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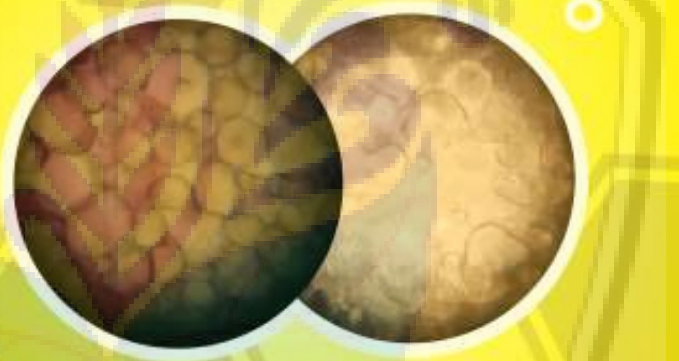

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Research Article

Formulation of Insulin Self Nanoemulsifying Drug Delivery System and Its *In Vitro-In Vivo* Study

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ABSTRACT

Particulate delivery system can be used for improving the efficacy of protein and peptide drug. In addition to a polymer-based particulate delivery system, self-nanoemulsifying drug delivery system (SNEDDS), a lipid-based delivery system, is currently developed for either less water-soluble or soluble drugs. This study aims to design SNEDDS for oral insulin administration and its *in vitro-in vivo* study. The SNEDDS template was designed using D-optimal mixture design and was analyzed using software Design Expert 7.1.5. The obtained optimum template was loaded with insulin and evaluated for its transmittance percentage, emulsification time, particle size, zeta potential, stability, the amount of insulin *in vitro* diffused across rat intestine, and insulin serum concentration after oral administration. The study results revealed that the optimum template of SNEDDS formula consisted of 10% (w/w) Miglyol 812N, 65% (w/w) Tween 80, and 25% (w/w) propylene glycol. These optimum template then was loaded with insulin and characterized. SNEDDS insulin has particle size of 12.0±1.7 nm, zeta potential of +0.16mV, transmittance of >90%, and emulsification time of < 60 seconds. The stability study showed that SNEDDS insulin was stable from both precipitation and phase separation. The amount of insulin transported from SNEDDS formula *in vitro* was 32.45±2.03% and non-SNEDDS formula was 10.44±5.04%. *In vivo* study of SNEDDS insulin produced a significantly increased Cmax, AUC, and F value than insulin non SNEDDS ($p < 0.05$). In brief, SNEDDS formulation in this study is a promising approach to increase the effectiveness of oral insulin. Insulin is better given orally in SNEDDS formulation than in non SNEDDS formulation.

Keywords: SNEDDS, insulin, D-optimal mixture design, *in vitro* diffusion study, *in vivo* study

INTRODUCTION

Oral insulin has not been commercially available due to the low bioavailability of insulin in the gastrointestinal tract (Sadrzadeh *et al.*, 2007). The enzymatic degradation in the gastrointestinal tract and low permeability of intestinal membrane result in the low bioavailability of per-oral insulin (Almaeda and Souto, 2007).

The approach for oral protein formulation is the use of specific excipients such as absorption enhancers, enzyme inhibitors, mucoadhesive polymers, and other formulations enabling protein protection against extreme environment in the gastrointestinal (Park *et al.,* 2011) like encapsulation of various delivery system including nanoparticles (Sonaje *et al.,* 2009; Nair *et al.,* 2017; Kunasekaran and Krishnamoorthy, 2015), microemulsion (Sharma *et al.,* 2010), self-nanoemulsifying drug delivery system (SNEDDS) (Ma *et al.*, 2006; Li *et al.,* 2012; Zhang *et al.,* 2012; Sakloetsakun *et al.,* 2013; Rao *et al.,* 2008; Rachmawati *et al.,* 2010), liposome (Wu *et al.,* 2011), and mixed with an aqueous extract obtained from Desmodium Gangeticum roots (Kurian *et al.,* 2010). Among those preparations, SNEDDS is potential to be developed as a protein delivery system. SNEDDS is a homogenous complex system which consists of oil, surfactant, co-surfactant, and co-solvent (Patel *et al.,* 2013). The system is also named as emulsion pre-concentrate. By light agitation in aqueous media leads to the formation of translucent emulsion (Mishra *et al.,*

2014). In some studies, SNEDDS is proven to be superior rather than the lipid solution due to the surfactant availability in its formulation; it is homogenous, the drug absorption is more consistent, it protects drugs against gastrointestinal environment, the bioavailability gets increased, and the efficiency of absorption becomes higher (Kaur and Harikumar, 2013).

SNEDDS has been applied to deliver hydrophobic drugs such as coenzyme Q10 (Khattab *et al.,* 2016), halofantrine (Michaelsen *et al.,* 2013), simvastatin (Thomas *et al.,* 2013), vitamin E-rutin (Khan *et al.,* 2015), and cyclosporine A (Jain *et al.,* 2015). Some studies reveal that SNEDDS is also used for protein and peptide drug such as BSA (Rachmawati *et al.,* 2002; Winarti *et al.,* 2016a; Winarti *et al.,* 2016b), β-lactamase (Rao *et al.,* 2008), Insulin (Ma *et al.,* 2006; Li *et al.,* 2012; Zhang *et al.,* 2012, Sakloetsakun *et al.,* 2013).

SNEDDS insulin prepared by previous researchers (Li *et al.,* 2012; Zhang *et al.,* 2012) using phospholipid to produce insulin phospholipid complex. The resulted insulin-phospholipid complex improved the insulin solubility in oil. Other reseracher prepared SNEDDS for mucus permeating by initially processing the insulin through hidrophobic ion pair of insulin/dimyristoyl phosphatidylglycerol method to improve the insulin solubility in the system and prevent from burst release (Karamanidou *et al.,* 2015). This formulation of SNEDDS insulin resulted in higher in vitro and in vivo permeability.

In this study, insulin was dissolved in glycerol and incorporated in optimum SNEDDS template optimized using D-optimal mixture design. The optimum SNEDDS template loaded insulin was characterized and evaluated for in vitro diffussion study and in vivo study. The study using rat was approved by the Ethics Commission of Integrated Reserach and Testing Laboratory, Universitas Gadjah Mada no. 00077/04/LPPT/X/2016.

The approach applied in this research is said to be able to succeed the generation of effective insulin delivery system. In addition, this study suggests interesting possibilities for other proteins formulated by SNEDDS. Studies using insulin as active ingredient that apply this approach have not been established. Therefore, this study aims to develop optimum SNEDDS template for potential oral insulin delivery.

MATERIAL AND METHODS

This study used Bovine Insulin from Beijing Top Science Biotechnology Co., Ltd., Miglyol 812N from CREMER OLEO GmbH & Co.KG, Tween 80, Span 20, Span 85 from Sigma Aldrich, Chremophor EL 40 was a gift from Shanghai Terppon China, propylene glycol from Bratachem Indonesia, glycerol from Merck, Bradford reagen kit from BioRad, Female Wistar rats obtained from Pharmacology and Pharmacy Clinic Laboratory of Pharmacy Faculty University of Gadjah Mada, and ELISA Bovine Insulin Kit from ALPCO USA.

Compatibility study of oils-surfactants-co-surfactant mixture

Various oil components consist of Miglyol 812N, Span 85, and oleic acid, surfactants (Tween 80, Tween 20, and Cremophor EL 40), and co-surfactants (Span 20, and propylene glycol) used for SNEDDS component. The compatibility of oil: surfactants: co-surfactants (1:1:1, 1:2:1, 1:3:1, 1:4:1, 1:5:1, 1:6:1, 1:7:1, 1:8:1, 2:1:1, 2:2:1, 2:3:1, 2:4:1, 2:5:1, 2:6:1, 2:7:1, 2:8:1, 3:1:1, 3:2:1, 3:3:1, 3:4:1, 3:5:1, 3:6:1, 3:7:1, 3:8:1) was visually observed for three days. The mixtures of the components with the largest miscibility area and with the highest emulsion transparency produced at a short emulsification time were used to construct the ternary phase diagram and to optimize the composition of SNEDDS templates.

Construction of pseudoternary phase diagram

Based on the compatibility study, the mixtures of the components that fulfilled the evaluation criteria were used to construct the pseudoternary phase diagram.

Optimization of SNEDDS template with D-optimal

The optimization using D-optimal mixture design was performed on three independent variables which are oil (Myglyol 81) 10-25%, surfactant (Tween 80) 50-80%, and co-surfactant (propylene glycol) 10-25%, and it was also on two dependent variables which are % transmittance (Y1) and emulsification time (Y2).

Optimum formula verification

The optimum formula verification was done to determine the suitability of the predicted value with the value of the observation (actual value).

Preparation of insulin SNEDDS

Insulin was dissolved in glycerin and was stirred in the mixture of surfactant (Tween 80), co-surfactant (propylene glycol) and Myglyol 812N. Each gram of SNEDDS template was added with 100µL of glycerin containing insulin.

Determination of emulsion droplet size and zeta potential

SNEDDS Insulin was added with distilled water (1:1000) in a test tube. The particle size was measured and the polydispersity index (PDI) of the formulated nanoemulsion was analyzed using DelsaTM Nano Beckman Coulter.

Evaluation of emulsification time

SNEDDS insulin of 250.0µL was quickly dripped into a baker glass using 250.0mL distilled water, simulated gastric fluid PH 1.2 and phosphate buffer pH 6.8 at 37±0.5°C. The medium was stirred at a speed of 100rpm (Weerapol *et al.,* 2014). The time to form nanoemulsion was recorded as emulsification time.

Transmittance percentage

SNEDDS insulin of 100µL was added to a vial containing 10mL and 100mL double distilled water, Simulated Gastric Fluids pH 1.2, and phosphate buffer pH 6.8 at the room temperature, stirred for a minute and measured for its transmittance using SpectroVis at λ 650 nm (Reddy and Sowjanya, 2015).

Procedure of *in vitro* difussion study (Ussing Chamber)

The diffusion study was conducted using Ussing Chamber and intestine of male Wistar rats put on a chip chamber. SNEDDS insulin (1mL) was dispersed in AIF (Artificial Intestinal Fluids) at pH 6.8 and put into the mucosal compartment. The non-SNEDDS insulin was used as the comparator. Phosphate buffer saline pH 7.4 was added into serosal compartment. The Ussing chamber was set on the water bath at 37±0.5°C. The oxygen was distributed at the speed of ±100 bubbles per minute to keep the membrane function. Sampling technique was performed by taking 1 mL solution of the serosal at the 0^{th}, 15^{th}, 30^{th}, 45^{th} , 60^{th}, 90^{th}, 120^{th}, 180^{th}, 240^{th}, and 300^{th} minute. To keep the sinking condition, the solution was changed to 1 mL of serosal media. The sample obtained was centrifuged at a speed of 3.000rpm for 5min to eliminate intestinal debris. The content detection was performed with visible spectrophotometer through validated micro Bradford Assay. This method was carried out by reacted 160µL sample solution with 40µL Bradford Reagent, then allowed to stand for at least 5min, and no more than 1h. Absorbance was measured at maximum wavelengths against blanks.

In vivo pharmacokinetic study

Experimental animal

The experimental animal used in the in vivo test was treated based on the approved procedure by the Ethics Commission of LPPT UGM no. 00077/04/LPPT/X/2016. The animals used are healthy 1.5–2-month-old female Wistar rats (150-250g). The rats were kept in light cage for 12h and in dark cage for 12 h, and were given standard diet and sufficient water access (*ad libitum*).

Induction of diabetes

The induction of diabetes in rats were performed through the injection of Intraperitoneal Streptozotocin (48mg/kg) in 10mM citrate buffer (pH 4.5) of rats fasted for 14h with water access (*ad libitum*). The rats for the following test were selected based on the glucose level >250mg/dL after five days of streptozotocin induction.

Treatment of experimental animals

The study for blood insulin profile was conducted by randomly dividing 28 rats into seven groups; each group consists of 4 rats. The experimental groups of the study are: Grup I was given 5.0mL/200g blank SNEDDS (oral); Grup II was given 43.39IU/KgBW SNEDDS Insulin (1mL oral); Grup III was given 108.47IU/KgBW SNEDDS insulin (2.5mL oral); Grup IV was given 216.94IU/KgBW SNEDDS insulin (5mL oral), Grup V was given 5.0 mL/200gr PBS pH 7.4 (oral); Grup VI was

given 200 IU/KgBW non-SNEDDS insulin (oral), and Grup VII was given 10 IU/KgBW subcutaneous insulin.

Sample Analysis

The blood sample (0.5mL) was obtained from the eye orbital sinus at the 0th, 15th, 30th, 45th, 60th, 90th, 120th, 240th, 480th, and 600th minute. The serum insulin concentration (25µL) was measured using Bovine Insulin ELISA Kit.

Statistical Analysis

The differences of each treatment group were statistically analyzed with $p<0.05$ indicating significantly different.

RESULTS AND DISCUSSION

Development of SNEDDS templates

The component screened for SNEDDS templates shows that Miglyol 812N: Tween 80: propylene glycol produced the mixtures fulfilling the designed criteria. Oleic acid and Span 85 tend to form less transparent emulsion than Miglyol 812N. Mygliol 812 is medium chain triglyceride with the HLB value 15.36 (Kawakami *et al.*, 2002), while the HLB of Span 85 is 1.8 and HLB of oleic acid is 1.0. It was reported that lipid with higher polarity is easier to form nano-emulsion (Hong *et al.*, 2006). Oil that has long hydrocarbon chain like oleic acid and Span 85 (C18) is difficult to form nano-emulsion; Miglyol 812N has such medium-long hydrocarbon chain that is emulsified easily (Anton and Vandamme, 2009; Sadurní *et al.*, 2005). Span 85 is a sorbitan trioleate, a long hydrocarbon chain, resulting in higher viscosity (200-300mpas) than Miglyol 812 has (27-33mpas) and oleic acid (25.6mpas); Span 85 has less spontaneous nano-emulsifying and tends to from bigger-sized droplets.

Tween 80 is able to form nano-emulsion with Miglyol 812 due to its higher HLB value than of Cremophor EL 40; although HLB of Tween 80 is lower than of Tween 20, it can form better nano-emulsion than Tween 20 (Chinwong *et al.*, 2012; Macedo *et al.*, 2006).

Phase Diagram of SNEDDS Formulation

Pseudoternary phase diagram was constructed to estimate the concentration in which SNEDDS templates can form nanoemulsion when added to water. The diagram consists of Miglyol 812N: Tween 80: propylene glycol (Figure 1). Red squares showed the nanoemulsion.

Optimization of SNEDDS template with D-optimal

Response of % transmittance

The % transmittance is one of SNEDDS characteristics that needs to be evaluated for its use to predict the size of emulsion droplets (Nasr *et al.*, 2016). The equation for % transmittance using D-optimal Design (pseudo components) is as follow:

$$Y1 = -8.43*A+9.8*B+8.89*C+28.79*A*B+2.07*A*C+2.67*B*C \quad (1)$$

Remarks: Y1= √ transmittance; A= Miglyol 812N composition; B= Surfactant (Tween 80) composition; C= Co-surfactant (propylene glycol) composition

Based on the equation 1, oil reduced the % transmittance due to the improving amount of oil composition leading to the increased droplet size. It results in the decreased value of % transmittance (Desmukh and Kulkarni, 2014). In contrast, surfactants increase the value of % transmittance as they will be absorbed on the oil surface so fast that the oil changes into small-sized droplets in continuous phase. Co-surfactants support surfactants to reduce the surface tension into negative value and to modulate the drop size to nanometer by decreasing the interfacial bending stress and increasing the flexibility of an interfacial film (Nasr *et al.*, 2016). Consequently, due to the synergic function, the increased amount of co-surfactants results in the increased value of % transmittance.

The oil-surfactant interaction has the biggest influence on % transmittance for the viscosity of oil-surfactant combination lower than of surfactant; it results in easier penetration of water in the nano-emulsion formation process (Ittiqo *et al.*, 2016).

Response of emulsification time

Emulsification time is essential parameter in evaluating the efficiency of self nanoemulsion formation (Basalious *et al.*, 2010; Costa *et al.*, 2012).

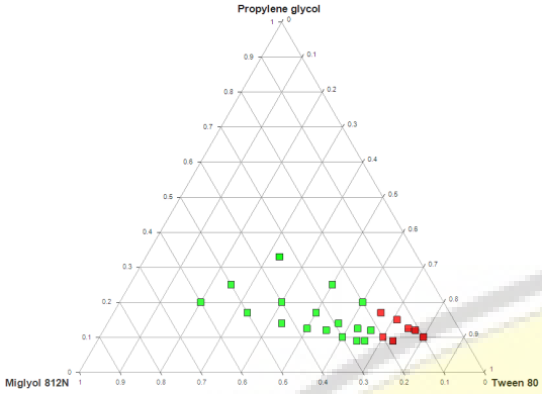

Figure 1. Pseudoternary phase diagram of SNEDDS template consist of Miglyol 812N:Tween 80:Propylene glycol

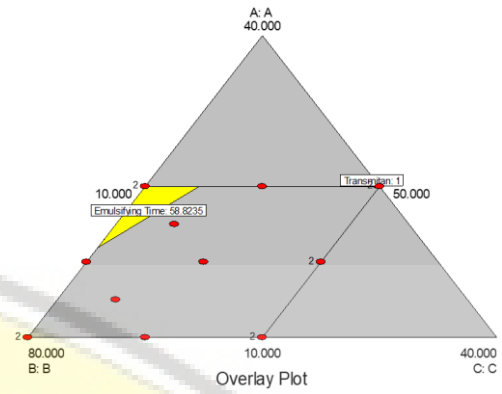

Figure 2. Overlay plot of % transmitan and emulsification time

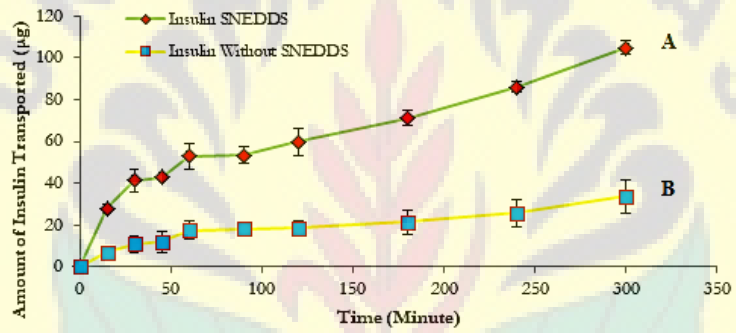

Figure 3. Amount of insulin transported across rat gut in vitro for 5h (average±sd) (a) insulin SNEDDS, (b) insulin non SNEDDS

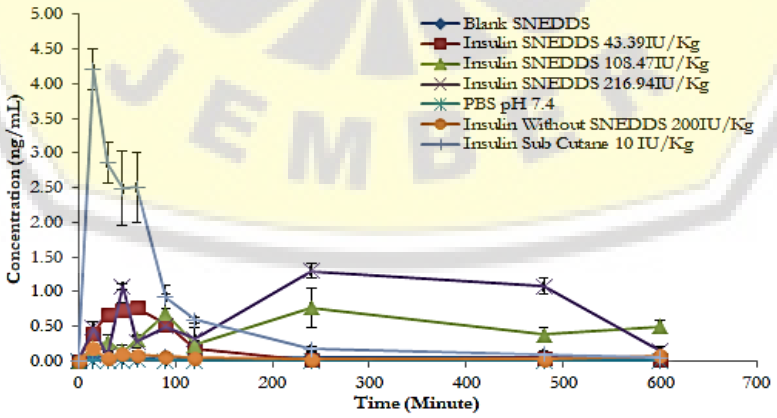

Figure 4. Profile of serum insulin concentration (Average±SEM, n=4)

Table I. Pharmacokinetic parameters after oral administration

Treatment	Cmax (ng/mL)	Tmax (min)	AUC_{0-10} (min.ng.mL^{-1})	F
Insulin non SNEDDS 200IU/Kg BB	0.52±0.51	15-60	27.34±10.00	0.004±0.003
Insulin SNEDDS 43.39IU/Kg BB	0.82±0.19	45-60	85.86±16.10	0.062±0.024
Insulin SNEDDS 108.47IU/Kg BB	0.77±0.17	240	293.23±42.76	0.084±0.027
Insulin SNEDDS 216.94 IU/Kg BB	1.31±0.19	240	506.75±32.75	0.073±0.008
Insulin Sub Cutane 10IU/Kg BB	4.21±0.57	15	324.60±26.33	1

The obtained equation of D-optimal Design (pseudo components) for response of emulsification time is as follow:

Y2 = -0.029*A+0.036*B+0.15*C……...(2)

Remarks: Y2= 1/*Emulsification time*; A= Miglyol 812N composition; B= Surfactant (Tween 80) composition; C= Co-surfactant (propylene glycol)

Oil increase the emulsification time while both surfactant and co-surfactant decreases the emulsification time. Oil prolong the emulsification time due to different phase of oil and water that results in high surface tension prohibiting the water penetration in forming nano-emulsion spontaneously (Ruan *et al*., 2010).

The optimization result shows the optimal ratio of Miglyol 812: Tween 80: propylene glycol is 10:65:25 (%w/w) with the desirability of 0.97. Figure 2 shows mixed overlay plots resulted by two responses.

Verification of optimal formula

The verification result showed no significant difference between the prediction value and observation value of Y1 and Y2 with p>0.05 therefore that the prediction value experimentally fits with the observation value.

Particle size and zeta potential

The analysis result of particle size indicated that the droplet size of insulin nano-emulsion is 12.0±1.7nm with narrow distribution size (polydispersity index = 0.243) and the zeta potential is +0.16mV.

Visual observation of emulsification time

Emulsification time of SNEDDS insulin occurs fast in three media, with emulsification time <60s, it belongs to grade A for less than one-minute emulsification time, and it has transparent or clear bluish appearance (Kaur *et al*., 2003).

The % transmittance

The % transmittance of SNEDDS insulin (>90%) indicates transparency formula or ability to form nano-emulsion in the used media.

Diffusion test with ussing chamber

Validation process to determines the level of insulin transported during diffusion study has been successfully carried out according to ICH Guideline Q2 (R1). Validation parameters include selectivity, linearity, LOD, LOQ, accuracy, and precision. Microbradford assay used in this study was selective, linear, accurate and precise. LOD and LOQ were obtained 0.49µg/mL and 1.64µg/mL. The result of diffusion test with Ussing Chamber shows that the amount of transported insulin of SNEDDS preparation (32.45±2.03%) is significantly different (p=0.001) from non-SNEDDS insulin (10.44±5.04%) (Figure 3). The test result reveals that SNEDDS significantly influences the increase of flux and amount of in vitro transported insulin.

Analysis of blood insulin level

The method used to determine insulin levels in serum is Sandwich ELISA. Color intensity after the addition of TMB substrate (3, 3', 5, 5'-tetramethylbenzidine) and stop solution measured using ELISA reader at the wavelength of 450 nm. Before use, ELISA kit was verified to know its linearity, accuracy, precision, and suitability with the internal quality controls. The verification results show that the standard curve is linear (R2 = 0.9998), accurate, precise, and in accordance with internal controls.

Protein activity depent on the intergrity of three dimensional structure. ELISA results show that SNEEDS are able to preserve biological activity of entrapped Insulin. After oral administration, SNEDDS formulation can increase in AUC, C max, and an F value of insulin (Figure 4). These caused by some factors including lymphatic transport, high surfactant content, and paracellular transport of tight junction (Georgakopoulos *et al.,* 1992). The lipid given orally will be digested and absorbed in intestinal lymphatic. SNEDDS insulin forms nano-sized droplet system that will experience intestinal uptake through lymphoid follicles and Peyer's patches GALT and be transported to spleen either directly or through macrophage phagocytosis effect (Reddy and Murthy, 2002). Insulin also transported to intestinal lymphatic due to its big-sized molecule and resistance to portal circulation absorption (Reddy and Murthy, 2002).

Insulin absorption can be reached using enhancer (Muranishi, 1990) and *lipid based vehicles* (Porter and Charman, 2001). Long chain lipid will tend to be transported to spleen rather than to portal circulation. The oil used in SNEDDS system is Miglyol 812N, medium chain triglycerides that will be transported to intestinal lymphatic due to its combination with Tween 80 consisting of oleic acid, long chain fat (C18). Tween 80 is an enhancer that increases the permeability of cell membrane. Tween 80 also has reversible effects in opening tight junction through interaction with polar parts of lipid bilayers (Selvam *et al*., 2013).

The fastest Cmax reached by Insulin SNEDDS 1mL than Insulin SNEDDS 2.5 mL adn 5mL (Figure 4). These can be seen from Insulin SNEDDS given orally as much as 2.5 mL and 5mL reached Cmax after 240 minutes while Insulin SNEDDS 1mL reached Cmax after 45-60min. These caused by delayed gastric emptying time (Cooke, 1975) and the slow dispersing process of SNEDDS into nanoemulsion in limited gastric media (Porter and Charman, 2001; Pouton, 2000). Fat has a long residence time in the stomach (Cooke, 1975) which delayed gastric emptying time leading to delayed absorption.

Compared with non-SNEDDS insulin, the SNEDDS insulin is absorbed more. This occurs as non-SNEDDS insulin is unstable to pH changes of gastrointestinal tract leading to unpredictable speed of insulin absorption. Subcutaneous insulin was used as positive control of blood insulin level. Cmax of this insulin is reached fast, in 15min, with 4.21±0.57ng/mL of level. The Cmax of SNEDDS insulin of the highest dosage remains smaller than of subcutaneous insulin; this occurs due to bigger insulin hindrance to enter blood through oral route. The protein given subcutaneously will move slowly from tissues to capillary, and it generally reach the bloodstream through lymphatic vessels; the protein given orally must be resistant to the extreme pH environment and protease that can destroy protein, and it must be able to penetrate the intestinal epithelial membrane to enter the bloodstream.

The result of Pharmacokinetic test reveals that insulin is better given in SNEDDS preparation than in non-SNEDDS preparation; this explains that it is highly possible to enhance the amount of absorbed insulin using SNEDDS preparation.

CONCLUSION

The resulted design of SNEDDS templates reveals that the optimal SNEDDS template after being loaded with insulin provides nano-emulsion characteristic resulting in bigger amount of in vitro diffused and in vivo absorbed insulin than of non-SNEDDS insulin. This enables the designed SNEDDS formula to be used in per-oral insulin delivery.

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