observations are in line with studies reported by Date et al. (2007). Therefore, Transcutol HP® and PEG 400 were selected as cosurfactants due to their superior solubilizing potential for SEN and ability to improve the emulsification of the selected surfactants.

3.1.2 Ternary phase diagram study

Based on the solubility and emulsification ability studies (Fig. 1, S1), ternary diagrams were constructed to identify the self-(nano)emulsification regions and to select a suitable ratio of oil, surfactant and cosurfactant for SNEDDS development. The phase diagrams were studied for the following combinations: Capryol PGMC®-Cremophor-EL®-Transcutol HP® (F1), Capryol PGMC®-Cremophor-EL®PEG 400 (F2) and Capryol PGMC®-Tween® 80-Transcutol HP® (F3). The area where the ternary mixture showed a clear dispersion with a low droplet size (< 200 nm) was selected for further

optimization studies. As shown in Fig. 2 (A-C), all three diagrams possessed a (nano)emulsification area, and these areas decreased as the ratio of cosurfactant increased. Furthermore, the sizes of the nanoemulsion regions were compared; the larger the size is, the greater the self-(nano)emulsification efficiency is. The largest nanoemulsion area was observed with a Smix ratio of 2:1 compared to 1:1, 3:1 and 3:2.

Cremophor-EL®-based systems with Transcutol HP® had larger nanoemulsification areas than those with PEG 400. This result could be attributed to the high aqueous partition of PEG 400, limiting its cosolvent efficacy compared to that of Transcutol HP® (Memvanga et al., 2013).

The nanoemulsion area of the Tween®80-based system was small compared to that of the CremophorEL®-based systems, and there was no increase in the cosolvent used (data not shown). Moreover, Cremophor-EL®-based systems could form nanoemulsions for the compositions that had surfactant concentrations as high as 75% (w/w), compared 55% for the Tween® 80-based systems.

In view of the current investigations, F1, F2 and F3 (table 2 and Fig. 2) were chosen because of their resulting nanoemulsion droplet size and polydispersity index (PDI). With these components and proportions of oil/Smix, stable nanoemulsions could be formed spontaneously by gentle agitation following water dilution, and the preconcentrated SNEDDSs could be used for further studies by filling them into capsules.

3.1.3 Maximum drug content determination

The saturation solubility of SEN in the preconcentrated SNEDDS formulations was 20 ± 1 mg/g, 24 ± 0 mg/g and 28 ± 0 mg/g for F1, F2 and F3, respectively. The oil, surfactants and cosurfactants used along with their amounts could have contributed to the significant ($p<0.05$) difference in solubility. For a more direct comparison among the three formulations, and following SEN pharmacokinetics studies (Ataga et al., 2009), 10 mg of SEN was loaded into 600 mg of each SNEDDS formulation.

3.2 Characterization of the optimized formulations

3.2.1 Transmittance percentage

Since SNEDDSs are defined as mixtures of oils, surfactants, cosurfactants and drugs that form oil-inwater optically isotropic and kinetically stable nanoemulsions upon mild agitation, the transmittance percentage is a useful tool to assess the isotropic properties of the resulting nanoemulsions. As presented in table 2, all the selected formulations exhibited high transmittance values (≥ 95%), suggesting the clarity of the dispersions.

3.2.2 Viscosity measurement

Preconcentrated liquid SNEDDSs are generally filled into capsules. Low-viscosity SNEDDSs face leakage issues, whereas more high viscous formulations are difficult to fill into gelatin capsules owing to pourability issues (Parikh et al., 2019). The viscosity was found to be 41.6 ± 1.4 mPa, 67.8 ± 2.4 mPa and 27.3 ± 1.7 mPa for F1, F2 and F3, respectively. The high viscosity of the Cremophor-EL®-based systems compared to the Tween® 80-based systems was expected and was previously reported by Li et al. (2019). Moreover, the rheogram for the three formulations showed a straight line, indicating Newtonian systems (data not shown), which showed that a change in shear stress will not induce a variation in viscosity during capsule machine filling operation (Parikh et al., 2019).

3.2.3 Emulsification time

The emulsification time is an important parameter for assessing the spontaneity of the self(nano)emulsification of formulations without the aid of any external thermal or mechanical energy (Parmar et al., 2011). The emulsification time was less than 80 s for the three optimized formulations, which indicated their ability to disperse completely and quickly when subjected to aqueous dilution under mild agitation (Basalious et al., 2010). The quick emulsification process was correlated with the ease of water penetration into the complex colloidal structure formed on the surface of the droplets (Parmar et al., 2011). As shown in table 2, the Tween®80-based system showed a lower emulsification time (27 s) than that of the Cremophor®-EL-based systems. This result could be correlated with a lower amount of oil and a higher amount of cosurfactant, which resulted in a lower viscosity of the Tween®80 -based system.

3.2.4 Droplet size analysis

The pH of the dispersion medium and drug incorporation may have a considerable impact on the behavior of SNEDDS formulations. Thus, the pH effect of the dispersion medium on the selfnanoemulsifying formulations was investigated (table 3). The mean droplet size of the diluted blank (F1 and F2) SNEDDS was < 60 nm, with a narrow distribution (PDI <0.4). However, the diluted blank F3 showed a mean droplet size > 100 nm with a distribution (PDI) >0.4. The high particle size could be attributed to the high aqueous partition of Tween® 80, probably owing to its high HLB value (HLB=15), whereas the high observed PDI appears to correlate with a higher amount (>65%) of relatively hydrophilic components (Tween® 80 and Transcutol® HP) and lower (<35%) amount of the oily phase (Capryol PGMC®). It was reported that the droplet size and the PDI of the nanoemulsion formed upon SNEDDS dispersion in aqueous environments depend on the type and amount of components (surfactant and cosurfactant) used. This observation is in agreement with studies reported by Zupančičet al. (2016).

The incorporation of SEN into SNEDDSs led to an increase in the droplet size of nanoemulsions compared to drug-free compositions, indicating its successful incorporation into the SNEDDS droplet (Menzel et al., 2018). The increased globule size of nanoemulsions could be attributed to the alteration of surfactant-oil interactions at the interface in the presence of SEN molecules.

Furthermore, the assessment of the *in vitro* precipitation was very important to eliminate the formulations that could potentially precipitate SEN at this development step. Even after storage for 48 h at 37°C in deionized water or buffers, SEN-SNEDDSs were clear in appearance and did not show any signs of flocculation, phase separation or drug precipitation.

The zeta potential is responsible for repulsion between adjacent, similarly charged and dispersed droplets. As shown in table 2, all formulations exhibited zeta potential values of approximately -7 mV, which complies with the zeta potential requirement and indicates the stability of SNEDDS formulations (Xue et al., 2018).

3.2.5 Thermodynamic stability studies

To check the stability, formulations were subjected to heating and cooling cycles, freeze-thawing cycles and centrifugation. As shown in supplementary table S2, all the SNEDDSs remained stable and no phase separation was observed.

3.3 *In vitro* dissolution profile

The *in vitro* dissolution profile of SEN from SNEDDSs is presented in Fig. 3. The pure drug showed a maximum drug release of up to 26.2% in 20 min. The amount of SEN released from the three SENSNEDDSs filled in hard gelatin capsules was over 80% within 20 min. Interestingly, F3 provided the highest release among all formulations (> 92%). It is obvious that SEN release from SNEDDSs was primarily governed by its solubility because SEN release was independent of the SNEDDS droplet size. SEN was released from the SNEDDS formulations in 60 min (F3>F2>F1) ($p< 0.05$), which was consistent with the increased solubility of SEN in the SNEDDS formulations (F3>F2>F1) ($p< 0.05$), indicating a good correlation between SEN solubility and its release. The solubility-dependent dissolution has also been reported earlier by Qian et al. (2017).

3.4 *In vitro* lipolysis

When administered orally, SNEDDSs are prone to digestion by pancreatic lipase. It has been reported that SNEDDS digestion in the GI tract is crucial for drug dissolution and absorption: it can be beneficial (drug solubilization) or deleterious (drug precipitation after digestion of the oil phase). The *in vitro* lipolysis of formulations was studied to understand the impact of the formulations on the lipolysis process. The consumption of NaOH during the experiment, reflecting the progress of lipolysis, is depicted in Fig. 4A. As presented in Fig. 4B, F1 and F2 show high SEN solubilization in the aqueous phase after 60 min of *in vitro* lipolysis. The larger quantities of lipids in those formulations could explain the higher SEN solubilization compared to that in F3 ($p < 0.05$). In agreement with previous studies (Alayoudi et al., 2018), increasing the quantity of lipid in the formulation reduced drug precipitation during digestion.

3.5 Transport study through the Caco-2 cell monolayers

3.5.1 Cytotoxic assessment of the SNEDDS formulations

The potential cytotoxicity of SNEDDS formulations to Caco-2 cells was tested to find the highest no/low toxic concentration to be used in transport experiments. Fig. 5 presents the concentration versus percent viability data of cells incubated with free SEN and unloaded-SNEDDS. Caco-2 cell viability exceeded 80% following exposure to all SNEDDS formulations (from 0.3 mg/mL to 1.25 mg/mL). Moreover, the IC50 values of F1, F2 and F3 were 1.6 mg/mL, 1.7 mg/mL and 2.9 mg/mL, respectively.

3.5.2 The transport of senicapoc across Caco-2 cell monolayers

Based on the cytotoxicity studies, Caco-2 monolayers were incubated for 120 min at 37°C with 1 mg/mL of each SEN-SNEDDS corresponding to 16.6 µg/mL SEN. No significant change in TEER values before and after incubation with the formulations was observed ($p >0.05$). The apical to basolateral transport of SEN from each formulation was in the following order: F1>F2>F3>control group (Fig. 6). The quantity of SEN transported across the Caco-2 monolayers ranged between 2.2 and 2.6% (0.57 to 0.66 µg) of the donor SEN-SNEDDS. The P_{app} values of SEN from F1, F2 and F3 were 115- ($p < 0.01$), 105- ($p < 0.01$) and 99-fold ($p < 0.01$) higher than those for the free drug, respectively, demonstrating that the P_{app} of SEN could be significantly enhanced using SNEDDS formulations. Compared to the Tween®80-based system, the Cremophor-EL®-based systems yielded the highest P_{app} ($p < 0.01$). It was previously reported that Cremophor-EL® could affect membrane fluidity and increase the permeation of lipophilic drugs (Yin et al., 2009). Compared to that for F2, the significantly high P_{app} observed for F1 ($p < 0.01$)

could come from the incorporation of Transcutol HP®. As reported in the literature (Alvi et al., 2014), the P_{app} values of several poorly water-soluble model compounds were improved by Transcutol, and its permeation-enhancing effect on Caco-2 monolayers was much stronger than that of PEG 400. Thus, Transcutol HP® present in F1 showed a higher SEN absorption enhancing effect than did PEG 400 in F2.

4. Conclusion

In this study, three optimized SNEDDS formulations were prepared and evaluated to improve the solubility and cell permeation of SEN. The screening of surfactants and cosurfactants helped to identify the most suitable components, whereas the pseudoternary phase diagrams gave an idea about the ratio of SNEDDS excipients that should be used to achieve optimized formulations. The formulated SNEDDSs showed a high release profile, and at least 80% of SEN remained solubilized after the *in vitro* lipolysis of the SNEDDS formulations. Additionally, the *in vitro* transport study across Caco-2 cell monolayers revealed that the SNEDDSs could significantly enhance the permeation of SEN. Overall, the present and previous investigations showed the potential of SNEDDSs to enhance the solubility and permeation of insoluble drugs such as SEN, although further preclinical studies are required before clinical trials can be conducted.

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Declaration of interest

All authors have no competing interest to declare

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Figure legends

Fig. 1 Solubility of SEN (mg/mL) in various oils, surfactants and cosurfactants at 37°C. Data are expressed as the mean ± SD, n=3.

Fig. 2 Phase diagrams of (A) Capryol PGMC®-Cremophor EL®-Transcutol HP® (F1), (B) Capryol PGMC®Cremophor EL®-PEG 400 (F2), Capryol PGMC®-Tween® 80-Transcutol HP® (F3).

Fig. 3 Dissolution profile of formulations in USP buffer pH 1.2 using paddle apparatus at 37± 0.5 °C. Data are expressed as the mean ± SD, n=3.

Fig. 4A NaOH consumption of SNEDDSs formulations during *in vitro* lipolysis. Data are expressed as the mean ± SD, n=3.

Fig. 4B SEN content in the aqueous phase during *in vitro* lipolysis. Data are expressed as the mean ± SD, n=3.

Fig. 5 Cell viability of Caco-2 cells following SEN suspension or SEN-SNEDDSs treatment. Each data point represents the mean ± SD (n= 6).

Fig. 6 The apparent permeability (P_{app}) values of SEN across the Caco-2 cell monolayer for each formulation after 2 h (A to B transport study). Each value is the mean ± SD of three separate determinations. **$p < 0.01$

Table 1
Emulsification study of surfactants for oils

Surfactant	% Transmittance	
	Capryol PGMC®	Lauroglycol 90®
Tween® 20	94.4 ±0.55	64.7 ± 0.42
Tween® 80	95.1 ±0.79	63.4 ± 0.49
Labrasol ALF®	60.5 ± 0.86	55.0± 0.36
Cremophor-EL®	99.3 ± 0.69	73.6 ± 0.15

Table 1

Composition and characterization of SNEDDS formulations. Data are expressed as the mean ± SD, n=3.

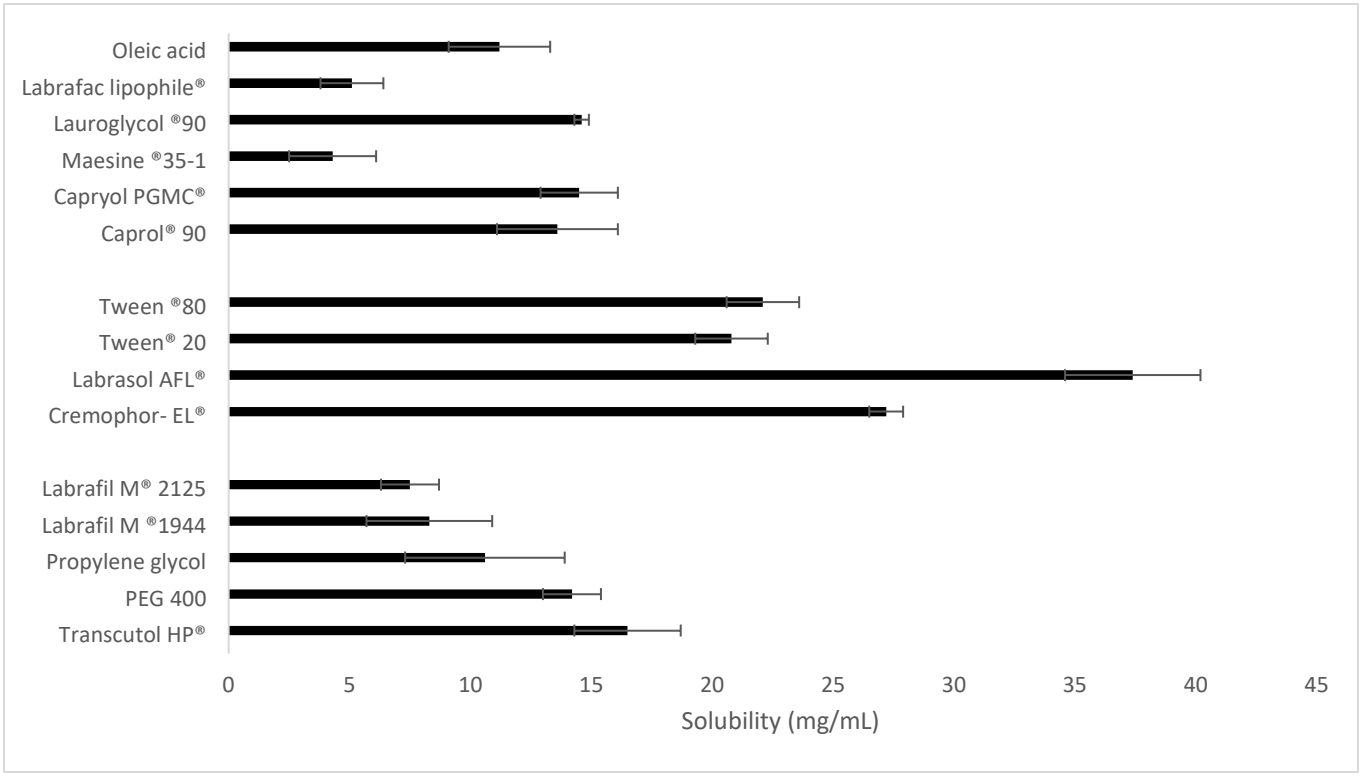
F-x	Sample composition (mg)			Emulsification time (s)	Zeta potential (mV)	Senicapoc solubility (mg/g)	% transmittance
	Oil	SA	Co-SA				
F1	260[a]	240[b]	100[d]	58 ± 2	-7.6± 3	20 ± 1	99 ± 0
F2	300[a]	240[b]	60[e]	64 ± 3	-7.4± 0	24 ± 0	96 ± 1
F3	180[a]	240[c]	180[d]	27 ± 6	-6.8 ± 2	28 ± 0	95 ± 1

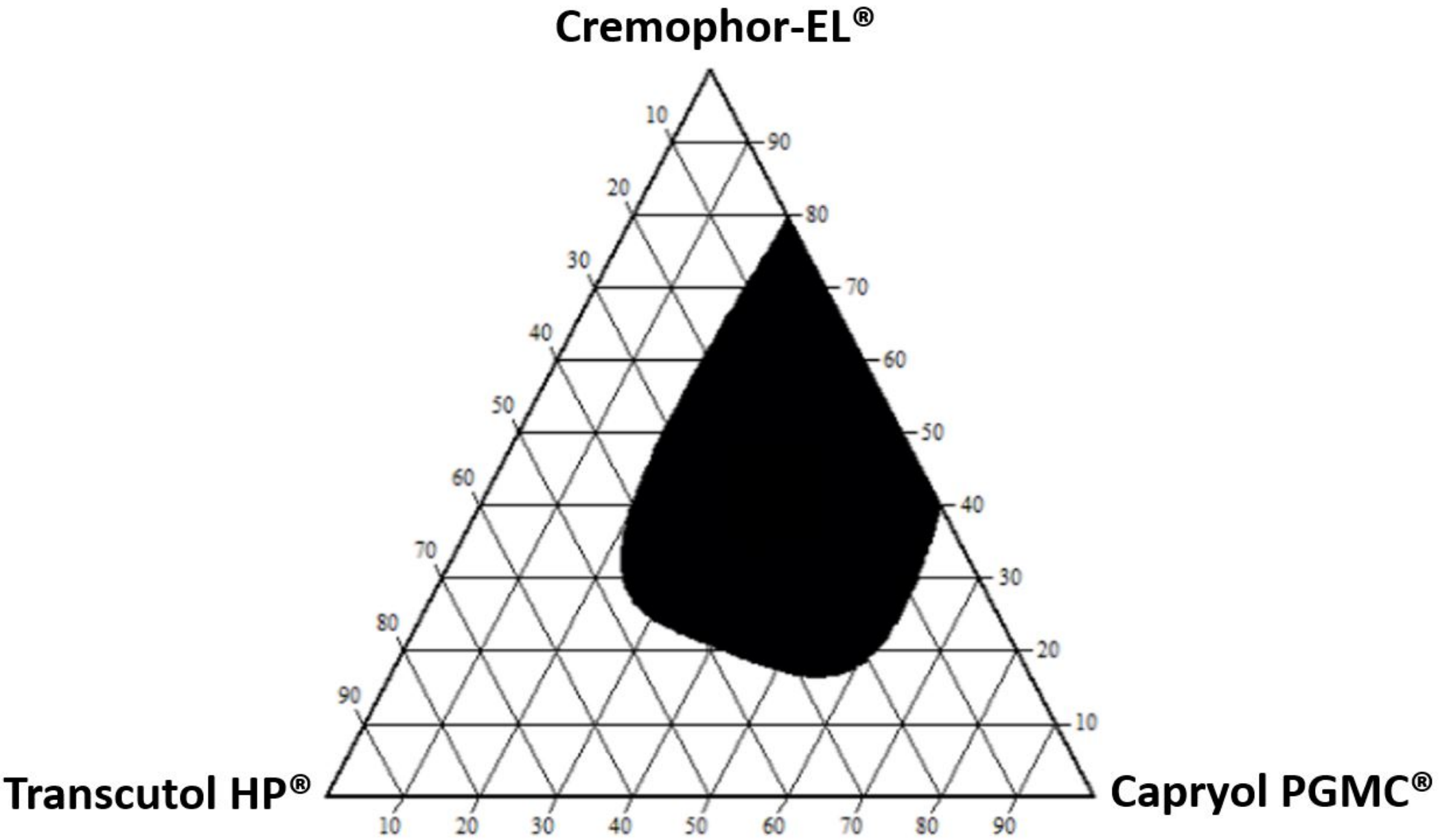
a Capryol PGMC® b Cremophor-EL® c Tween®-80 d Transcutol HP® e PEG 400

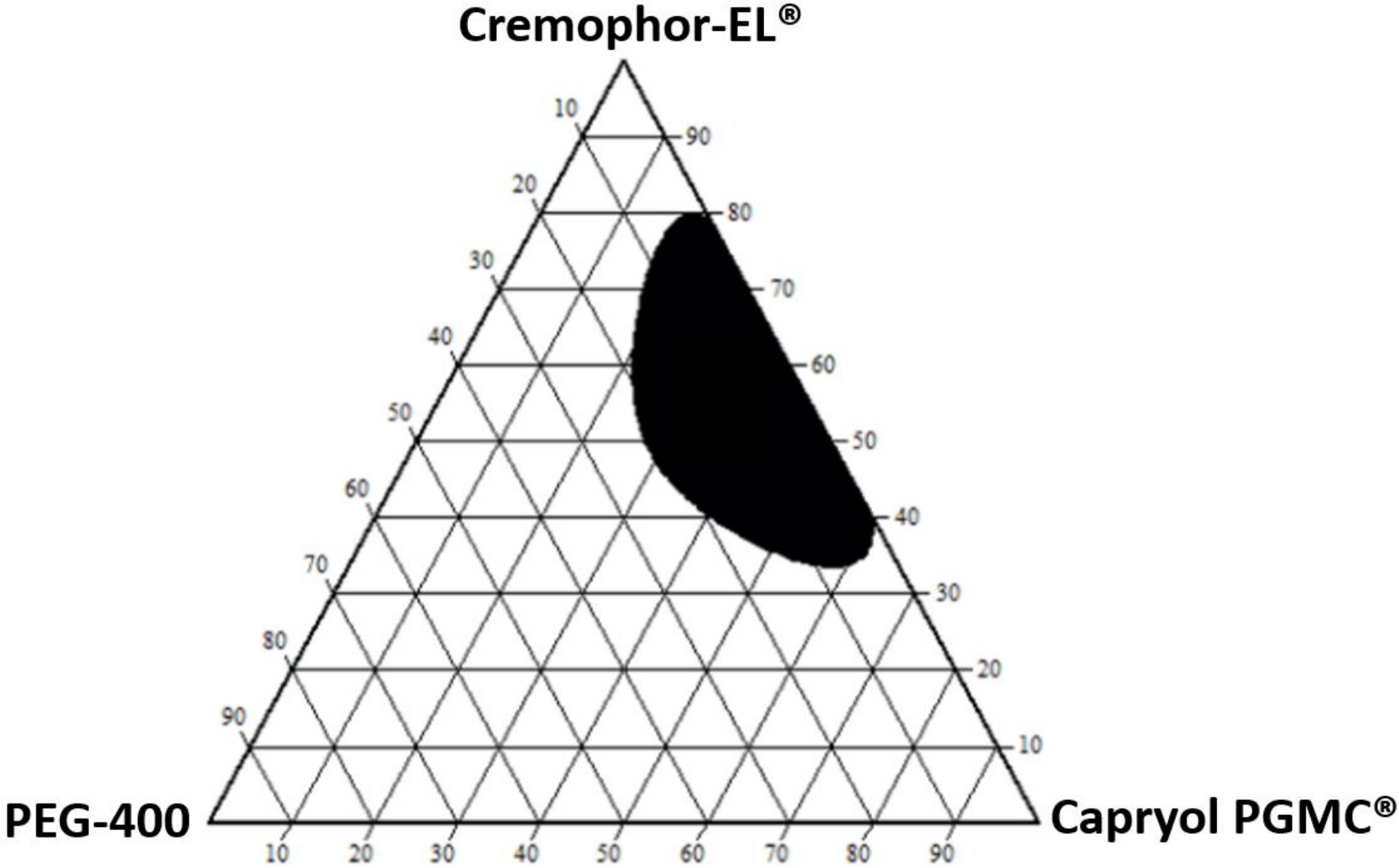
Table 2
Globule size and polydispersity index of formulations before and after SEN incorporation diluted with various buffers as dispersion medium.
Data are expressed as the mean ± SD, n=3.

	Hydrochloric acid buffer pH 1.2 Deionized water[a]		PBS[a] pH 6.8		FaSSGF[a] pH 1.6		FaSSIF[a] pH 6.8	
	Globule size (nm)	PDI	Globule size (nm)	PDI	Globule size (nm)	PDI	Globule size (nm)	PDI
F1[a]	41 ± 1	0.19	28 ± 0	0.17	31 ± 0	0.13	60 ± 2	0.13	39 ± 0	0.27
[b]	62 ± 1	0.24	41 ± 1	0.22	63 ± 0	0.25	78 ± 1	0.33	57 ± 1	0.18
F2 [a]	33 ± 1	0.32	38 ± 2	0.39	60 ± 2	0.36	33 ± 0	0.36	34 ± 2	0.38
[b]	41 ± 0	0.25	52 ± 1	0.15	81 ± 1	0.44	48 ± 1	0.40	86 ± 2	0.33
F3 [a]	111 ± 2	0.58	123 ±3	0.45	105± 2	0.48	125 ± 2	0.41	130 ± 3	0.42
[b]	136 ± 0	0.44	152 ±1	0.33	111 ± 4	0.46	130 ± 4	0.23	145 ± 3	0.38

a Before SEN incorporation **b** After SEN incorporation


Oleic acid
Labrafac lipophile®
Lauroglycol ®90
Maesine ®35-1
Capryol PGMC®
Caprol® 90
Tween ®80
Tween® 20
Labrasol AFL®
Cremophor- EL®
Labrafil M® 2125
Labrafil M ®1944
Propylene glycol
PEG 400
Transcutol HP®
0
5
10
15
20
25
30
35
40
45
Solubility (mg/mL)


Cremophor-EL®
Transcutol HP®
Capryol PGMC®
10
20
30
40
50
60
70
80
90


Cremophor-EL®
10
20
30
40
50
60
70
80
90
90
80
70
60
50
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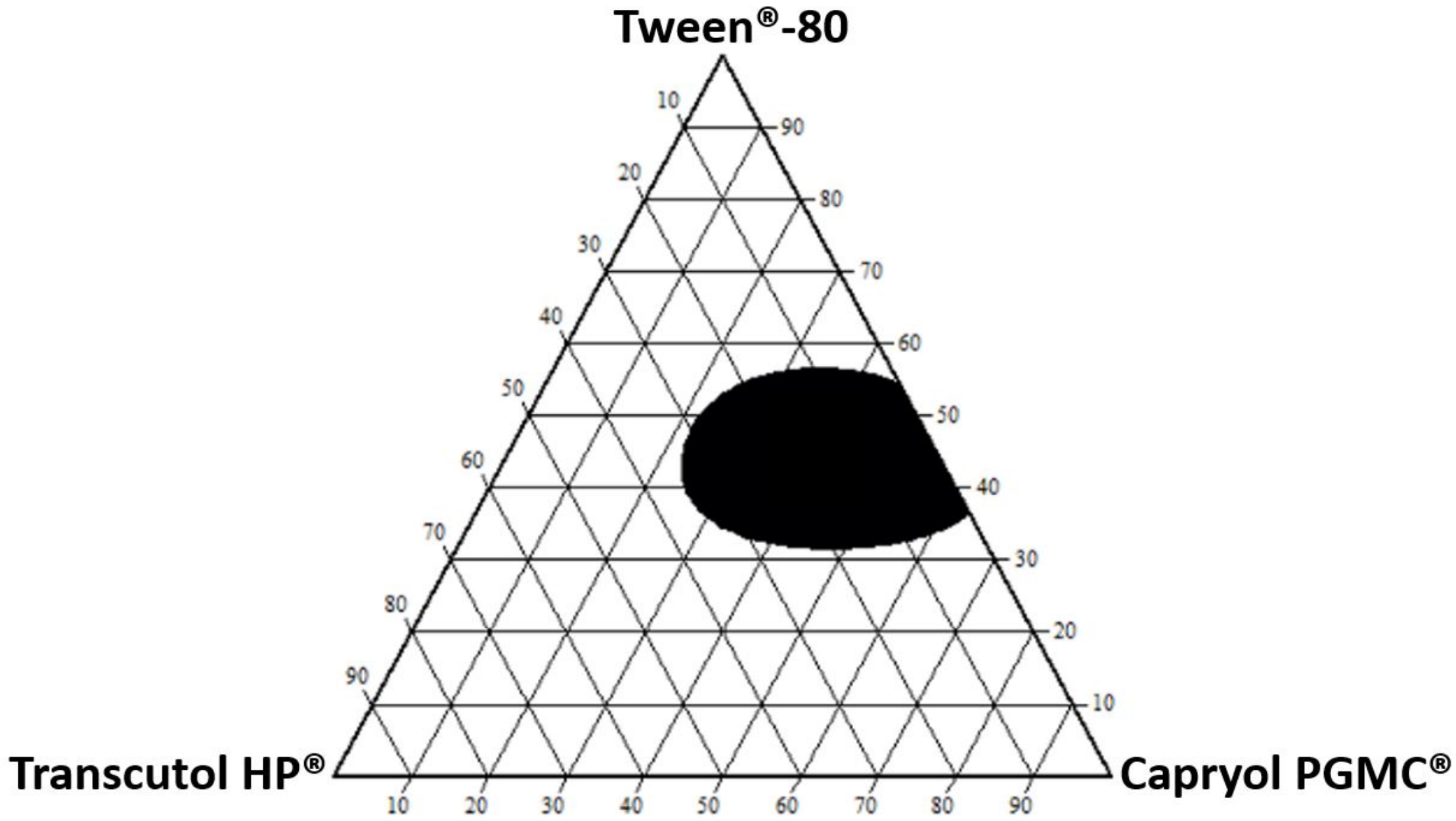
30
20
10
10
20
30
40
50
60
70
80
90
PEG-400
Capryol PGMC®


Tween®-80
10
90
20
80
30
70
40
60
50
50
60
40
70
30
80
20
90
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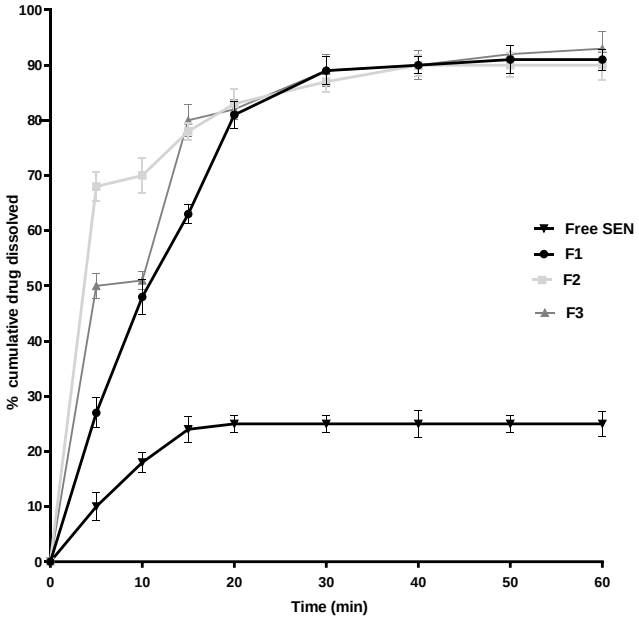
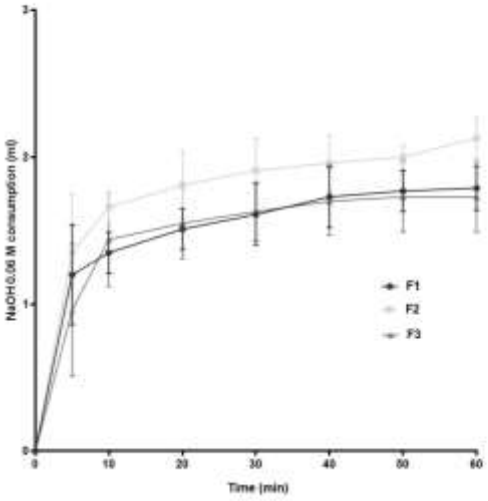
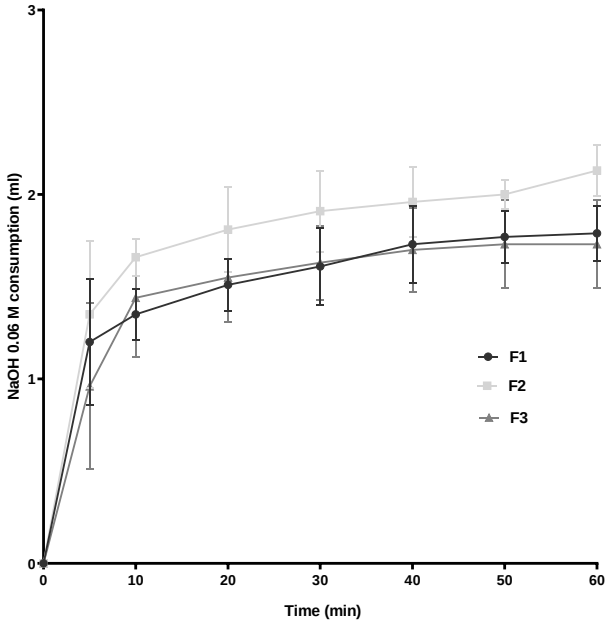
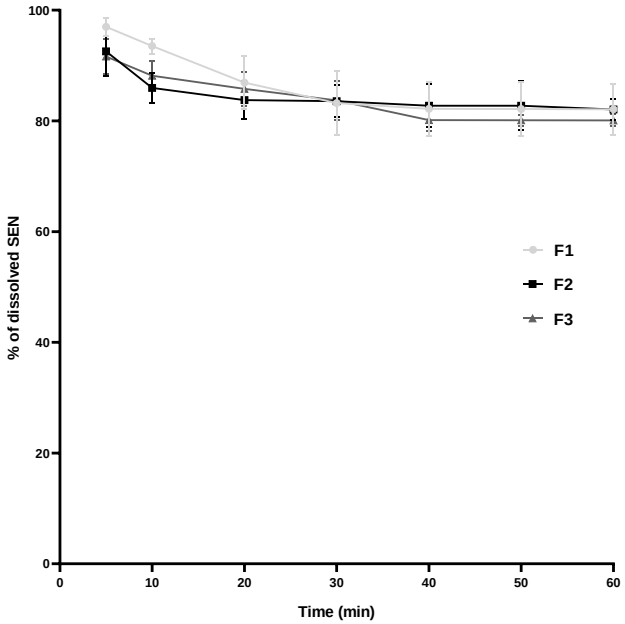
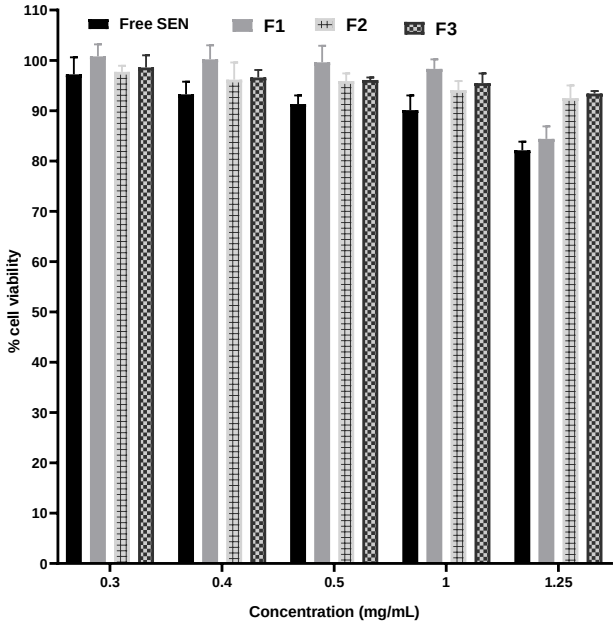
10
20
30
40
50
60
70
80
90
Transcutol HP®
Capryol PGMC®


100
90
80
70
60
50
40
30
20
10
0
% cumulative drug dissolved
0
10
20
30
40
50
60
Time (min)
Free SEN
F1
F2
F3


NaOH 0.05 M consumption (ml)
Time (min)
F1
F2
F3


NaOH 0.06 M consumption (ml)
Time (min)
F1
F2
F3
0
1
2
3
0
10
20
30
40
50
60


100
80
60
40
20
0
% of dissolved SEN
0
10
20
30
40
50
60
Time (min)
F1
F2
F3


Free SEN
F1
F2
F3
% cell viability
110
100
90
80
70
60
50
40
30
20
10
0
0.3
0.4
0.5
1
1.25
Concentration (mg/mL)

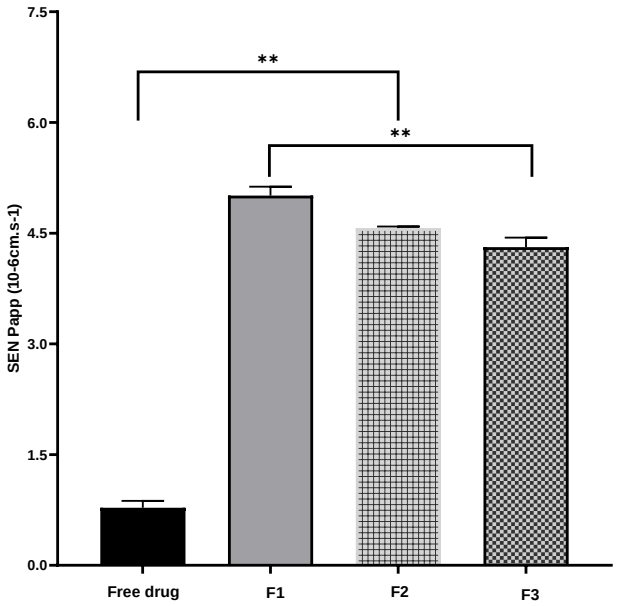

7.5
6.0
4.5
3.0
1.5
0.0
SEN Papp (10-6cm.s-1)
**
**
Free drug
F1
F2
F3

Table S1

Emulsification study of surfactant/co-surfactant combinations

Co-surfactant	% Transmittance	
	Cremophor-EL®	Tween® 80
Transcutol HP®	99.8 ± 0.85	95.5 ± 0.67
Propylene glycol	99.4 ± 0.45	88.2 ± 0.76
Polyethylene glycol 400	99.6 ± 1.62	85.4 ± 0.89
Labrafil M® 1944 CS	98.3 ± 0.65	94.4 ± 0.25
Labrafil M® 2125 CS	89.4 ± 0.15	78.4 ± 0.60

Table S2

Thermodynamic stability studies

Formulation	Heating-cooling cycles	Freeze-thaw stress cycles	Centrifugation
F1	No phase separation	No phase separation	No phase separation
F2	No phase separation	No phase separation	No phase separation
F3	No phase separation	No phase separation	No phase separation