International Journal of Pharmaceutics 362 (2008) 2–9

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Self-nanoemulsifying drug delivery systems (SNEDDS) for oral delivery of protein drugs
I. Formulation development

Sripriya Venkata Ramana Rao, Jun Shao*

Biotechnology and Drug Delivery Laboratory, Department of Pharmacy and Administrative Sciences, College of Pharmacy and Allied Health Professions, St. John's University, 8000 Utopia Parkway, Queens, NY 11439, USA

ARTICLE INFO

Article history:
Received 19 September 2007
Received in revised form 14 May 2008
Accepted 15 May 2008
Available online 27 May 2008

Keywords:
Self-nanoemulsifying drug delivery systems (SNEDDS)
Solid dispersion
protein delivery
β-Lactamase

ABSTRACT

The global aim of this research project was to develop a self-nanoemulsifying drug delivery system (SNEDDS) for non-invasive delivery of protein drugs. The specific aim of this study was to develop SNEDDS formulations. An experimental design was adopted to develop SNEDDS. Fluorescent labeled β-lactamase (FITC-BLM), a model protein, was loaded into SNEDDS through solid dispersion technique. The experimental design provided 720 compositions of different oil, surfactant, and co-surfactant at various ratios, of which 33 SNEDDS prototypes were obtained. Solid dispersion of FITC-BLM in SoyPC prepared was able to dissolve in 16 SNEDDS prototypes (approximately 2200 mU BLM in 1 g SNEDDS). SNEDDS NE-12-7 (composition: Lauroglycol FCC, Cremophor EL and Transcutol; ratio: 5:4:3) formed O/W nanoemulsion with mean droplet size in the range of 22–50 nm when diluted with various pH media and different dilution factor with PBS (pH 7.4). The phase diagram of NE-12-7 indicated a broad region of nanoemulsion. BLM-loaded SNEDDS (NE-12-7) stored at 4 °C for 12 weeks indicated 10% loss of BLM activity. A SNEDDS was developed to load FITC-BLM into the oil phase which can spontaneously form O/W nanoemulsion upon the addition of water.

1. Introduction

Oral delivery of proteins/peptide drugs (hereafter called as proteins) usually result in poor bioavailability due to two major reasons: low permeation of the protein due to its large size and hydrophilicity, and extensive degradation in the harsh environment of the gastro-intestinal tract (GIT) (Van Hoogdalem et al., 1989; Sarciaux et al., 1995; Toorisaka et al., 2005; Almeida and Souto, 2007). A well-known fact is that oils are usually effectively absorbed. The oils may be digested before absorption, and meanwhile, the oils are emulsified to form fine O/W droplets which can also be effectively absorbed. The lipid absorption mechanisms include pinocytosis, passive diffusion or endocytosis, or absorption through lymphatic system (Georgakopoulos et al., 1992). In general, after oral administration, the oils are partially digested by the gastric lipase in the stomach and emulsified by the shear forces when passing through the pylorus. Subsequent to entering into the intestine, the oils are further emulsified, and part of the oils may be digested by pancreatic lipases and co-factor co-lipase. The digestion results in breakdown into di-/mono-glycerides and fatty acids which are incorporated into vesicles or micellar structure due to the presence of biliary salts, phospholipids and cholesterol to form micelles, mixed micelles, uni- and multi-lamellar vesicles (Porter et al., 2008). In brief, the oils can be sufficiently absorbed as the fine oil droplets in O/W emulsions or as the digestion products. Therefore, the hypothesis of the present research is that the proteins loaded inside fine oil droplets (nanometer range) may be effectively absorbed when the oil droplets are absorbed. The present goal is to develop such a delivery system in the form of O/W nanoemulsion.

Nanoemulsions (also called as microemulsions in many previous publications) are thermodynamically stable systems with the droplet size usually less than 100 nm. Nanoemulsions have been widely studied to increase the bioavailability of water-insoluble drugs (Constantinides, 1995; Gao et al., 1998; Lawrence and Rees, 2000; Kawakami et al., 2002; Araya et al., 2006; Ghosh et al., 2006). The water-insoluble drugs are usually easily dissolved in the oil phase. The mechanisms for the increase in the bioavailability are

Abbreviations: BLM, β-lactamase; CoSA, cosurfactant; EDTA, ethylene diamine tetracetic acid; FITC, fluorescein isothiocyanate isomer I; PBS, phosphate buffer saline; SA, surfactant; SNEDDS, self-nanoemulsifying drug delivery system; TCA, tricholoro acetic acid.

* Corresponding author. Tel.: +1 718 990 2510; fax: +1 718 990 6316.
E-mail address: shaoj@stjohns.edu (J. Shao).

0378-5173/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2008.05.018

due to improvement of drug solubilization, protection against enzymatic hydrolysis, the increased specific surface area of droplets that lead to wide distribution of the drug in the gastro-intestinal tract as well as surfactant-induced permeability changes (Ke et al., 2005).

Nanoemulsions have also been previously studied as a delivery system for hydrophilic drugs such as proteins. In these studies, the hydrophilic drugs are always present in the aqueous phase regardless whether it is a W/O nanoemulsion (Sarciaux et al., 1995; Constantinides et al., 1994; Cilek et al., 2005) or O/W microemulsion (Swenson and Curatolo, 1992; Ho et al., 1996). These studies have demonstrated the increased oral bioavailability of the hydrophilic macromolecules, but to a limited extent. For example, the oral bioavailability of insulin was found to be 0.2% when administered as W/O microemulsion (Cilek et al., 2005). In addition to the long-term stability issue, there are several disadvantages associated with these delivery systems where the proteins are loaded in the aqueous phase. For O/W microemulsion, the proteins can be lost into the environmental aqueous media *in vivo* when the microemulsion is administered and diluted by the GI fluid since the proteins are in the external phase. For W/O microemulsion, phase inversion may happen *in vivo* when the preparation is diluted with a relatively large amount of GI fluid, which will lead to the loss of the proteins to the environment aqueous media. Also, oral administration of a W/O microemulsion may have patient compliance issue due to the oily nature of the preparation.

Therefore, the present study aims at to develop an O/W nanoemulsion for proteins, in which the proteins are loaded in the oil phase. It is well known that the solubility of hydrophilic proteins in oils is almost zero, which presents the major barrier to load the proteins into the oil phase. To overcome this problem, the present study has employed a solid-dispersion technique for loading proteins into the oil phase. The protein is first dispersed at the single-molecular level into an amphiphile such as phospholipids, and then such a solid dispersion is dissolved into oil. The rationale is that the amphiphile will surround the protein to form a micelle structure in the oil, so that the protein can be dissolved in the oil.

Self-nanoemulsion drug delivery systems (SNEDDS) are isotropic mixtures of oil, surfactant and cosurfactant and when mixed with water, an O/W nanoemulsion will be spontaneously formed (Nazzal et al., 2002; Shah et al., 2007). These nanoemulsions are thermodynamically stable systems with the droplet size in the range of 50 nm (Nazzal et al., 2002). The small droplet size warrants the highly efficient absorption of these oil droplets. SNEDDS also can provide other advantages such as: an efficient, convenient and more patient compliant approach as SNEDDS can be filled in hard gelatin capsules due to their anhydrous nature.

Therefore, our primary objective is to develop a SNEDDS for a model protein β-lactamase (BLM, 29 kDa), in which BLM is loaded in the oil phase, in order to increase its oral bioavailability. This article describes the formulation development of such a system. The subsequent two articles report the *in vitro* transport and *in vivo* oral absorption studies on the BLM-loaded SNEDDS.

2. Materials and methods

2.1. Materials

β-Lactamase (BLM) type II lyophilized powder (29 kDa; 1.26 mU/μg; from *Bacillus cereus* EC 3.5.2.6), oleic acid, polysorbate-80, polysorbate-20, propylene glycol (PG) and polyethylene glycol-400 (PEG-400) were purchased from Sigma (St. Louis, MO). Free samples of Peceol®, Labrasol®, Caproyl-90®, Transcutol HP® and Lauroglycol FCC® were generously provided by Gattefosse Corp. (Paramus, NJ). Free samples of Cremophor EL® and Cremophor RH40® were generously provided by BASF Corp. (Florham Park, NJ). Hydrogenated soy phosphatidyl choline (SoyPC) was purchased from Avanti Polar Lipid (Alabaster, AL). Fluorescein isothiocyanate isomer I (FITC), Fluorotag kit and all other reagents and chemicals were purchased from Sigma (St. Louis, MO).

2.2. Preparation of fluorescent labeled BLM (FITC-BLM)

Fluorescein isothiocyanate isomer I (FITC) labeled BLM (FITC-BLM) was used in the formulation development studies. The FITC labeling of BLM was done with the Sigma (St. Louis, MO) kit Fluorotag according to manufacturer's instructions. Briefly, 10 ml of FITC solution (5 mM) prepared in 0.1 M sodium carbonate–bicarbonate buffer (pH 9.0) was added to 1 ml of BLM solution (0.172 mM) of the same buffer. The mixture was gently stirred for 2 h at room temperature in a glass vial with aluminum foil as a cover to protect from light. The unbound FITC was removed via gel filtration using a Sephadex G25M column with a capacity of 10 ml volume. Phosphate buffer saline solution (PBS, pH 7.4) was used as the elution phase. Fractions of 0.5 ml each were collected and scanned using a UV spectrophotometer in the wavelength range of 230–500 nm. The fractions that had peak absorbance at both (i) 280 nm (due to the amino acids of BLM) and (ii) 485 nm (due to FITC) were deemed to contain FITC-BLM, and were combined together and was lyophilized and then stored at 4 °C and protected from light with an aluminum cover. The yield of FITC-BLM was determined using a UV spectrophotometer.

2.3. Stability of FITC-BLM

FITC-BLM stability in PBS was studied. FITC-BLM solutions (1000 mU/ml) in PBS were stored at 4 °C, 25 °C or 37 °C, with or without the protection from light. In addition, BLM solid material stored at 4 °C in a refrigerator up to 3 months was analyzed for stability. Samples were taken at pre-determined time points and subjected to an HPLC assay and a fluorimetric assay (see Section 2.9). The HPLC method examines the cleavage of FITC from FITC-BLM and the fluorimetric method examines the stability of fluorescent intensity values of FIT-BLM. The HPLC system consisted of a Waters 600E system controller, a Waters 717 Autosampler, and a Waters 470 Scanning fluorescence detector set at an excitation and emission wavelength of 485 nm and 535 nm, respectively. The column used was a Grace Vydac Protein C4 column. The mobile phases were A: 0.1% (v/v) trifluoro acetic acid (TFA) in water and B: 0.1% (v/v) TFA in 95% acetonitrile and 5% water set to run at flow of 1.5 ml/min. The gradient composition was 75% A and 25% B set initially and to switch over gradually to 25% A and 75% B over a 20-min period. The injection volume was 10 μl. A peak at 5 min would indicate the presence of free FITC in the solution.

2.4. Preparation of solid dispersion of FITC-BLM

SoyPC was evaluated as the carrier for the solid dispersion of FITC-BLM. Aqueous dispersion of SoyPC in PBS was mixed with FITC-BLM solution in PBS at the weight ratio of 4:1 (SoyPC: BLM). Then this mixture was lyophilized by a Labconco bench top lyophilizer unit at a condensation temperature of −50 °C. The resultant freeze-dried powder was the solid dispersion of FITC-BLM.

2.5. Development of SNEDDS prototypes

SNEDDS prototypes are an oil solution consisted of an oil, surfactant and a cosurfactant which will spontaneously form nanoemulsion when mixed with water. Four oils, five non-ionic surfactants (SA) and three cosurfactants (CoSA) were used in this study for the formulation development of SNEDDS prototypes (Table 1).

Table 1
SNEDDS components evaluated

Component	Name	Chemical name	HLB value
Oils	Oleic acid	Octadecenoic acid	1
	Peceol®	Glyceryl mono oleate	3
	Lauroglycol FCC®	Propylene glycol mono laurate	4
	Caproyl-90	Propylene glycol mono caprylate	6
Surfactants	Labrasol®	Caprylocaproyl macrogol glycerides	14
	Cremophor EL®	Polyoxy ethylene 35 castor oil	12–14
	Cremophor RH40®	Polyoxy ethylene 40 hydrogenated castor oil	14–16
	Polysorbate-80	Polyoxyethylene 20 sorbitan mono oleate	15
	Polysorbate-20	Polyoxyethylene 20 sorbitan mono laurate	15
Cosurfactants	Transcutol HP®	Diethylene glycol monoethyl ether	–
	PEG 400	Polyethylene glycol 400	–
	PG	Propylene glycol	–

$2\hat{3}$ Full Factorial with 4 Center/Pure Error Points experimental design was adopted to determine the ratios of oil, SA and CoSA to be evaluated. To each oil/SA/CoSA system (1 g) taken in a beaker set up with gentle magnetic stirring (50 rpm), PBS (100 ml) was added dropwise to see whether nanoemulsion was formed spontaneously. Nanoemulsion was characterized as clear, transparent liquid with or without slight bluish tint. The oil/SA/CoSA systems that formed nanoemulsion spontaneously were "SNEDDS prototypes" and were further studied.

2.6. Preparation of FITC-BLM-loaded SNEDDS

FITC-BLM was loaded into SNEDDS by dissolving the solid dispersion of FITC-BLM into SNEDDS prototype. Fixed amount of FITC-BLM solid dispersion (4 mg) was added to 2 g of SNEDDS prototype in a tube. The tube was shaken for 2 h and then gently vortexed and placed in the sonicator (Branson, Model 2210) maintained at 25 °C for 60 min. If a clear oily solution was obtained, it indicated that the FITC-BLM solid dispersion was soluble in that particular SNEDDS prototype system, otherwise additional 1 g of the respective SNEDDS prototype was added to the tube and then shaken, gently vortexed and sonicated as mentioned above. If it still did not result in a clear oily solution, it was concluded that the FITC-BLM solid dispersion was not soluble in that particular SNEDDS prototype, and that SNEDDS prototype was excluded from further studies.

2.7. Selection of best FITC-BLM-loaded SNEDDS nanoemulsion

To 2 g of a FITC-BLM-loaded SNEDDS, 4 ml of PBS was added. The mixture was then gently vortexed to form nanoemulsion. The droplet size of the nanoemulsion was measured. The various nanoemulsions were screened for their enhancement of FITC-BLM transport through the MDCK cell monolayer *in vitro* to select the best FITC-BLM-loaded SNEDDS nanoemulsion. Nanoemulsion NE-12-7 was found to give the high transport rate, and hence was further studied.

2.8. Characterization of BLM-loaded SNEDDS NE-12-7

2.8.1. Phase diagram and nanoemulsion type

Lauroglycol FCC (oil for SNEDDS NE-12-7) was mixed with Cremophor EL/Transcutol HP (4:3, the SA and CoSA for NE-12-7) at various ratios to form the oil phases. Then to a fixed amount of these oil phases taken in a beaker at gentle stirring (50 rpm), PBS was added dropwise. Then the mixture was examined visually after each dropwise addition to see whether clear and transparent nanoemulsion was formed. The nanoemulsions formed were further evaluated for O/W or W/O type by the measurement of conductivity with a VWR conductivity meter (Traceable).

2.8.2. Droplet size

The droplet size of the BLM-loaded SNEDDS NE-12-7 nanoemulsion was determined using a Nicomp 380 ZLS laser diffraction sizer (PSS Nicomp, Santa Barbara, CA, USA). The effect of dilution factors (SNEDDS: water ratio), the pH and bile salt (deoxycholate) of the aqueous media on the droplet size of nanoemulsion was determined. The various aqueous media used were: deionized water, 0.1N HCl (pH 1), acetate buffer (pH 5.5), PBS (pH 7.4), and 0.5% sodium deoxycholate in PBS. The dilution factors of 2, 10 and 100 times were studied in PBS.

2.8.3. Stability of BLM loaded in SNEDDS NE-12-7

BLM-loaded SNEDDS NE-12-7 was stored at 4 °C in a refrigerator. Then at 1, 2, 4, 8 and 12 week intervals, samples were taken to analyze the enzyme activity of BLM. Freshly prepared BLM-loaded SNEDDS was considered as time zero and was used as a standard to estimate the degradation in enzyme activity of BLM after being stored for different time periods.

2.8.4. Leakage of FITC-BLM from the oil phase

The leakage of FITC-BLM from the oil phase into the aqueous phase in the nanoemulsion NE-12-7 was studied by a gel filtration method. Briefly, FITC-BLM-loaded SNEDDS NE-12-7 was mixed with PBS (pH 7.4) at the ratio of 1:4 to form nanoemulsion containing 360 mU/mL BLM. Then 0.5 ml of this nanoemulsion was loaded on a Sephadex G-100 column of 10-ml capacity. PBS was used as the elution phase. Fractions of 0.5 ml each were collected and scanned by a UV spectrophotometer in the wavelength range of 230–500 nm. The fractions that had peak absorbance at both (i) 280 nm (due to the amino acids of BLM) and (ii) 485 nm (due to FITC) were deemed to contain FITC-BLM. The concentration of FITC-BLM in these fractions was analyzed by the fluorimetry (see Section 2.9). FITC-BLM free solution in PBS (360 mU/ml) and the nanoemulsion prepared by the mixing of FITC-BLM free solution in PBS with blank SNEDDS NE-12-7 at the ratio of 4:1 (360 mU/ml in the nanoemulsion) were used as the controls.

2.9. Assay of FITC-BLM

FITC-BLM was assayed with a spectro-fluorimeter (TRIAD multi mode reader, Dynex Technologies), at excitation and emission wavelength of 485 nm and 535 nm, respectively. A linear standard curve of FITC-BLM in PBS was generated in the concentration range of 0–1300 mU/ml. The assay method was validated in terms of within-day variation, day-to-day variation, and precision.

2.10. Assay of BLM enzymatic activity

The enzyme activity of BLM was determined by the modified Baker's fluorimetric assay method (Shao and Kaushal, 2004), wherein BLM converts ampicillin (substrate) to ampiciolloate, which can be further processed to produce a fluorescent product. Briefly, 0.2 ml of BLM-loaded SNEDDS was mixed with 0.8 ml of ampicillin (11.4 mM) in PBS at 37 °C. After exactly 10 min, the reaction was terminated by adding 0.1 ml of trichloroacetic acid (TCA) at 4 °C. The mixture was then diluted to 5 ml with acetate buffer (pH 4) containing ascorbic acid (0.5 mg/ml) and EDTA (50 mM), and was

heated at 100 °C for exactly 30 min. After cooling to room temperature for 1 h, the samples were placed in 96 well plate (100 μl/well), and the fluorescence was read by a microplate reader at an excitation wavelength of 365 nm and an emission wavelength of 455 nm. The activity of BLM was then calculated according to the standard curves. Blank SNEDDS was used as control. This assay method was validated in terms of within-day variation, day-to-day variation, and precision.

3. Results

3.1. Preparation of FITC-BLM

FITC-BLM was prepared by mixing solutions of FITC and BLM in 0.1 M sodium carbonate–bicarbonate buffer (pH 9) for 2 h and then passed the solution mixture through the Sephadex G-25 column along with PBS. Ten fractions of 0.5 ml each collected in tubes were scanned using a UV spectrophotometer from 230 nm to 500 nm range. Fractions 1 and 2 were discarded as they did not show any absorbance, which indicated that they were just plain buffer. Fractions 3–7 had two peaks, one at 280 nm corresponding to the amino acids of BLM and another at 485 nm corresponding to FITC. These fractions containing FITC-BLM were combined and lyophilized. The lyophilized powder of FITC-BLM was thus prepared. The yield was 83 ± 2%. Fractions 8–10 showed only one peak at 485 nm which indicated that it was free FITC. None of the fractions collected showed only one peak at 280 nm, which indicated that un-labeled BLM was not present.

3.2. Stability of FITC-BLM

Based on the fluorescent measurement, FITC-BLM solid material stored at 4 °C was stable when protected from light for up to 3 months. No assay was done beyond 3 months storage. FITC-BLM in PBS solution when protected from light maintained its fluorimetric intensity at 90% up to 72 h, 24 h and 12 h at 4 °C, room temperature and 37 °C, respectively. So in all the experiments where FITC-BLM solution was used, the containers were covered with aluminum foil and the experiments were completed within 12 h after FITC-BLM solution was prepared.

When FITC solution was injected into HPLC, a peak corresponding to FITC was obtained at 5 min. This peak was not seen in the HPLC chromatograms of (1) FITC-BLM stored (as a solid) for 3 months at 4 °C; (2) FITC-BLM solution in PBS for 72 h at 4 °C; (3) FITC-BLM solution in PBS for 24 h at room temperature; and (4) FITC-BLM solution in PBS for 12 h at 37 °C. The results indicate that FITC-BLM did not degrade to yield free FITC in these samples.

3.3. Preparation of solid dispersion of FITC-BLM

In order to load BLM, a highly hydrophilic protein into SNEDDS, a solid dispersion of FITC-BLM prepared with SoyPC was prepared. The SoyPC dispersion of BLM was soluble in 16 various SNEDDS prototypes. The actual loading of BLM per mg of BLM-SoyPC dispersion was found to be 166.65 ± 1.0 μg (~2100 mU) of BLM per 1 mg of dispersion for 10 batches. This result indicates that the recovery of BLM in this SoyPC dispersion was 83.3 ± 0.5%. In addition, BLM-SoyPC physical mixture in the ratio of 1:4 was found to be insoluble in the 16 different SNEDDS prototypes.

3.4. Development of SNEDDS formulations

The experimental design involved assigning low, mid and high values for the oil, SA and CoSA. Then based on the coded (−1, 0, +1) design shown in Table 2, 12 different run #s were obtained, which indicated the ratios at which the oil, SA and CoSA (NE-#) should be mixed with each other to evaluate self-nanoemulsion formation. The different run #s covered the following SA: CoSA ratios of 4:1, 4:2, 4:3 and 3:2. It also covered the following oil: surfactant mixture (SA + CoSA) ratios of 3:5, 3:7, 4:5, 5:3 and 5:5. Since there were four oils, five surfactants and three cosurfactants, it resulted in 720 (60 combinations × 12 ratios) potential SNEDDSs of different compositions. A total of 33 SNEDDS prototypes out of the 720 potential SNEDDSs were able to spontaneously form nanoemulsions upon the addition of aqueous phase (compositions not shown).

Table 2
Experimental design runs tested for SNEDDS evaluation

Run #	Oil	Surfactant (SA)	Cosurfactant (CoSA)
1	−1	−1	1
2	0	0	0
3	1	1	−1
4	1	−1	−1
5	−1	−1	−1
6	0	0	0
7	1	1	1
8	−1	1	1
9	0	0	0
10	1	−1	1
11	−1	1	−1
12	0	0	0

$2\hat{3}$ Full Factorial with four Center/Pure Error Points experimental design was adopted to determine the ratios of oil, SA and CoSA to be evaluated for SNEDDS formation. Each component was evaluated at three levels: low (−1), medium (0) and high (+1).

Out of these 33 SNEDDS prototypes, only 16 SNEDDS prototypes were able to dissolve the FITC-BLM–SoyPC solid dispersion. These 16 FITC-BLM-loaded SNEDDS prototypes are listed in Table 3. The amount of FITC-BLM (mU) loaded in each 1 g of the SNEDDS prototypes was in the range of 1953 ± 37.5–2454.6 ± 34.1 mU/1 g of SNEDDS. The 16 FITC-BLM-loaded SNEDDS spontaneously formed nanoemulsion with a mean droplet size in the range of 8–28 nm upon the addition of PBS at the ratio of 1:2 (SNEDDS: PBS).

All the 16 nanoemulsions showed increased BLM transport through MDCK monolayer as compared to the free solution form. SNEDDS NE-12-7 nanoemulsion resulted in the highest transport rate of BLM across the cell monolayer. Transport data is presented in the subsequent manuscript. Therefore, NE-12-7 was considered as the best formula and was further characterized.

3.5. Characterization of SNEDDS NE-12-7 nanoemulsion

3.5.1. Phase diagram and nanoemulsion type

The pseudo ternary phase diagram for SNEDDS NE-12-7 in Fig. 1 indicates the nanoemulsion region. The nanoemulsion region covers (oil/SA/CoSA): water range of 0.4: 99.6–97.5: 2.5. This indicates that with just 2.5% of water added to SNEDDS NE-12-7 is sufficient to form nanoemulsion. Also the nanoemulsion region covers oil:SA/CoSA mix in the range of 1:1.5 ratio to 1:9. The sigmoid curve of conductivity values of the nanoemulsion plotted against the aqueous fraction of the nanoemulsion is indicated in Fig. 2. It can be seen that the inversion from W/O to O/W occurred when the fraction of the aqueous phase increased to about 40%.

3.5.2. Droplet size

The results of droplet size measurement of NE-12-7 nanoemulsion shown in Fig. 3 indicate that the droplet size did not change upon increasing dilution factor from 2 to 100-fold. Hence the dilution factor had no effect on the droplet size and the self-nanoemulsifying behavior, indicating that NE-12-7 nanoemulsion is very robust to aqueous volume of the environment.

Table 3
Composition of FITC-BLM-loaded SNEDDS capable of spontaneously forming nanoemulsion

SNEDDS prototypes	Composition O/SA/CoSA	Ratio of O/SA/CoSA	Amount of FITC-BLM (mU)/g of SNEDDS prototype (mean ± S.D., $n = 3$)
Lauroglycol FCC-based SNEDDS			
NE-12-5	Lauroglycol FCC/Cremophor EL/Transcutol HP	3:2:1	2273.3 ± 25.3
NE-12-7	Lauroglycol FCC/Cremophor EL/Transcutol HP	5:4:3	2410.0 ± 9.6
NE-13-2	Lauroglycol FCC/Cremophor RH40/Transcutol HP	4:3:2	2312.6 ± 28.4
NE-13-3	Lauroglycol FCC/Cremophor RH 40/Transcutol HP	5:4:1	1998.3 ± 17.1
NE-13-7	Lauroglycol FCC/Cremophor RH40/Transcutol HP	5:4:3	2217.3 ± 37.0
NE-13-8	Lauroglycol FCC/Cremophor RH40/Transcutol HP	3:4:3	2023.3 ± 45.8
NE-13-11	Lauroglycol FCC/Cremophor RH40/Transcutol HP	3:4:1	1953.0 ± 37.5
Caproyl-90-based SNEDDS			
NE-17-2	Caproyl-90/Cremophor EL/Transcutol HP	4:3:2	2394.0 ± 72.0
NE-17-3	Caproyl-90/Cremophor EL/Transcutol HP	5:4:1	2379.3 ± 25.6
NE-17-5	Caproyl-90/Cremophor EL/Transcutol HP	3:2:1	2454.6 ± 34.1
NE-17-7	Caproyl-90/Cremophor EL/Transcutol HP	5:4:3	2352.3 ± 48.0
NE-18-8	Caproyl-90/Cremophor RH40/Transcutol HP	3:4:3	2302.6 ± 57.1
NE-37-5	Caproyl-90/Cremophor EL/PEG-400	3:2:1	1979.6 ± 31.6
NE-57-2	Caproyl-90/Cremophor EL/Propylene glycol	4:3:2	2189.0 ± 18.02
NE-57-3	Caproyl-90/Cremophor EL/Propylene glycol	5:4:1	2175.0 ± 34.5
NE-57-7	Caproyl-90/Cremophor EL/Propylene glycol	5:4:3	2089.3 ± 53.5

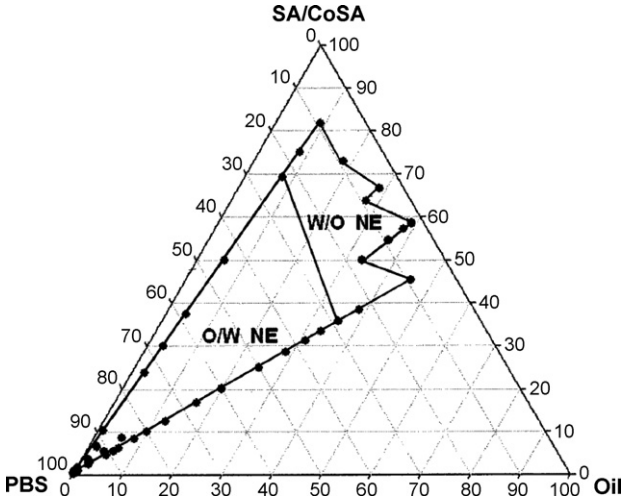

Fig. 1. Pseudo-ternary phase diagram showing O/W and W/O nanoemulsion region of NE-12-7 (Oil: Lauroglycol FCC; SA: Cremophor EL; CoSA: Transcutol HP and water) at room temperature. The ratio of SA/CoSA is 4:3.

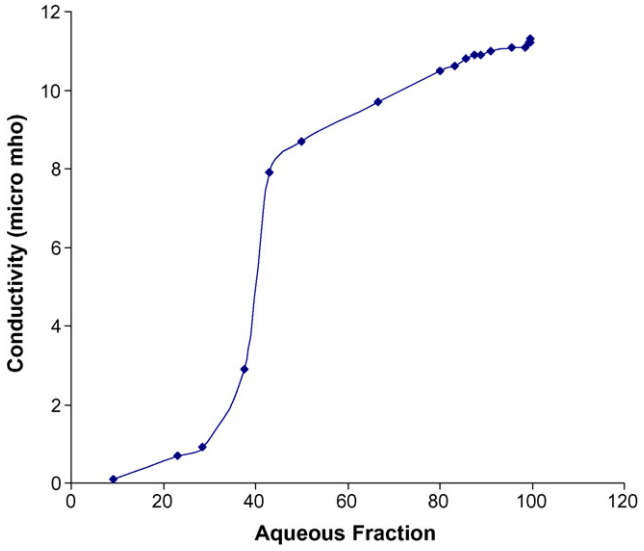

Fig. 2. Conductivity values of NE-12-7 nanoemulsion vs. the aqueous fraction of the nanoemulsion.

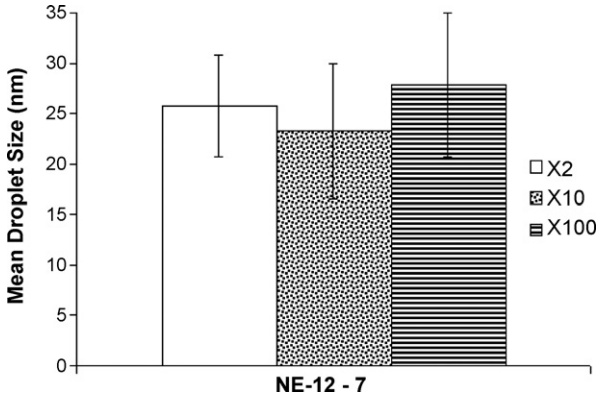

Fig. 3. Effect of the dilution factor on the droplet size of NE-12-7 nanoemulsion. SNEDDS NE-12-7 was mixed with water at the ratios of 1:2, 1:10 and 1:100 to form nanoemulsion, and then the droplet size was measured (mean ± S.D., $n = 3$).

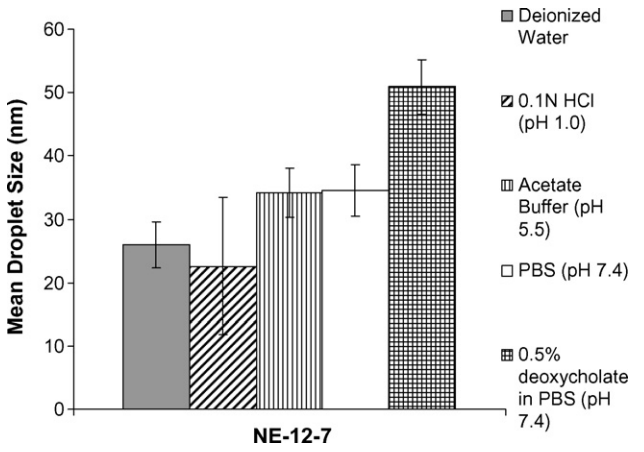

Fig. 4. Effect of pH and deoxycholate on the droplet size of NE-12-7 nanoemulsion. SNEDDS NE-12-7 was mixed with various aqueous media at the ratio of 1:100 to form nanoemulsion. Then the droplet size of the nanoemulsion was measured (mean ± S.D., $n = 3$).

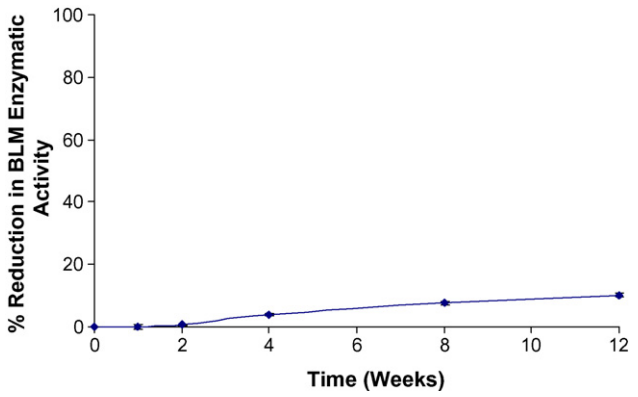

Fig. 5. Stability of BLM in SNEDDS NE-12-7. BLM-loaded SNEDDS NE-12-7 was stored at 4 °C, and the enzymatic activity of BLM was assayed at certain time periods and compared to the initial value (mean ± S.D., $n = 3$).

The mean droplet size of NE-12-7 nanoemulsion formed upon 100 times dilution with various pH media (1, 5.5 and 7.4) is shown in Fig. 4. It can be clearly seen that the droplet size of the resultant nanoemulsion formed did not change significantly by the pH and or the ionic strength of the diluent. However the media containing the bile salt increased the droplet size of the nanoemulsion by 48%, compared to PBS as the media.

3.5.3. Stability of BLM in SNEDDS NE-12-7

The stability of BLM in SNEDDS NE-12-7 was measured based on BLM enzymatic activity assay. The decrease of enzymatic activity of BLM in SNEDDS NE-12-7 stored at 4 °C was less than 10% at 12 weeks (Fig. 5).

3.5.4. Leakage of FITC-BLM from the oil phase

In order to determine whether FITC-BLM remained in the oil phase of the nanoemulsion, a gel filtration analysis was used. The method was based on the elution time of FITC-BLM in the different samples. The results are presented in Fig. 6. When free solution of FITC-BLM (Sample I) was loaded onto the column, FITC-BLM was eluted out in Fractions 6–9. For the nanoemulsion prepared by free FITC-BLM solution with blank SNEDDS (Sample II), the FITC-BLM was eluted out in Fractions 8–11. In both the cases of Sample I and II, only one peak was observed in the chromatograms. However, when the nanoemulsion prepared by the FITC-BLM-loaded SNEDDS (Sample III) was analyzed, there were two peaks in the chromatogram: the first peak consisted of Fractions 9–11 and the second peak consisted of Fractions 13–15. This first peak is very much similar as the peak from Sample II where the FITC-BLM existed in the aqueous phase of the nanoemulsion. Therefore, the first peak of Sample III represents the FITC-BLM leaked out of the oil phase, and the second peak represents the FITC-BLM remaining in the oil phase. The results of the fluorimetric measurement of the fractions show that there was 49% of the FITC-BLM remaining in the oil phase of the nanoemulsion NE-12-7.

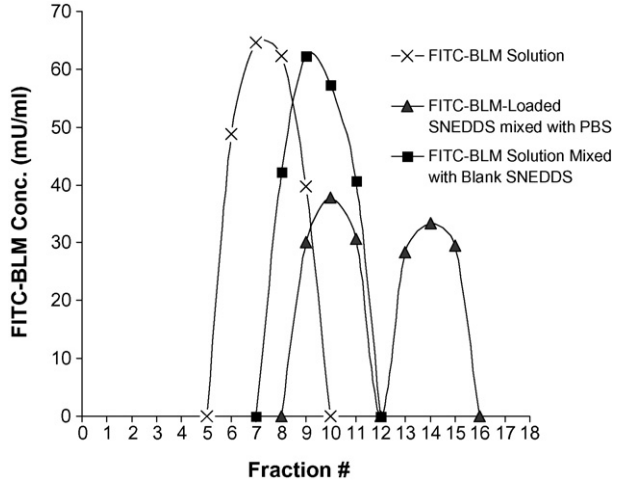

Fig. 6. Gel filtration chromatograms of FITC-BLM from different samples. The samples were loaded onto a Sephadex G-100 column, and eluted by PBS (pH 7.4). The elution fractions were collected and assayed for FITC-BLM by the fluorimetry.

4. Discussion

Self-microemulsification has been shown to be specific to the nature of oil, nature of SA/CoSA pair, SA/CoSA concentration, and that only very specific combination of these ingredients could result in efficient self-microemulsification (Gursoy and Benita, 2004). Therefore, an effort was made in this study to select the right composition for SNEDDS. The four oils, five SA and three CoSA screened in this study provided 60 combinations ($4 \times 5 \times 3$) of potential SNEDDS systems and these 60 combinations had to be evaluated at different ratios of oil, SA and CoSA, which would result in construction of thousands of phase diagrams. So, in an effort to minimize the number of phase diagrams to be constructed, an experimental design, namely $2\hat{3}$ Full Factorial with 4 Center/Pure Error Points was adopted to determine the ratios at which oil, SA and CoSA to be evaluated for SNEDDS composition. This experimental design allowed the systematic evaluation of the ratios of oil, SA and CoSA for SNEDDS formation with 720 experimental runs.

In this study, two oils, Lauroglycol FCC and Caproyl-90, with HLB values greater than the other two oils, oleic acid and Peceol produced successful SNEDDS formulations. Lauroglycol FCC and Caproyl-90 are saturated medium chain fatty acids of carbon chain length 15 and 11, respectively. Both oleic acid and Peceol are unsaturated long chain fatty acid having carbon chain length of 18 and 21, respectively. It seems that oils of medium carbon chain length and higher HLB values are better than oils of longer chain length and lower HLB values to form SNEDDS. However, it is still possible to develop SNEDDS formulations with long chain fatty acids with some suitable surfactants. But no efforts were made in this study to proceed in that direction.

In general, the surfactants for SNEDDS should be very hydrophilic with HLB value in the range of 15–21 (Eastoe et al., 1996). The results show that the surfactants Cremophor EL and Cremophor RH 40 can be used with the oils Lauroglycol FCC and Caproyl-90 to form SNEDDS. In terms of the other three SAs tested, polysorbate-80 provided only one SNEDDS prototype and neither polysorbate-20 nor Labrasol provide any SNEDDS prototype. These results indicate that HLB is not the only factor to determine the surfactant's ability to form SNEDDS since all the five SAs have similar HLB value. The structure of the surfactant also played a role. Cremophor is polyethoxylated castor oil which is a mixture of ricinoleic acid, polyglycol ester, glyerol polyglycol esters, and polyglycols whereas polysorbate is a derivative of polyoxylated sorbitol and oleic acid. Cremophors have branched alkyl structure whereas polysorbates have linear chain alkyl structure. It has been indicated that alkyl chain structure of surfactant imparts an effect on the penetration of oil onto the curved surfactant film (Eastoe et al., 1996) thus resulting in the self-nanoemulsion formation. Based on the present study results, it seems that branched

alkyl structure of a SA is better for self-nanoemulsion formation.

Many previous studies have shown that CoSA can help the SA to form SNEDDS (Kang et al., 2004; Date and Nagarsenker, 2007). CoSA are amphiphilic with an affinity for both the oil and aqueous phases and partitions to an appreciable extent into the SA interfacial monolayer present at the oil–water interface (Lawrence and Rees, 2000). CoSA provide very low interfacial tension required for the stability and formation of nanoemulsion; modify the curvature and increases the fluidity of the interfacial film. The alternate of SA and CoSA molecules induce a dramatic decrease of interfacial tension. The length of the CoSA molecule as well as the medium ionic strength plays a very important role in the size of the nanoemulsion existence field (Georgakopoulos et al., 1992). All the three CoSA used in this study were capable of forming nanoemulsion in combination with Cremophor EL as the SA. And, Transcutol HP, a glycolic ether was capable of forming nanoemulsion with Cremophor EL and Cremophor RH 40 in various combinations. Based on the lipid formulation classification system (Pouton, 2006), the 16 SNEDDS formulations developed are Type IIIA lipid formulations because these are self-microemulsifying delivery systems that contain water soluble surfactants with HLB values >12 and cosolvents such as transcutol/PEG-400/propylene glycol.

BLM, as a hydrophilic molecule, cannot be dissolved into the oil phase of the SNEDDS by itself. To solve this problem, our approach is to first prepare a solid dispersion of BLM with an amphiphilic carrier, SoyPC, and then dissolve this solid dispersion into the oil phase of the SNEDDS. In such a way, BLM, in single molecule status, is surrounded by SoyPC, which has certain affinity with the oil and thus bring the BLM into the oil phase. The physical mixture of BLM and SoyPC was not able to dissolve BLM into the oil phase. It is very obvious that the pre-dispersion of BLM into SoyPC at a single molecule status is critical to dissolve BLM into the oil phase. This method can be applied to other hydrophilic drugs as well.

Since the present SNEDDS preparation is intended mainly for oral delivery of protein drugs, the potential effect of GI tract conditions on the nanoemulsion was evaluated *in vitro*. The dilution volume had no effect on the droplet size and self-nanoemulsifying behavior of NE-12-7, indicating that the preparation will be very robust upon *in vivo* administration. The results also demonstrated that the pH in the range of 1.0–7.4 did not have any effect on the droplet size. This is probably due to the SA Cremophor EL and the oil Lauroglycol FCC are non-electrolytes and their functions are insensitive to pH and or ionic strength changes. But when the bile salt solution, namely 0.5% sodium deoxy cholate solution in PBS, was used as the diluent, the droplet size increased by 48%. This indicates that the presence of bile salts may have caused the aggregation of the nanoemulsion droplets, but only to a limited extent, since the resultant emulsion is still a nanoemulsion with droplet size less than 50 nm. This increase in droplet size in the presence of bile salts is consistent with the previous study (Ritschel, 1991) which suggests that the *in vivo* emulsification of the nanoemulsion by bile salts might lead to an increase in the droplet size.

The present study results indicate that approximately 50% of BLM loaded into SNEDDS leaked out of the oil phase into the aqueous phase when the SNEDDS was mixed with PBS to form nanoemulsion. Our hypothesis is that only when the protein loaded inside the oil droplets, can the absorption of the protein be significantly increased. Therefore, this kind of leakage of protein from the oil phase into aqueous phase will decrease its absorption. How to retain the protein inside the oil phase becomes an important issue to be addressed in the future studies.

5. Conclusion

A SNEDDS was successfully developed with Lauroglycol FCC, Cremophor EL and Transcutol HP as the components. BLM, a hydrophilic protein, was loaded into the SNEDDS oil phase by solid dispersion technique.

Acknowledgements

The authors wish to thank Gattefosse Corp. and BASF Corp. for providing the chemicals.

References

Almeida, A.J., Souto, E., 2007. Solid lipid nanoparticles as a drug delivery system for peptides and proteins. Adv. Drug Deliv. 59, 478–490.
Araya, H., Tomita, M., Hayashi, M., 2006. The novel formulation design of O/W microemulsion for improving the gastrointestinal absorption of poorly water soluble compounds. Int. J. Pharm. 305, 61–74.
Cilek, A., Celebi, N., Tirnaksiz, F., Tay, A., 2005. A lecithin-based microemulsion of rh-insulin with aprotinin for oral administration: investigation of hypoglycemic effects in non-diabetic and STZ-induced diabetic rats. Int. J. Pharm. 298, 176–185.
Constantinides, P.P., Scalart, J.P., Lancaster, C., Marcello, J., Marks, G., Ellens, H., Smith, P.L., 1994. Formulation and intestinal-absorption enhancement evaluation of water-in-oil microemulsions incorporating medium-chain glycerides. Pharm. Res. 11, 1385–1390.
Constantinides, P.P., 1995. Lipid microemulsions for improving drug dissolution and oral absorption: physical and biopharmaceutical aspects. Pharm. Res. 12, 1561–1572.
Date, A.A., Nagarsenker, M.S., 2007. Design and evaluation of self-nanoemulsifying drug delivery systems (SNEDDS) for cefpodoxime proxetil. Int. J. Pharm. 329, 166–172.
Eastoe, J., Hetherington, K.J., Sharpe, D., Dong, J., 1996. Mixing of alkanes with surfactant monolayers in microemulsions. Langmuir 12, 3876–3880.
Gao, Z.G., Choi, H.G., Shin, H.J., Park, K.M., Lim, S.J., Hwang, K.J., Kim, C.K., 1998. Physicochemical characterization and evaluation of a microemulsion system for oral delivery of cyclosporin A. Int. J. Pharm. 161, 75–86.
Georgakopoulos, E., Farah, N., Vergnault, G., 1992. Oral anhydrous non-ionic nanoemulsions administered in soft gel capsules. Bull. Tech. Gattefosse, 11–19.
Ghosh, P.K., Majithiya, R.J., Umrethia, M.L., Murthy, R.S.R., 2006. Design and Development of microemulsion drug delivery system of acyclovir for improvement of oral bioavailability. AAPS PharmSciTech. 7, 77.
Gursoy, N.R., Benita, S., 2004. Self-emulsifying drug delivery systems (SEDDS) for improved oral delivery of lipophilic drugs. Biomed. Pharm. 58, 173–182.
Ho, H.O., Hsiao, C.C., Sheu, M.T., 1996. Preparation of microemulsions using polyglycerol fatty acid esters as surfactant for the delivery of protein drugs. J. Pharm. Sci. 85, 138–143.
Kang, B.K., Lee, J.S., Chon, S.K., Jeong, S.Y., Yuk, S.H., Khang, G., Lee, H.B., Cho, S.H., 2004. Development of self-microemulsifying drug delivery systems (SMEDDS) for oral bioavailability enhancement of simvastatin in beagle dogs. Int. J. Pharm. 274, 65–73.
Ke, W.T., Lin, S.Y., Ho, H.O., Sheu, M.T., 2005. Physical characterizations of microemulsion systems using tocopheryl polyethylene glycol 1000 succinate (TPGS) as a surfactant for the oral delivery of protein drugs. J. Control. Release 102, 489–507.
Kawakami, K., Yoshikawa, T., Moroto, Y., Kanaoka, E., Takahashi, K., Nishihara, Y., Masuda, K., 2002. Microemulsion formulation for enhanced absorption of poorly soluble drugs. I. Prescription design. J. Control. Release 81, 65–74.
Lawrence, M.J., Rees, G.D., 2000. Microemulsion-based media as novel drug delivery systems. Adv. Drug Deliv. Rev. 45, 89–121.
Nazzal, S., Smalyukh, I.I., Lavrentovich, O.D., Khan, M.A., 2002. Preparation and *in vitro* characterization of a eutectic based semisolid self-nanoemulsified drug delivery system (SNEDDS) of ubiquinone: mechanism and progress of emulsion formation. Int. J. Pharm. 235, 247–265.
Porter, C.J.H., Pouton, C.W., Cuine, J.F., Charman, W.N., 2008. Enhancing intestinal drug solubilisation using lipid-based delivery systems. Adv. Drug Del. Rev. 60, 673–691.
Pouton, C.W., 2006. Formulation of poorly water-soluble drugs for oral administration: physicochemical and physiological issues and the lipid formulation classification system. Eur. J. Pharm. Sci. 29, 278–287.
Ritschel, W.A., 1991. Microemulsions for improved peptide absorption from the gastrointestinal tract. Methods Find. Exp. Clin. Pharmacol. 13, 205.
Sarciaux, J.M., Acar, L., Sado, P.A., 1995. Using microemulsion formulations for oral drug delivery of therapeutic peptides. Int. J. Pharm. 120, 127–136.
Shah, R.B., Zidan, A.S., Funck, T., Tawakkul, M.A., Nguyenpho, A., Khan, M.A., 2007. Quality by design: characterization of self-nano-emulsified drug delivery systems (SNEDDs) using ultrasonic resonator technology. Int. J. Pharm. 341, 189–194.

Shao, J., Kaushal, G., 2004. Normal flora: living vehicles for non-invasive protein drug delivery. Int. J. Pharm. 286, 117–124.
Swenson, E.C., Curatolo, W.J., 1992. Intestinal permeability enhancement for proteins, peptides and other polar drugs: mechanisms and potential toxicity. Adv. Drug Deliv. Rev. 8, 39–92.
Toorisaka, E., Hashida, M., Kamiya, N., Ono, H., Kokazu, Y., Goto, M., 2005. An enteric-coated dry emulsion formulation for oral insulin delivery. J. Control. Release. 107, 91–96.
Van Hoogdalem, E.J., De Boer, A.G., Breimer, D.D., 1989. International drug absorption enhancement: an overview. Pharmacol. Ther. 44, 407–443.