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Superolein Based Intravenous Lipid Emulsion 20% w/w Physicochemical Characterization, Stability and Its Effect on Liver Status

(Superolein berasaskan Emulsi Lipid Intravena 20% w/w Pencirian Fizikokimia, Kestabilan dan Kesannya pada Keadaan Hati)

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ABSTRACT

Long chain triglycerides (LCT) from soya oil have been reported to cause hepatic abnormalities among patients receiving parenteral nutrition. A new source for LCT is needed to support current therapy. The aims of this study were to develop a good and stable superolein oil based intravenous lipid emulsion 20% (SoLE), and to determine its effect on liver status of an animal model. The emulsion was prepared using superolein oil and medium chain triglyceride oil (1:1), 1.2 g lecithin and homogenized by high pressure homogenization method. The physical stability study was performed based on real-time, accelerated, force degradation and environmental degradation. Chemical stability was measured based on fatty acids and vitamin E contents, peroxide value and acidity value. As the animal model, three rabbits were administered with 1.5 g/kg SoLE for 5 h and repeated daily for three days. The SoLE was found to have good physicochemical characters: 229.07±1.57 nm of particle size; 0.00% of particle > 5 μm; 342.00±1.00 mOsm/kg of osmolarity; -46.67±4.40 mV of zeta potential; 1.72±0.04 cP of viscosity; 7.83±0.06 of pH; 0.80±0.06 kcal/g calorie; 1.74±0.05 mEqO$_2$/kg of peroxide value; and 0.03±0.01% of acidity value, and excellent physicochemical stabilities for up to six months of storage at various temperatures and conditions. The level of plasma lipid and plasma liver enzymes profiles of the rabbits remained statistically unchanged. This study has successfully developed a stable SoLE 20% and it was found to be safe to the liver of the animal model.

Keywords: Animal model; intravenous lipid emulsion; physicochemical; stability; superolein oil

ABSTRAK

Trigliserida rantaian panjang (LCT) daripada minyak kacang soya telah dilapor menyebabkan keabnormalan hepatik dalam kalangan pesakit yang menerima nutrisi parenteral. Sumber baharu untuk LCT diperlukan bagi menyokong terapi semasa. Tujuan kajian ini adalah untuk membangunkan emulsi lipid intravena 20% (SoLE) berasaskan minyak superolein yang baik dan stabil dan menentukan kesannya terhadap status hati model haiwan. Emulsi telah disediakan dengan menggunakan minyak superolein dan minyak trigliserida rantaian sederhana (1: 1), 1.2 g lesitin dan dihomogenkan dengan kaedah penghomogenan tekanan tinggi. Kajian kestabilan fizikal dijalankan berdasarkan masa sebenar, dipercepat, degradasi paksa dan degradasi persekitaran. Kestabilan kimia dihitung berdasarkan kandungan asid lemak, kandungan vitamin E, nilai peroksida dan nilai keasidan. Sebagai model haiwan, tiga ekor arnab diberikan 1.5 g/kg SoLE selama 5 jam dan diulang setiap hari selama tiga hari. SoLE didapati mempunyai sifat fizikokimia yang baik iaitu 229.07±1.57 nm saiz zarah, 0.00% zarah bersaiz melebihi 5 μm, 342.00±1.00 mOsm/kg osmolariti, -46.67±4.40 mV potensi zeta, 1.7 ±0.04 cP kelikatan, 7.83±0.06 pH, 0.80±0.06 kcal/g kalori, 1.74±0.05 mEqO$_2$/kg nilai peroksida dan 0.03±0.01% nilai keasidan serta kestabilan fizikokimia pula adalah cemerlang sehingga enam bulan penyimpanan pada pelbagai suhu dan keadaan. Tahap profil lipid plasma dan enzim hati plasma haiwan yang dikaji kekal tidak berubah secara statistik. Kajian ini telah berjaya membangunkan SoLE 20% yang stabil dan selamat kepada hati model haiwan.

Kata kunci: Emulsi lipid intravena; kestabilan fizikokimia; minyak superolein; model haiwan

INTRODUCTION

Currently, most of the long chain triglycerides (LCT) supply are sourced from soya oil and almost all of the intravenous lipid emulsion (IVLE) are soya oil based (Arrigo et al. 2015; Fell et al. 2015; Zhu et al. 2016). LCT has been claimed to be the cause of hepatic abnormalities, especially among patients with long term parenteral nutrition (PN) infusion (Anez-Bustillos et al. 2016; Ren et al. 2013). Thus, a new source for LCT such as superolein palm oil is needed to support current therapy and requirements for IVLE in pharmaceutical and medical practice (Manzanares et al. 2013; Vanek et al. 2012).

Superolein oil (SO) is produced from the double fractionation processes of *Elaeis guineensis* fruit, and has abundant palmitic acids and equivalent composition of saturated and unsaturated fatty acids (Mba et al. 2015). In

addition, SO is also rich with triglycerides, tocotrienols, tocopherols, beta-carotene, and oleic acid. Its antioxidant activities provide benefits for both pharmaceutical and clinical aspects such as providing a protective effect to the liver of PN patients (Narang et al. 2016, 2004). An earlier study has shown the benefit of palm oil based IVLE to serve as a secondary energy provider and also as vehicle for poorly water soluble drugs (Jufri et al. 2012).

A stable pharmaceutical product is a formulation with the ability to remain within the physical, chemical, toxicological, microbiological, protective and informational specifications in a specific container (Bajaj et al. 2012). As IVLE possesses the inherent thermodynamic instability of oil in water emulsions, it is prone to become physically and chemically unstable (Driscoll 2017). The early signs of IVLE destabilization are creaming, flocculation and coalescence and it can be detected through the changes of particle size diameter and distribution (Stevens et al. 2016; Tadros 2013). This instability may increase the technical and clinical risks to the recipients. Thus, stability testing is required to evaluate the influence of storage conditions and environments on the quality of the IVLE product produced (Blessy et al. 2014).

The aims of this study were to develop a good and stable superolein oil based intravenous lipid emulsion 20% (SoLE), and to determine its effect on the liver status of an animal model.

Materials & Methods

MATERIALS

The main components for this superolein oil based intravenous lipid emulsion 20% (SoLE) are superolein oil from FELDA IFFCO Sdn. Bhd. (Malaysia) and coconut MCT oil (Enersos ®) from Pharm-D Sdn. Bhd. (Malaysia). Lecithin (Lipoid E80®) from Lipoid AG (German) was used as the emulsifier. Glycerol from EMD Millipore Corp. (Billerica, MA, USA), sodium oleate and sodium hydroxide from Sigma Aldrich Chemie GMbH (German) were also used for the preparation.

PREPARATION OF INTRAVENOUS LIPID EMULSION

All of the ingredients used were measured accordingly. Superolein oil (10 g), MCT oil (10 g) and lecithin (1.2 g) were mixed in a 500 mL beaker (oil phase). Sodium oleate (20 mg), glycerol (2.5 g) and distilled water (to 80 g) were also mixed in 200 mL beaker (aqueous phase). Both phases were incubated and stirred with a magnetic stirrer in a 70°C water bath for 10 min. Subsequently, both phases were mixed by adding the oil phase slowly into the constantly stirred aqueous phase. The mixture was stirred continuously using an Ultra Turrax T25 Basic (IKA, German) for another 5 min with increasing speed from 3000 rpm to 18000 rpm.

The coarse emulsion produced was further homogenized using an APV High Pressure Homogenizer 2000 (APV, Denmark) with 800 psi pressure for 7 cycles to produce a fine lipid emulsion. Then, the emulsion was transported to a grade C clean room for the adjustment of pH with NaOH 1 M to 8.0 ± 0.5. The fine lipid emulsion was then transferred into 100 mL clear glass vials, purged with nitrogen gas for 5 min, closed with a rubber stopper and sealed with an aluminum vial cap in a grade B clean room. The ILE was sterilized using an autoclave at 121°C for 10 min.

PHYSICOCHEMICAL CHARACTERIZATION OF THE IVLE

Particle size, polydispersity index and zeta-potential IVLEs' particle sizes (Z-average), polydispersity index (PDI) and zeta potential (ZP) were measured by dynamic light scattering (DLS) method using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Capillary cuvettes were used for ZP measurement. Zetasizer was operated at 633 nm on 25°C with a scattering angle of 173°. The IVLE used was diluted with de-ionized water (1:100) to avoid the effect of multiple scattering. Triplicate measurements were made for every sample (Garcia-Moreno et al. 2016).

Particle distribution IVLEs' particles distribution ($PFAT_5$) was measured by laser diffraction (LD) method using the Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK). The Mastersizer was operated at 633 nm on 25°C and equipped with a backscatter detector angle of 173°. The detection used a solid state light (blue light) and also a helium neon laser (red light). IVLE was slowly added to 800 mL of re-circulating de-ionized water at 1800 rpm until it reached an ideal obscuration range of 10-20%. The refractive index of super-olein oil used for the measurement was 1.458. Triplicate measurements were made for every sample (Garcia-Moreno et al. 2016).

Rheological behavior The IVLEs' viscosity was measured using a Brookfield Digital Rheometer DVIII viscometer (Brookfield Engineering Laboratories Inc., USA). Approximately 6 mL of samples were placed in SC4-13 sample jars and SC4-18 spindles were attached to the rheometer prior to analysis. Triplicate measurements were made for every sample.

Osmolarity The IVLEs' osmolarity was measured using a Micro-Osmometer Model 332 (Advanced Instruments Inc., USA). Approximately 20 μL samples were pipetted and placed into the osmometer. Results were reported as mOsm/kg. Triplicate measurements were made for every sample.

Measurement of pH The IVLEs' pH value was measured using a digital FiveEasyPlus pH meter FEP20 (Mettler-Toledo AG Analytical, Switzerland). The pH meter was calibrated prior to measurement. Triplicate measurements were made for every sample.

Calories content The IVLEs' calorie content was measured by calorimetric method using a Bomb Calorimeter c5003 (IKA-Werke GmbH & Co., German). Approximately 0.5 g IVLE sample was placed in an acetobutyrate capsule prior to the analysis. The results were reported as kcal/g. Triplicate measurements were made for every sample. Calorie (H) calculation was based on the (1).

$$H = \left(\frac{C \times \Delta T - \Sigma Q}{m}\right), \quad (1)$$

where C represents the heat capacity of calorimeter systems; ΔT is the temperature increment of calorimeter systems; ΣQ is the extra energy produced (J); and m is IVLE sample weight (g).

Transmission electron microscopy (TEM) The IVLEs' morphology was observed by transmission electron microscopy using a Philips CM12 TEM (Koninklijke Philips N.V., Netherlands). The formulation was diluted at 20 times fold with deionized water. One drop of the diluted samples was placed on a carbon-coated 400 mesh copper grid and left for 30 min in a fume hood to dry the sample. The grid was then observed with TEM.

OBSERVATION OF THE PHYSICAL STABILITY

Real-time physical stability study Freshly prepared IVLEs were stored at different storage temperatures and the physical stability was monitored over a period of six months or until physical instability was observed. Three vials of 100 mL IVLE were stored at 4ºC in a National NR-B37TA (National, Japan) chiller and at 25ºC in a Protech Incubator SS-380 (Protech Electronic, France). The dependent variables monitored were Z-average, $PFAT_5$, polydispersity index, zeta potential, viscosity and pH value.

Accelerated stability study For the accelerated physical stability, three vials of 100 mL IVLE were stored at 40ºC with a relative humidity of 75% in a Climacell CLC 707 (MMM Medcenter Einrichtungen GmbH-MMM Group, German) over a period of six months or until physical instability was observed. The dependent variables monitored were Z-average, $PFAT_5$, polydispersity index, zeta potential, viscosity and pH value.

Force degradation study For the force degradation study, three vials of 100 mL IVLE were stored at 80ºC with a relative humidity of 75% in a Climacell CLC 707 (MMM Medcenter Einrichtungen GmbH-MMM Group, German) for 3 days (Blessy et al. 2014). The IVLEs' physical stability was monitored at day 0, 1, 2 and 3. The dependent variables monitored were Z-average, $PFAT_5$, polydispersity index and zeta potential.

Environmental degradation study Approximately 10 mL of IVLE sample was placed in three centrifuge tubes and centrifuged using a Zentrifugen Universal 320R (Hettich Lab Technology, German) with 4000 rpm speed at 25°C for 15 min (Cheong & Nyam 2016). The process was repeated daily for three consecutive days. The IVLEs' physical stability was monitored at day 0, 1, 2 and 3. The dependent variables monitored were Z-average, $PFAT_5$, polydispersity index and zeta potential.

Creaming rate The IVLEs' creaming rate and shelf-life were measured using a LUMiFuge centrifuge (L.U.M. GmbH, German). A combination method of low speed centrifugation and optoelectronic measurement system was applied and analyzed by the SepView Version 5.1 Build 0.804 software (L.U.M. GmbH, German). Approximately 0.5 mL sample was placed in a PC cell, which was then placed horizontally in the LUMiFuge and centrifuged at 3025 rpm for 19.2 h at 5°C. The sample was analyzed every 271 s.

OBSERVATION OF THE CHEMICAL STABILITY

Extraction of fatty acids Approximately 10 mL of IVLE sample was mixed with 80 mL of organic solvent consisting of chloroform: methanol: NaCl 0.85% (2:1:1) in a separatory funnel. The mixture was homogenized and left for 24 h for the separation to take place. Later, the chloroform layer at the bottom of the mixture was removed, filtered using filter paper and air-dried in a fume hood until the solvent smell disappeared (Kang et al. 2017; Jufri et al. 2012; Mulet-Cabero et al. 2017). Then, 50 mg of the extracted dry sample was diluted with 1 mL of hexane in a micro tube and vortexed for 30 s. Later, 1 mL of 2.5 M sodium methoxide in anhydrate methanol solution was introduced into the micro tube and centrifuged for 60 s at 2000 rpm. The preparation was left for 5 min to allow for chemical reactions to take place and then centrifuged again at 2000 rpm for 5 min. Once the process was completed, 0.5 μL of supernatant was transferred into a clear glass vial, capped and proceeded with gas chromatography analysis (RamLi et al. 2009).

Fatty acids contents A Shimadzu GC-2010 (Shimadzu Corporation, Japan) gas chromatography machine equipped with flame ionization detector was used to measure the fatty acids contents and the column used was the polar cyanosiloxane SP-2380, 30 m × 0.25 mm × 0.20 µm (Supelco Incorporation, USA). The oven temperature was heated to 140°C and maintained for 2 min. It was then heated again to 250°C at 4°C per min and maintained for 15 min. The detector and injector were set at 250°C. Nitrogen was used as the carrier gas and its flow rate was set at 60 cm^3 per s. The fatty acids contents were identified by comparing the retention time of the samples' fatty acids methyl ester (FAME) and the standard FAME (Supelco Incorporation, USA). The percentage for every fatty acid identified (xp) was calculated based on (2).

$$xp\,\% = \frac{ap}{a1 + a2 + a3 + \ldots + ap} \times 100 \quad (2)$$

where *ap* represents the chromatograms' peak area of the identified fatty acids *p* and (*a1* + *a2* + *a3* + … + *ap*) is the sum of chromatograms' peak area for all of the identified fatty acids. The measurements were done in triplicate for every sample.

Vitamin E and its' isomers contents High performance liquid chromatography was used for the separation and to measure vitamin E and its isomers. The instrument was equipped with a Waters 1515 isocratic pump, Waters 2487 dual λ absorbance detector, Waters 717 plus auto injector and a XBridge C18 column (5.0 μm, 4.6 mm × 250 mm) (Waters Corporation, USA). The analysis was performed with methanol:water (93:7) as the mobile phase, 1.0 mL/min flow and 10 min run time. Sample identification and quantification were performed at the wavelength of 292 nm. Vitamin E isomers contents were identified by comparing the retention time of the samples and the standard (Supelco Incorporation, USA).

Peroxide contents & free fatty acids values Both peroxide (PV) and acidity values (FFA) were determined based on Palm Oil Tester method (CDR Palm Oil Tester, Italy). A cuvette was filled with 1 mL of alcohol and chromogenous compounds (peroxide reagent) and then incubated in the Palm Oil Testers' incubation cells for 5 min. Then, 10 μL of sample and 10 μL of redox solution were added into the cuvette, which was then gently inverted several times and then incubated again for 3 min. Finally, the cuvette was inserted into the reading cell to measure the peroxide contents at 505 nm. For acidity test, the cuvette was filled with 1 mL of alcohol and chromogenous compounds (acidity reagent) and then incubated for 5 min. Then, 5 μL of sample was added and the cuvette was gently inverted several times. Finally, the acid content was measured at 630 nm.

ANIMAL STUDY

Animal study was approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (FF/2017/AHMAD FUAD/24-MAY/847-JULY-2017-FEB.-2018). All procedures were performed on the rabbits under anaesthesia. Three male New Zealand white rabbits weighing between 2.26 and 2.98 kg were used in this study, and were purchased from the Laboratory Animal Resource Unit (LARU), Faculty of Medicine, Universiti Kebangsaan Malaysia. The rabbits were individually caged for two weeks for acclimatization in the Animal Room, Faculty of Pharmacy, Universiti Kebangsaan Malaysia. The temperature and humidity of the room were set at 25°C and 55 ± 10%, respectively, with a light-dark cycle for 12 h each. The rabbits were given access to rabbit pellet and water freely during the acclimatization and study periods. Heart rate and respiratory rate were monitored on a daily basis.

A dose of 0.2 mL/kg intravenous (IV) KTX (ketamine/Zoletil-50/Xylazine) (LARU, Universiti Kebangsaan Malaysia) were used as the anaesthetic agent to restrain the animals. Subsequently, 0.1 mL/kg KTX was supplied every 30-45 min as additional doses. KTX was administered via the marginal blood vessels of the rabbit ear using a 30G × 4/8" injection needle attached to a 1 mL syringe. Rabbits were infused with 1.5 g/kg SoLE 20% for 5 h continuously per day, and this was repeated daily for 3 days. SoLE 20% were infused using a winged infusion set (Surflo® 23G size, 0.65 mm outer diameter, 19 mm long) (Terumo Corporation, Japan) connected to an infusion pump (Infusomat® Space P) (BBraun Melsungen, Germany) through the marginal vein of the ear.

About 10 mL of blood samples were withdrawn from the rabbit marginal ear artery before the start of infusion on day 1 and placed in an EDTA-containing test tube. The same procedure was repeated on days 2 and 3. Blood samples were also taken on day 4 to monitor the effect of the last dose. The analysis were performed using ADVIA 2400 machine (Siemens Healthineers GmbH, Germany) within 24 h of the blood samples taken. The biochemical analysis performed included the plasma lipid profile: triglyceride (TG), cholesterol (CHOL), low density lipoprotein (LDL) and high density lipoprotein (HDL); and the plasma liver enzyme profile: alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

RESULTS AND DISCUSSION

CHARACTERIZATION OF THE IVLE

Superolein oil based intravenous lipid emulsion 20% (SoLE 20%) was successfully developed using a high pressure homogenization technique. The emulsion had a milky white and homogenous visual appearance (Figure 1). Table 1 shows the physical appearance of this new lipid emulsion with advantage of internal vitamin E contents. Based on the criteria listed in the USP Chapter <729> (Driscoll 2007), this new lipid emulsion complies with the requirements for an intravenous lipid emulsion and is pharmaceutically equivalent with other intravenous lipid emulsion 20% in the market.

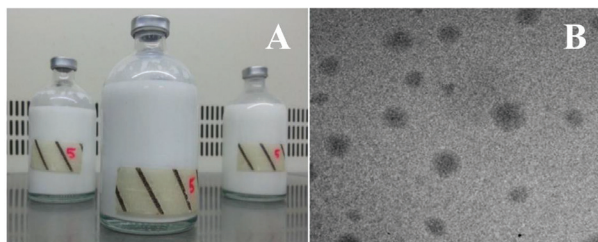

FIGURE 1. (A) SoLE 20% in 100 mL glass vials and (B) Distribution of SoLE 20% particle analyzed by transmission electronic microscopy

TABLE 1. Physicochemical characteristics of SoLE 20%

Characters	SoLE 20 %
Z-average (nm)	229.07 ± 1.57
$PFAT_5$ (%)	0.00
Polydispersity index	0.09 ± 0.02
Zeta potential (mV)	-46.67 ± 4.40
Osmolarity (mOsm/kg)	342.00 ± 1.00
Viscosity (cP)	1.72 ± 0.04
pH value	7.83 ± 0.06
Calorie (cal/g)	804.00 ± 60.10
Peroxide value (mEqO_2/kg)	1.74 ± 0.05
Acidity value (mEq/mL)	0.03 ± 0.01

OBSERVATIONS OF THE PHYSICAL STABILITY

As the IVLE might be stored for a period of time prior to use, real-time stability studies on physical aspects were carried out to confirm their characteristics are of good quality. The IVLE was observed for any phase separation due to coalescence or creaming while particle size, particle distribution, polydispersity index, zeta potential, viscosity and pH value were measured for six months at different temperatures and environments.

The destabilization of IVLE was characterized by the increment of particle size and particle size distribution (Stevens et al. 2016). Both are the crucial factors in the development of coalescence and Ostwald ripening processes (Kaur et al. 2017). Particle-size distribution limit for all commercial IVLE has been established to assist in developing a stable pharmaceutical product. Those limits include a Z-average of below 500 nm and a $PFAT_5$ less than 0.05% (Anez-Bustillos et al. 2016; Driscoll 2015). The current study showed that the IVLE Z-average still complied with the physical stability requirements for 6 months at 4°C and 25°C. Its' also stable at the accelerated temperature of 40°C. The particle size range was 226.83 ± 11.58 to 243.90 ± 3.04 nm with 0.00% $PFAT_5$ (Table 2). IVLE with $PFAT_5$ less than 0.05% are also known as fine emulsion (Driscoll 2015).

The IVLE particle distribution homogeneity and width were measured as the polydispersity index. The acceptable PDI values for IVLE should be in a range of 0.00 up to 0.25 (Zainol et al. 2012). A PDI range from 0.0 to 0.4 indicates monodispersity and 0.5 to 1.0 indicates a relatively broad distribution (Alayoubi et al. 2015; Tan et al. 2016). In this study, the PDI for IVLE ranged from 0.03 ± 0.04 to 0.13 ± 0.04 after 6 months of storage at 4°C, 25°C and 40°C (Table 2). Therefore, all of the formulations stored at the different temperatures were within the monodisperse range and indicated a narrow droplet size distribution. A small PDI value could also be influenced by the low addition rate of the oil phase into the aqueous phase during the pre-homogenization process (Tan et al. 2016).

Long term stability of IVLE was measured by the zeta potential and ranges between -30 and -50 mV. A minimum value of -30 mV is required to have a sufficient electrostatic repulsion between droplets to maintain physical stability and to prevent coalescence from occurring (Cheong & Nyam 2016; Muller et al. 2012; Zainol et al. 2012). Meanwhile, a ZP higher than -60 mV will show good stability for the long term (Muller et al. 2012). In the current study, the IVLEs' ZP range was -40.77 ± 0.64 to -58.30 ± 1.80 mV within 6 months of storage at 4°C, 25°C and

TABLE 2. Real-time physical stability and accelerated physical stabilities studies for SoLE 20%

Character	Temp. (°C)	Duration (month)					
		0	1	2	3	6	
Z-average (nm)	4	229.07 ± 1.57	235.90 ± 5.30	233.07 ± 11.64	241.53 ± 1.01	230.50 ± 4.92	0.18
	25	229.07 ± 1.57	228.20 ± 0.46	226.83 ± 11.58	231.90 ± 0.70	234.83 ± 7.07	0.54
	40	229.07 ± 1.57	243.90 ± 3.04	233.80 ± 1.14	232.07 ± 3.10	231.63 ± 3.62	0.00
$PFAT_5$ (%)	4	0.00	0.00	0.00	0.00	0.00	1.00
	25	0.00	0.00	0.00	0.00	0.00	1.00
	40	0.00	0.00	0.00	0.00	0.00	1.00
PDI	4	0.09 ± 0.02	0.08 ± 0.03	0.12 ± 0.02	0.05 ± 0.03	0.13 ± 0.04	0.38
	25	0.09 ± 0.02	0.08 ± 0.03	0.06 ± 0.02	0.05 ± 0.01	0.06 ± 0.07	0.59
	40	0.09 ± 0.02	0.07 ± 0.05	0.08 ± 0.02	0.03 ± 0.04	0.10 ± 0.03	0.21
ZP (mV)	4	-46.67 ± 4.40	-49.40 ± 2.43	-53.33 ± 0.81	-51.43 ± 1.96	-40.77 ± 0.64	0.00
	25	-46.67 ± 4.40	-49.40 ± 2.95	-51.87 ± 1.00	-55.91 ± 1.85	-41.27 ± 2.16	0.00
	40	-46.67 ± 4.40	-50.43 ± 2.17	-58.23 ± 1.36	-58.30 ± 1.80	-47.43 ± 0.57	0.00
Vc (cP)	4	1.72 ± 0.04	1.93 ± 0.01	1.50 ± 0.06	1.55 ± 0.05	1.58 ± 0.03	0.00
	25	1.72 ± 0.04	1.86 ± 0.05	1.52 ± 0.14	1.52 ± 0.15	1.73 ± 0.03	0.00
	40	1.72 ± 0.04	1.83 ± 0.07	1.61 ± 0.06	1.61 ± 0.07	1.84 ± 0.07	0.00
pH value	4	7.83 ± 0.06	7.84 ± 0.05	7.90 ± 0.04	7.88 ± 0.02	7.6 ± 0.02	0.00
	25	8.00 ± 0.09	7.90 ± 0.02	7.90 ± 0.02	7.44 ± 0.02	7.38 ± 0.01	0.00
	40	7.83 ± 0.06	7.67 ± 0.08	7.44 ± 0.17	6.63 ± 0.03	5.97 ± 0.02	0.00

$PFAT_5$, particle size bigger than 5 μm; PDI, polydispersity index; ZP, zeta potential; Vc, viscosity

40°C (Table 2) and no separation occurred. This might be due to the negative charge of the phospholipids that create a mutual repulsion among the emulsion droplets. This environment keeps the droplets separated and stabilizes the emulsion (Staven et al. 2016).

Viscosity is a function of particle size of the disperse phase and the concentration (Muller et al. 2012) and is a measure of the injectability feature of IVLE. Vc will influence the stability of IVLE in the long term. A high Vc can effect the efficacy of the IVLE and results in a higher risk for coalescence due to the large particle size (Zainol et al. 2012). From a clinical aspect, a recent study reported that 1 - 20 cP pharmaceutical solutions are well tolerated without pain when injected subcutaneously (Berteau et al. 2015). In the present study, the Vc values of the IVLE were between 1.50 ± 0.06 and 1.93 ± 0.01 cP after 6 months of storage at 4°C, 25°C and 40°C (Table 2). The use of MCT as part of the oil phase and the low percentage of oil phase were the main contributors to the low value of viscosity in our IVLE. The particle size, particle charge, colloidal interaction and dispersed phase volume fraction were the main factors that influence the Vc of IVLE (Kaur et al. 2017).

The pH value will influence the physical stability of a lipid emulsion (Kaur et al. 2017). A narrow particle size distribution of IVLE can probably be achieved with a low pH (Staven et al. 2016). In other words, it will reduce the IVLEs' $PFAT_5$ and lower the risk for lipid emulsion instability. The pH value of our newly developed IVLE ranged from 7.54 ± 0.01 to 8.33 ± 0.02 after 6 months of storage at 4°C, 25°C and 40°C (Table 2). Even though changes were detected for the lipid emulsion stored at 40°C, it was still within the accepted pH range for intravenous lipid emulsion, that is within pH 6 and 9 (Anez-Bustillos et al. 2016). Neither coalescence nor oiling out were seen in this IVLE group.

As a product, IVLE will be transported, stored and exposed to different environments. Hence, the forced degradation stability study and the environmental stability study were conducted to assess the quality of the emulsion post exposed to the simulated conditions (Blessy et al. 2014). In the current study, after exposing the intravenous lipid emulsion to the extreme temperature of 80°C and extreme acceleration of 4000 rpm for 15 min, both particle size and its distribution were still stable after 72 h. The polydispersity index and zeta potential were also stable after 72 h in both situations (Table 3).

Despite no visible changes to the IVLE after six months, its shelf-life stability was further investigated using a new method that measured the creaming behavior of an emulsion (Ng et al. 2013). By introducing centrifugal forces to the emulsion at 5°C, the creaming process was accelerated, which forced the dispersed oil molecules to the top of the cell (Figure 2). This phenomenon has led to a clarification of the cells' bottom. It took 4.6 h or equivalent to 5.75 months at earth gravity for the creaming to achieve the 30% of the maximum light transmission at the creaming velocity of 1.47% per h. It was suggested that the light transmission of 30% is acceptable for quantifying the lipid emulsion stability or separation tendency (Kuchler et al. 2006). Thus, the emulsion is stable and good for use for up to six months. Figure 2 shows the transmission profile of SoLE 20% at 5°C and the related phase.

OBSERVATION OF THE CHEMICAL STABILITY

An ideal IVLE for PN is able to provide sufficient calories and supply an appropriate amount of essential fatty acids (FA) to avoid a deficiency of essential fatty acids (Ren et al. 2013). Based on the GC analysis, SoLE contains all of the fatty acids from superolein oil and MCT oil (Derawi et al. 2014). The four major components of SoLE are caprilic acid and capric acid from MCT oil and oleic acid and palmitic acid from superolein oil. Other FAs include lauric acid, myristic acid, stearic acid, linoleic acid, α-linolenic acid and arachidic acid. SoLE has an advantage against peroxidation as the formulation has low unsaturated FA (30%) and high monounsaturated FA (24%). Lipid peroxidation is one of the important mechanisms involved in inflammation, atherosclerosis and cancer (Ren et al. 2013). Fewer double bonds in the structure of FA will reduce the risk for oxidation and its implications (Khanum & Thevanayagam 2017). Monounsaturated FA

TABLE 3. Force degradation and environmental degradation stability studies for SoLE 20%

Parameters	Duration (days)				
	0	1	2	3	p
Force degradation					
Z-average (nm)	224.23 ± 3.06	217.77 ± 2.29	221.07 ± 0.45	222.03 ± 1.37	0.03
$PFAT_5$ (%)	0.00	0.00	0.00	0.00	1.0
PDI	0.04 ± 0.04	0.11 ± 0.03	0.10 ± 0.01	0.09 ± 0.03	0.04
ZP (mV)	-44.63 ± 0.61	-46.07 ± 1.41	-47.23 ± 0.70	-46.80 ± 0.90	0.05
Environmental degradation					
Z-average (nm)	223.97 ± 2.23	221.10 ± 3.55	222.90 ± 2.48	222.67 ± 1.86	0.62
$PFAT_5$ (%)	0.00	0.00	0.00	0.00	1.00
PDI	0.07 ± 0.02	0.04 ± 0.02	0.04 ± 0.04	0.01 ± 0.00	0.08
ZP (mV)	-45.03 ± 1.60	-45.17 ± 0.64	-44.43 ± 1.85	-44.00 ± 0.80	0.69

$PFAT_5$, particle size bigger than 5 μm; PDI, polydispersity index; ZP, zeta potential

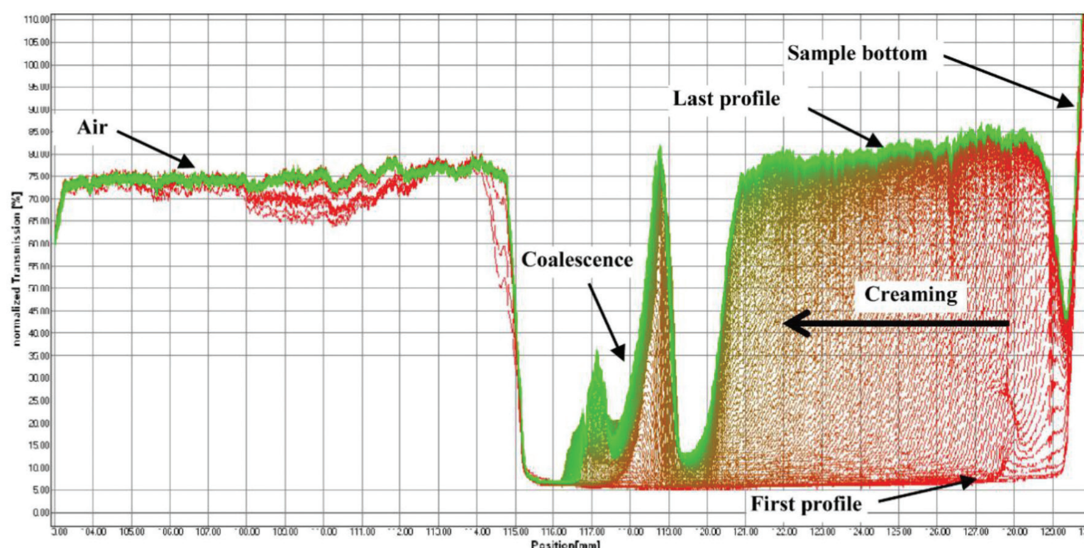

FIGURE 2. Evolution of transmission profile of superolein based intravenous lipid emulsion 20% at 5°C

has only one double bond thus, it has less inflammatory and peroxidation effects compared to polyunsaturated FA. As SoLE is also blended with MCT oil, it shares the MCT benefits of resistance to lipid peroxidation since it mainly contains saturated FAs, easily being metabolized due to its ability to cross the mitochondrial membrane passively and has less pro-inflammatory effects (Raman et al. 2017). Figure 3 shows the GC chromatogram of SoLE fatty acids contents. Furthermore, the fatty acids contents of SoLE were stable for over six months of storage at 4°C with only small changes (Table 4).

Separation of tocopherols and tocotrienols, collectively known as tocols, can be performed using normal or reverse phase HPLC. In the current study, the reverse phase method was used because it has a lower chemical risk due to the mobile phase being a non-dangerous liquid such as methanol and has a more repeatable retention time. This study used a mixture of methanol and distilled water (93:7) as the mobile phase. Hence, the most significant

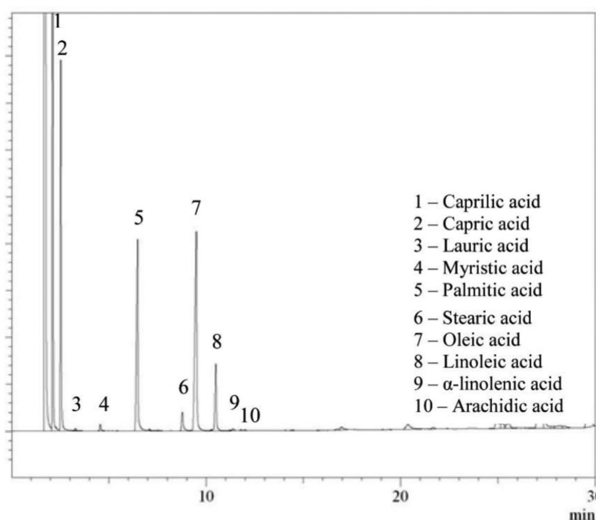

FIGURE 3. GC chromatogram of superolein based intravenous lipid emulsion 20% contents

TABLE 4. The fatty acids compositions of superolein based lipid emulsion 20%

Fatty acids		Contents (%)			p
		0 month	3 months	6 months	
Caprilic acid	(C8: 0)	26.23 ± 0.23	26.67 ± 0.44	27.51 ± 0.33	0.0161
Capric acid	(C10: 0)	21.80 ± 0.13	21.91 ± 0.23	21.59 ± 0.23	0.2373
Lauric acid	(C12: 0)	0.16 ± 0.00	0.16 ± 0.00	0.16 ± 0.00	0.0582
Myristic acid	(C14: 0)	0.50 ± 0.01	0.49 ± 0.02	0.53 ± 0.02	0.0321
Palmitic acid	(C16: 0)	18.27 ± 0.09	17.99 ± 0.28	18.06 ± 0.17	0.2352
Stearic acid	(C18: 0)	2.15 ± 0.07	2.35 ± 0.01	2.02 ± 0.04	0.0003
Oleic acid	(C18: 1n9)	24.24 ± 0.27	23.66 ± 0.39	23.80 ± 0.30	0.1380
Linoleic acid	(C18: 2n6)	6.42 ± 0.06	6.48 ± 0.13	6.07 ± 0.08	0.0022
α-linolenic acid	(C18: 3n3)	0.23 ± 0.01	0.30 ± 0.13	0.24 ± 0.00	0.0115
Arachidic acid	(C20: 0)	0.10 ± 0.01	0.12 ± 0.01	0.12 ± 0.00	0.0472
Saturated fatty acids		69.11 ± 0.33	69.56 ± 0.38	69.88 ± 0.39	
Monounsaturated fatty acids		24.24 ± 0.27	23.66 ± 0.39	23.80 ± 0.30	
Polyunsaturated fatty acids		6.65 ± 0.06	6.78 ± 0.02	6.32 ± 0.08	

issue related to this method is the difficulty to completely separate the β and γ components of tocols as both chemical structures have methyl groups at the chromanol rings and only differ on the location of the methyl group. However, there were reports of using C30 silica bonded column to achieve the complete separation of tocols' β and γ components (Lampi 2011).

The current study used a C18 XBridge column to separate the vitamin E isomers. Based on the findings, this method was capable of separating the tocols components. All isomers were detected and identified based on the comparisons of elution time with the standard tocols. Vitamin E isomers were separated by adsorption based on the polarity of the isomers, with the more polar isomer being eluted first. Two factors influence the polarity of the isomers which primarily are the methyl groups attached to the chromanol ring and also the degree of saturation on the side chain. Isomers with more methyl groups and more double bonds are more polar compared to other isomers. These factors made tocotrienols more polar compared to tocopherols and thus being eluted earlier (Pacifico et al. 2012). Figure 4 shows the RP-HPLC chromatogram of SoLE contents. Table 5 shows the vitamin E homologs of SoLE.

The present study managed to identify 5 isomers of tocols from the superolein based IVLE 20%. The elution sequence for the isomers was based on the polarity of the isomers with δ-tocotrienol (δ-T_3) being eluted first, followed by γ-tocotrienol (γ-T_3), β-tocotrienol (β-T_3), α-tocotrienol (α-T_3) and α-tocoferol (α-T) as the last isomer being eluted. The isomers sequence was similar to previous reports (Abu-Fayyad & Nazzal 2017; Gornas & Siger 2015; Ng & Choo 2012). α-T was the only tocopherol identified and accounted for 18.43% of the total tocols content. T_3 on the other hand had a higher tocols content with γ-T_3 being the highest tocol in the emulsion at 43.01%. The percentage of tocols contents identified from SoLE 20% was also in agreement with earlier reports (Mba et al. 2015; Ng et al. 2006) at 18 to 25% tocopherol and 75 to 82% tocotrienol with its isomers. Tocols contents of the SoLE 20% were stable over six months of storage at 4°C with only small changes (Table 5).

In the present study, the peroxide values of SoLE 20% was observed to increase from 1.74 ± 0.05 mEqO2/kg to 2.97 ± 0.16 mEqO2/kg (p<0.0001) within six month of storage at 4°C (Figure 5). This increase in peroxide value is due to the oxidation of unsaturated fatty acids in SoLE 20% but the value is still within normal limit (Eriksson 2016; Gharby et al. 2014; Yadav et al. 2018). ILE should have a PV of less than 5.00 mEq/kg (Ismail et al. 2016). The rancidity can be smelled as the PV increases to 30-40 mEq/kg (Gharby et al. 2014; Sarwar et al. 2016).

Acidity values also showed a similar trend as it increased from 0.02 ± 0.01 mEq/mL to 0.06 mEq/mL

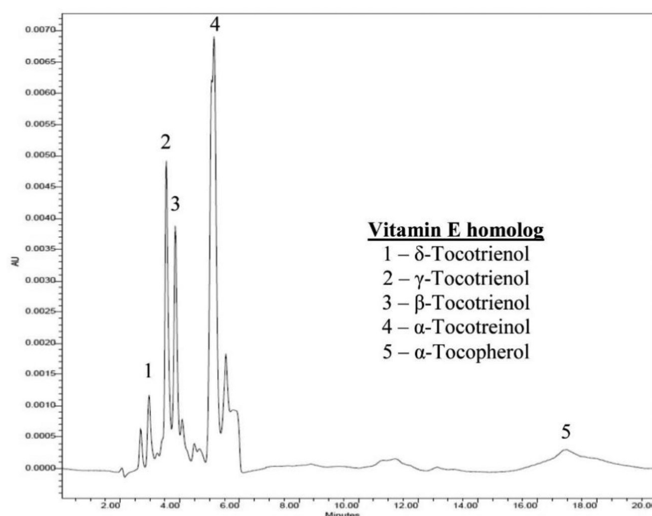

FIGURE 4. RP-HPLC chromatogram of superolein based intravenous lipid emulsion 20% contents

TABLE 5. Vitamin E isomers of superolein based intravenous lipid emulsion 20%

Vitamin E isomers		Contents (%)			p
		0 month	3 months	6 months	
α-Tocopherol	α-T	18.51 ± 0.00	18.35 ± 0.00	18.45 ± 0.01	< 0.0001
α-Tocotrienol	α-T_3	7.74 ± 0.25	8.55 ± 0.43	8.32 ± 0.54	< 0.0001
β-Tocotrienol	β-T_3	11.61 ± 0.10	12.23 ± 0.07	12.64 ± 0.10	< 0.0001
γ-Tocotrienol	γ-T_3	45.87 ± 0.64	44.72 ± 0.47	44.37 ± 0.34	< 0.0001
δ-Tocotrienol	δ-T_3	16.27 ± 0.01	16.15 ± 0.03	16.21 ± 0.01	< 0.0001

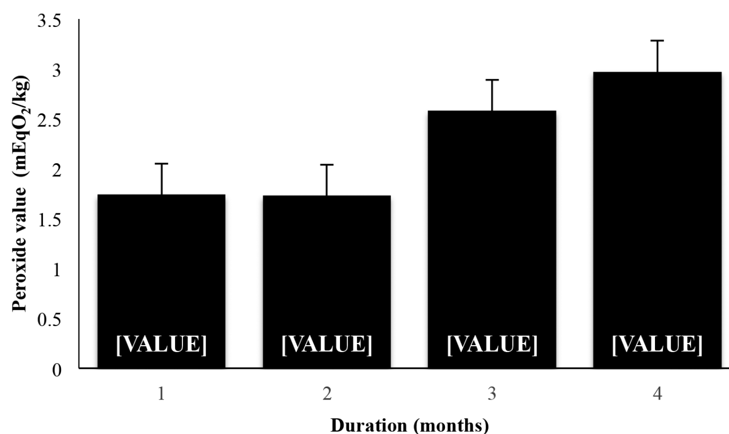

FIGURE 5. Peroxide value for SoLE 20% after six months of storing at 4°C

(p=0.0043) within six month of storage at 4°C (Figure 6). The acidity value indicated the accumulation of free fatty acid contents due to natural or stimulated degradation of the triglycerides in SoLE 20%. ILE should have an FFA of less than 0.07 mEq/mL (Driscoll 2006). The SoLE 20% FFA in the present study was still within the suggested range for ILE.

ANIMAL STUDY

Rabbits that were infused with SoLE 20% for 3 days survived during and after the administration without complications. Throughout the acclimatization and study period, weight, rectal temperature (39.1-40.3°C), heart rate (128.0-180.0 counts/min) and respiratory rate (32.0-64.0 counts/min) did not differ statistically (p>0.05). The results

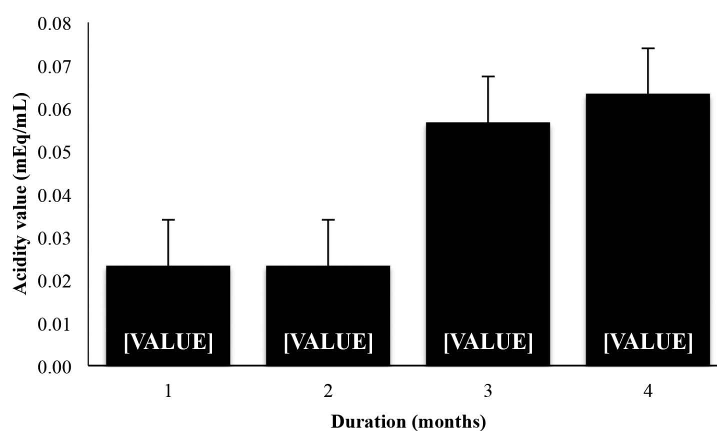

FIGURE 6. Acidity value for SoLE 20% after six months of storing at 4°C

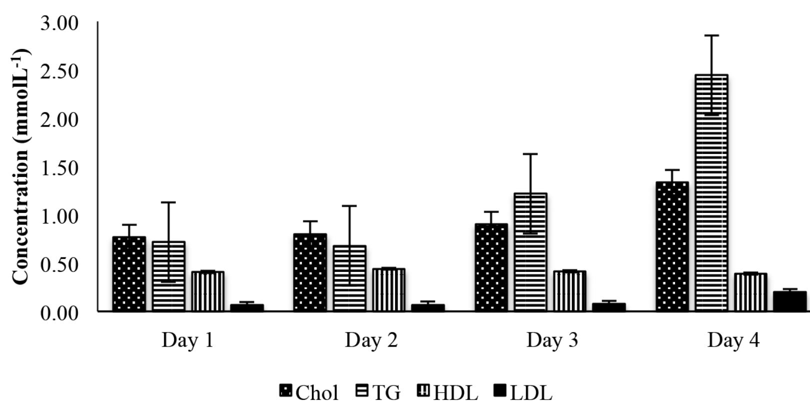

FIGURE 7. Plasma lipid profile prior to the administration of SoLE 20% for 3 days

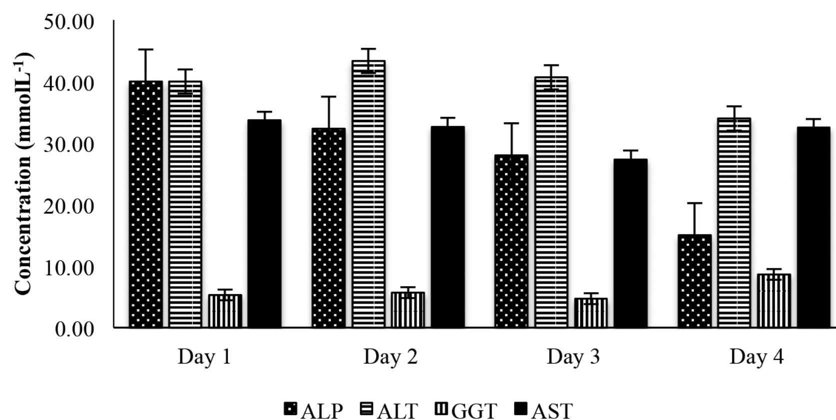

FIGURE 8. Plasma liver enzymes profile prior to the administration of SoLE 20% for 3 days

of the plasma lipid profile and plasma liver enzymes profile analyses showed no significant difference before and after SoLE 20% administration (p>0.05) (Figures 7 and 8).

The infusion of 1.5 g/kg/day SoLE 20% also did not cause abrupt changes to the lipid profiles of the rabbits. The level of plasma lipid profile; CHOL, TG, HDL and LDL, and plasma liver enzymes profile; ALP, ALT, GGT and AST, remained statistically the same (p>0.05). The normal range for cholesterol in rabbit plasma is 0.99-2.05 mmol/L, triglyceride is 0.82-1.51 mmol/L, high density lipoprotein is 0.45-0.76 mmol/L and low density lipoprotein is 0.18-0.52 mmol/L as reported by previous studies (Roche et al. 2012a, 2012b; Shahid et al. 2012). Meanwhile, the common range for ALP is 12-96 uL^{-1}, ALT is 55-260 uL^{-1}, GGT is 0.00-31.00 uL^{-1} and AST is 33.00-99.00 uL^{-1} (Mayada et al. 2015; Meredith & Rayment 2009; Onyesom & Anosike 2007; Ozkan et al. 2012; Shousha et al. 2017). These findings suggested that the infusion of 1.5 g/kg/d SoLE 20% is safe and will not alter the recipient plasma lipid profile and plasma liver enzymes profile since any issues or liver conditions caused by the SoLE will raise their levels (Arain et al. 2017; Malakouti et al. 2017).

CONCLUSION

The SoLE 20% was found to have good physicochemical characteristics and excellent physicochemical stability for up to six months at various temperatures and storage conditions. It also did not alter the plasma lipid and plasma liver enzymes profile of the animals studied. This study has successfully developed a stable SoLE 20% that is safe to the animal model liver. SoLE 20% has a potential to be a candidate for alternative IVLE in the future.

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