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Self-nanoemulsifying drug delivery system (SNEDDS) for oral delivery of protein drugs
III. *In vivo* oral absorption study

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ABSTRACT

To use self-nanoemulsifying drug delivery system (SNEDDS) to deliver hydrophilic proteins orally. β-Lactamase (BLM), a 29 kDa protein was used as a model protein, and formulated into the oil phase of a SNEDDS through solid dispersion technique. The oral absorption of BLM in rats when delivered by such a SNEDDS was investigated. Oral delivery of 4500 mU/kg of BLM in SNEDDS nanoemulsion resulted in the relative bioavailability of 6.34%, C_{max} of 1.9 mU/ml and mean residence time of 12.12 h which was 1.5-, 2.7- and 1.3-fold higher than that by free solution, respectively. Delivery of BLM in the aqueous phase of the nanoemulsion resulted in a PK profile similar to that by the free solution. BLM when loaded in oil phase of SNEDDS, can significantly enhance the oral bioavailability of BLM. SNEDDS has a great potential for oral protein delivery.

1. Introduction

Oral delivery systems remain the most attractive and widely used method for delivering most therapeutic agents including protein/peptide drugs (hereafter called as proteins) into the systemic circulation, as it avoids the pain and risk of infection associated with parenteral administration and thereby leads to greater patient compliance (Lyons et al., 2000). Two major problems prevent almost all proteins being delivered by non-invasive routes: poor permeability and extensive degradation. The biochemical barrier, composed of exo- and endo-peptidases, is well designed for the digestion of proteins to a mixture of amino acids and hence the oral intact absorption of proteins is difficult. The physical barriers: the unstirred water layer, mucous layer, apical and basal cell membranes and cell contents, tight junctions, basement membrane and the wall of lymph and blood capillaries are also very difficult for the proteins to pass through (Banga and Chien, 1988). The formulation that protects the protein from breakdown in the gut and increases the intestinal permeability will be likely to enhance the oral bioavailability of the protein.

It is known that oil droplets can be easily absorbed orally via lipid absorption mechanisms such as endocytosis, passive diffusion or pinocytosis (Georgakopoulos et al., 1992). We have hypothesized that proteins loaded in the oil droplets may be effectively absorbed when the oil is absorbed. To test this hypothesis, we have developed a self-nanoemulsifying drug delivery system (SNEDDS) in which the proteins are loaded in the oil phase.

SNEDDS is defined as isotropic mixtures of oil, surfactant and cosurfactant that rapidly form nanoemulsion upon mixing with water (Nazzal et al., 2002). The self-nano-emulsification process occurs spontaneously because the free energy required to form nanoemulsion is either low and positive or negative (Paul and Moulik, 1997). The formed nanoemulsion usually has a narrow droplet size distribution, which is typically less than 100 nm (Gursoy and Benita, 2004). The fine droplet size provides another major basis for high absorption efficiency for proteins when they are loaded in the oil droplets.

In our previous study (Venkata Ramana Rao and Shao, 2008), β-lactamase (BLM), a 29 kDa protein, has been formulated into a SNEDDS through a solid-dispersion technique. When mixed with water, the SNEDDS will spontaneously form an O/W nanoemulsion, and BLM is present in the oil phase. Our

Abbreviations: AUC, area under the curve; AUMC, area under the first-moment curve; BLM, β-lactamase; GIT, gastrointestinal tract; NE, nanoemulsion; MRT, mean residence time; PBS, phosphate buffer saline; SNEDDS, self-nanoemulsifying drug delivery systems; TCA, trichloroacetic acid.

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Table 1
Dosing design for the in vivo study on β-lactamase absorption in rats

Group	BLM preparation	Delivery route	No. of Rats
I.V.	BLM solution in PBS (600 mU/0.5 ml)	i.v. injection	4
Control 1	BLM solution in PBS (1800 mU/1.5 ml)	Oral gavage	4
Control 2	BLM solution (1800 mU/0.5 ml) coadministered with blank SNEDDS NE-12-7 (1 ml) (NE not formed before administration)	Oral gavage	4
Control 3	BLM solution (1800 mU/0.5 ml) mixed with blank SNEDDS NE-12-7 (1 ml) (NE formed before administration, BLM in aqueous phase)	Oral gavage	4
Test 1	BLM-loaded SNEDDS NE-12-7 (1800 mU/1 ml) co-administered with PBS (0.5 ml) (NE not formed before administration; BLM in oil phase)	Oral gavage	5
Test 2	BLM-loaded SNEDDS NE-12-7 (1800 mU/1 ml) mixed with PBS (0.5 ml) (NE formed before administration, BLM in oil phase)	Oral gavage	5

previous study (Rao et al., 2007) has also shown that the SNEDDS nanoemulsion system can increase the transport of BLM through MDCK monolayer *in vitro*, and the % BLM transported from the SNEDDS nanoemulsion was more than 20-fold higher than the solution form. The present study was carried out to test the concept of using SNEDDS as a protein delivery system *in vivo*, and to study the delivery efficiency by such a system.

2. Materials and methods

2.1. Materials

Ampicillin, β-lactamase (BLM) Type II lyophilized powder (29 kDa; 1.26 mU/μg; from *Bacillus cereus* EC 3.5.2.6), ascorbic acid, ethylenediamine tetraacetic acid (EDTA), trichloroacetic acid (TCA) and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Free samples of Lauroglycol-FCC® and Transcutol HP® were generously provided by Gattefosse Corp. (Paramus, NJ). Free sample of Cremophor EL® was generously provided by BASF Corp. (Florham Park, NJ). Hydrogenated soy phosphatidyl choline (SoyPC), was purchased from Avanti Polar Lipid (Alabaster, AL).

Sprague–Dawley rats weighing 400 ± 50 g, purchased from Taconic Farms (Germantown, NY), were used in this study. The rats were acclimated to their surrounding for at least 1 week before the experiment. The micro-isolator cages were used with two animals in each cage. The rats were housed on a 12 h light/dark cycle and a relative humidity of 40–60%, and animals were restrained from food and water for 12 h prior to the experiments.

2.2. Preparation of BLM-loaded SNEDDS NE-12-7

The formulation (NE-12-7) development and preparation of BLM-loaded SNEDDS were described in the first paper of the three sequential reports (7). SNEDDS NE-12-7, that consisted of an oil (Lauroglycol FCC), a surfactant (Cremophor EL) and a cosurfactant (Transcutol HP) in the ratio of 5:4:3, which spontaneously formed O/W nanoemulsion when mixed together with water was developed. SoyPC was evaluated as the carrier for the preparation of solid dispersion of BLM. Aqueous dispersion of the carrier prepared in PBS was mixed with FITC-BLM solution in PBS at the ratio of 4:1 (carrier: 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 BLM. Then the solid dispersion of BLM in SoyPC was dissolved into SNEDDS NE-12-7 (4 mg in 2 g of SNEDDS). The droplet size of the resultant nanoemulsion of BLM-loaded SNEDDS NE-12-7 was less than 50 nm.

2.3. β-Lactamase quantitation

A recently published HPLC method (Kaushal and Shao, 2006) was slightly modified and used for the determination of β-lactamase concentration in plasma. Briefly, to 0.2 ml of plasma sample, 0.8 ml of 2.86 mM ampicillin (substrate) was added. The reaction mixture was incubated at 37 °C for 30 min and then 0.1 ml of 60% TCA at 4 °C was immediately added to cease the reaction. The solution was centrifuged at 9000 × *g* for 5 min and 0.5 ml of the supernatant was added to 2 ml solution of 0.5 M acetate buffer (pH 4.0) containing ascorbic acid (0.5 mg/ml) and EDTA (50 mM). The resulting solution was heated at 100 °C for exactly 30 min and was allowed to cool down to the room temperature. The samples were then analysed by the HPLC method.

The HPLC system consisted of a Waters 600E system controller, a Waters 717 Autosampler, and a Waters 470 Scanning fluorescence detector. The separation was done on a μ Bondapak C18 cartridge column (3.9 mm × 300 mm i.d.). The injection volume was 10 μl. The mobile phase was 80% of 0.1 M phosphate buffer (pH 5.0) and 20% of acetonitrile with a flow rate of 1.5 ml/min. The column effluents were monitored at excitation and emission wavelength of 410 and 475 nm, respectively, for a run time of 15 min, and the peak of interest was seen at the retention time of 9 min. Two standard curves were constructed by analysis of the peak area against the concentration of the BLM in two different concentration ranges of 0.126–1.26 mU/ml and 1.26–12.6 mU/ml, which were prepared by spiking the blank plasma with BLM standard solution. The concentration of BLM in the plasma samples was determined by the standard curve method.

2.4. Oral absorption study

The research protocol was approved by Institutional Animal Care and Use Committee of the St. John's University and confirmed to the NIH guide for the use and care of laboratory animals. The rats fasted overnight for at least 12 h were used in this study. Four or five rats randomly placed in each group were each orally administered with 1.5 ml or injected through tail vein with 0.5 ml of various preparations. The IV dose was 1500 mU/kg and the oral dose was 4500 mU/kg. The detailed dosing schedule is shown in Table 1. The uneven number of rats in different groups was due to the shortage of the animals. No data was dropped. Blood samples of 0.5 ml were withdrawn from the tail at predetermined intervals and collected in eppendorf tubes containing 25 mg of anticoagulant, EDTA. Plasma was immediately separated by centrifugation at 13,000 × *g* for 15 min, and 0.2 ml of plasma was collected in a separate eppendorf tube. These plasma samples were assayed for BLM.

2.5. Pharmacokinetic parameters and statistics

The pharmacokinetic parameters were determined using PK Solutions version 2.0. The maximum plasma concentration (C_{max}) and the time (T_{max}) taken to reach the maximum plasma concentration was found by visual inspection from the β-lactamase plasma concentration–time profile. The area under the curve ($AUC_{0-\infty}$) and the area under the moment curve ($AUMC_{0-\infty}$) was calculated by non-compartmental analysis (Gibaldi and Perrier, 1982) using the trapezoidal method. All the values are expressed as mean ± S.D.

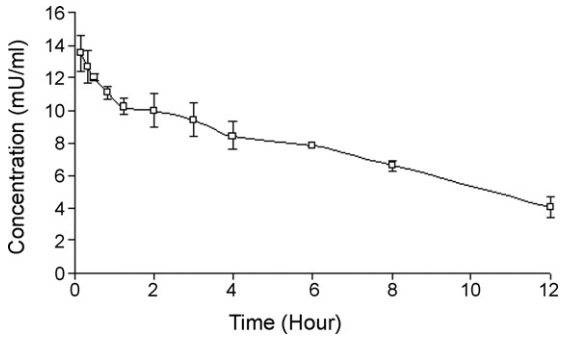

Fig. 1. BLM plasma concentration after the IV administration of 1500 mU/kg BLM in free solution (mean ± S.D., $n = 4$).

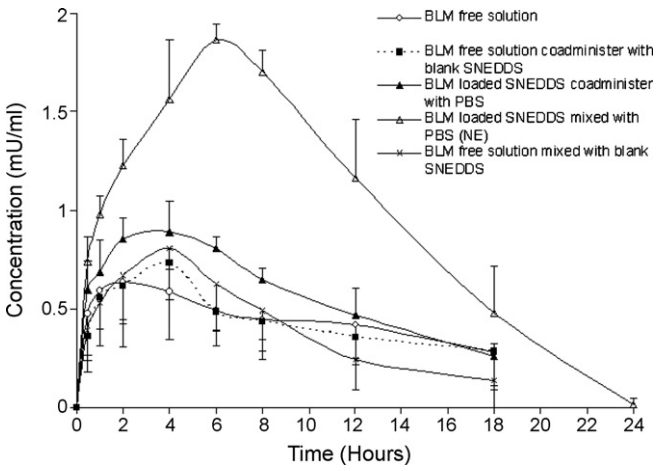

Fig. 2. BLM plasma concentration after the oral administration of 4500 mU/kg of BLM in various preparations (mean ± S.D., $n = 4/5$).

An one-way analysis of variance (ANOVA) followed by pair wise comparisons by Tukey–Kramer method was performed for the pharmacokinetic parameters, T_{max}, C_{max}, $AUC_{0-\infty}$ and MRT (mean residence time) using the software GraphPad Prism (San Diego, CA). A value of $p < 0.05$ was considered statistically significant.

3. Results

The plasma concentration profile of BLM after IV administration is shown in Fig. 1. The IV data was used to calculate the oral bioavailability. The plasma concentration profiles of BLM and the pharmacokinetic parameters after oral administration are shown in Fig. 2 and Table 2, respectively.

When BLM free solution (control 1) was given, the mean C_{max} was 0.68 mU/ml, T_{max} was between 1 and 2 h, and the mean bioavailability was 2.4%. There was no BLM detected in plasma at 24 h. Administration of BLM solution together with blank SNEDDS NE-12-7 (without forming nanoemulsion before administration, control 2), and BLM solution mixed with blank SNEDDS NE-12-7 (nanoemulsion formed, BLM in aqueous phase, control 3) resulted in C_{max} values of 0.76 and 0.81 mU/ml, respectively and T_{max} value between 2 and 4 h, the mean bioavailability of 2.05% and 2.13%, respectively. These results are similar to those from the free solution alone. Statistical analyse demonstrate that there was no significant difference in C_{max} and AUC among the three controls. Controls 2 and 3 resulted in longer T_{max} than control 1.

When BLM-loaded SNEDDS NE-12-7 was coadministered with PBS (without forming NE before administration, Test 1), the BLM plasma concentration profile was higher than that of the free solution (control 1), BLM solution coadministered with blank SNEDDS (control 2) and BLM solution mixed with blank SNEDDS NE-12-7 (control 3). This increase in plasma concentration profile of Test 1 is characterized by higher C_{max} of 0.98 mU/ml and higher mean bioavailability of 3.10% ($p < 0.05$). However, the T_{max} was between 2 and 4 h and BLM was not detected at 24 h after administration in Test 1 similar to all the controls 1, 2 and 3.

When BLM-loaded SNEDDS NE-12-7 nanoemulsion (SNEDDS mixed with PBS to form nanoemulsion before administration, Test 2) was administered, the mean C_{max} was 1.90 mU/ml (2.8 times of that by the free solution, $p < 0.05$), bioavailability was 6.34% (2.6 times of that by the free solution, $p < 0.05$), T_{max} was between 4 and 6 h and BLM was still detected in plasma at very low concentration (0.018 mU/ml) at 24 h. BLM-loaded SNEDDS NE-12-7 nanoemulsion (Test 2) showed maximum bioavailability and significant increase ($p < 0.05$) in plasma concentration profile over all the three controls 1, 2, 3 and Test 1.

4. Discussion

The oral BLM plasma concentration profile results indicate that the oral absorption of BLM in rats was significantly increased ($p < 0.05$) by administration in the SNEDDS NE-12-7 nanoemulsion compared with an aqueous solution. In addition, BLM absorption was also increased by administration of BLM-loaded SNEDDS NE-12-7 coadministered with PBS. However, nanoemulsion formed before dosing showed higher increase in absorption as compared to BLM-loaded SNEDDS NE-12-7 coadministered with PBS. These results demonstrate that the effect of increase in BLM absorption is partially dependent on premixing the BLM-loaded SNEDDS with PBS. The reason could be that there might not be sufficient emulsification of SNEDDS in the rat GIT. In a recent publication (Grove et al., 2006) it is indicated that when studying SNEDDS the animal model might be important to consider due to the dependency of the dynamic processes responsible for the emulsification process. From these results, it is obvious that nanoemulsion formation before dosing can warrant high bioavailability. This formation can be easily done with SNEDDS. Nanoemulsion will be formed by gentle

Table 2
Pharmacokinetic parameters of BLM in rats after oral administration (mean ± S.D., $n = 4/5$)

Group	Description	$AUC_{0-\infty}$ (mU h ml^{-1})	C_{max} (mU/ml)	T_{max} (h)	MRT (h)	F (%)
Control 1	BLM solution in PBS ($n = 4$)	9.08 ± 3.89	0.68 ± 0.23	1–2	8.95 ± 1.45	2.40 ± 1.0
Control 2	BLM solution coadministered with blank SNEDDS NE-12-7 ($n = 4$)	7.74 ± 2.1	0.76 ± 0.06	2–4	10.18 ± 1.75	2.05 ± 0.6
Control 3	BLM solution mixed with blank SNEDDS NE-12-7 ($n = 4$)	8.05 ± 0.95	0.81 ± 0.26	2–4	9.43 ± 2.54	2.13 ± 1.0
Test 1	BLM-loaded SNEDDS coadministered with PBS ($n = 5$)	11.7 ± 1.23	0.98 ± 0.05	2–4	11.8 ± 3.68	3.10 ± 0.4
Test 2	BLM-loaded SNEDDS NE-12-7 nanoemulsion ($n = 5$)	23.90[a,b,c,d] ± 2.48	1.90[a,b,c,d] ± 0.09	4–6[a,b]	12.12[a] ± 4.97	6.34[a,b,c,d] ± 0.5

[a] Significant difference from control 1 ($p < 0.05$).
[b] Significant difference from control 2 ($p < 0.05$).
[c] Significant difference from control 3 ($p < 0.05$).
[d] Significant difference from Test 1 ($p < 0.05$).

mixing of SNEDDS with water. This process can be easily carried out by patients prior to administration.

The oral absorption of BLM from free solution mixed with blank SNEDDS NE-12-7 or free solution coadministered with blank SNEDDS NE-12-7 was not significantly different from free solution administration alone. Hence, when BLM is in the aqueous phase of the nanoemulsion, the bioavailability is similar to the free solution, and when BLM is loaded into the oil phase of the nanoemulsion, the bioavailability is almost three times of that by the free solution. This increase in bioavailability can be attributed to the fact that when BLM is enclosed in the oil phase of the nanoemulsion, BLM is absorbed when the oil droplet is absorbed by the various lipid absorption mechanisms: passive diffusion, pinocytosis or endocytosis (Georgakopoulos, 1992), and whereas when BLM is in the aqueous phase of the nanoemulsion, BLM is similar to being in free solution fully exposed to the harsh environment of the GIT, and minimal absorption for a macro-hydrophilic molecule.

It is not surprising to see the T_{max} was delayed in controls 2 and 3 and Tests 1 and 2, as compared to the free solution (control 1). The presence of oil in the dose can cause the delay of gastric emptying time. In addition, oil droplets may be absorbed through lymphatic system, and then enter into the blood circulation after a certain time.

The previous studies in our laboratory have explored the feasibility of using normal flora to deliver protein/peptide drugs orally (Shao and Kaushal, 2004; Kaushal and Shao, 2006). These studies demonstrated the use of the probiotic bacteria, *Lactococcus lactis* (*L. lactis*), a genetically modified system capable of secreting BLM, for oral delivery of BLM. This normal flora system when administered orally to rats showed 2.6 times higher bioavailability than that by the solution control. In comparison, both normal flora and SNEDDS systems can result in enhanced oral bioavailability. However, these two approaches have their own advantages and disadvantages. The normal flora system can provide a sustained delivery mechanism since the bacteria can stay inside the intestine for a certain time period and continuously secrete/deliver the protein drugs. However, this approach cannot be used for the proteins which undergo post-translation process, and the issue of potential environment pollution by the genetically modified bacteria should be solved before it can be used clinically. In addition, the normal flora should be administered to areas where they normally reside but not to the other places in the body. In case of SNEDDS approach, it is relatively easy to develop the product and it may be applied through different delivery routes. But there are some issues such as how to increase the loading capacity so that the desired dose can be delivered with a limited amount of lipids should be addressed. In conclusion, we have preliminarily demonstrated that both the normal flora and SNEDDS approaches can be used to increase the oral bioavailability of protein/peptide drugs.

5. Conclusion

Compared to free solution delivery, SNEDDS NE-12-7 nanoemulsion in which BLM was in the oil phase of the nanoemulsion significantly increased the oral bioavailability. Therefore, in conclusion, SNEDDS can be an efficient oral delivery system for protein drugs or other poorly absorbed drugs.

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