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Development and Characterization of Self-Microemulsifying Drug Delivery System of Tacrolimus for Intravenous Administration

Vivek Baban Borhade, Hema Ajit Nair, and Darshana Deepak Hegde
Department of Pharmaceutics, Bombay College of Pharmacy, Kalina, Santacruz, Mumbai, India

Tacrolimus (FK 506), a poorly soluble immunosuppressant is currently formulated in nonaqueous vehicle containing hydrogenated castor oil derivative for intravenous administration. Hydrogenated castor oil derivatives are associated with acute anaphylactic reactions. This proposes to overcome the problems of poor aqueous solubility of the drug and the toxicity associated with currently used excipients by the development of a new parenterally acceptable formulation using self-microemulsifying drug delivery system (SMEDDS). Solubility of FK 506 in various oils, surfactants, and cosurfactants was determined to identify SMEDDS components. Phase diagrams were constructed at different ratios of surfactants : cosurfactant (K_m) to determine microemulsion existence area. Influence of oily phase content, K_m, aqueous phase composition, dilution, and incorporation of drug on mean globule size of microemulsions was studied. SMEDDSs were developed using ethyl oleate as oily phase and Solutol HS 15 as surfactant. Glycofurol was used successfully as a cosurfactant. Developed SMEDDS could solubilize 0.8% (wt/wt) FK 506 and on addition to aqueous phase could form spontaneous microemulsion with mean globule size <30 nm. The resulting microemulsion was iso-osmotic, did not show any phase separation or drug precipitation even after 24 h, and exhibited negligible hemolytic potential to red blood cells.

Keywords tacrolimus; self-microemulsifying drug delivery system; solubility; Solutol HS 15; glycofurol

INTRODUCTION

Tacrolimus (FK 506), a macrolide immunosuppressant is clinically used as primary agent in the prevention of organ rejection after liver, kidney, heart, lung, small bowel, and bone marrow transplantation (Christine & Susan, 2004; Spencer, Goa, & Gillis, 1997). It is more potent and efficacious than cyclosporine A (CyA) and proved to be effective as a "rescue" drug for patients for whom CyA- and steroid-based regimen fails to prevent persistent refractory rejections. These advantages of FK 506 coupled with its long-term tolerability have led to improved graft and patient survival rates indicating its clinical potential (Shapiro, 1999; Spencer et al., 1997). In transplantation therapy, it is necessary to achieve rapid immunosuppression during immediate postoperative period. However, during this period, the patient is not frequently able to take FK 506 orally or cannot tolerate its oral administration because of gastrointestinal dysfunctioning. Usually, it requires 4–5 days to start oral therapy (Christine & Susan, 2004; Spencer et al., 1997). Therefore, FK 506 is required to be administered as an intravenous (IV) infusion in early post-transplantation period and is continued till the initiation of oral treatment.

Successful development of formulation of FK 506 for IV administration is difficult because of its poor aqueous solubility (5–8 μg/mL), high lipophilicity, and neutral nature (Kino et al., 1987; Shigeki, Atsuo, Rinta, & Gordon, 2002). Moreover, though FK 506 has good solubility in organic solvents such as dehydrated alcohol, its alcoholic solution becomes turbid because of the precipitation immediately after dilution with aqueous phase (Namiki, Fujiwara, Kihara, Koda, & Hane, 1995). Therefore, its marketed IV formulation, Prograf® 5 mg/mL, is available as a preconcentrate in a nonaqueous vehicle containing dehydrated ethanol and Cremophor RH 60 (polyoxyl 60 hydrogenated castor oil). An appropriate solubilizer such as Cremophor is added to prevent its unfavorable precipitation on dilution. Commercially available IV formulation of FK 506, however, is reported to cause adverse reactions that are primarily attributed to Cremophor RH 60 rather than drug (Gelderblom, Verweij, & Sparreboom, 2001; Lee, 1995). Cremophor RH 60 is not an inert vehicle, and serious anaphylactic reactions in considerable population of patients receiving Prograf® have been reported (Spencer et al., 1997). Moreover, Cremophor group of surfactants are also reported to be responsible for the nephrotoxicity in products such as Sandimmune (CyA) (Volcheck & Dellen, 1998). It is also known to leach plasticizer (diethylhexyl phthalate) from polyvinyl chloride infusion bags and polyethylene-lined tubing sets, which can cause hepatic toxicity. This necessitates the use of plastizer-free containers

Address correspondence to Darshana Deepak Hegde, Department of Pharmaceutics, Bombay College of Pharmacy, Kalina, Santacruz, Mumbai 400098, India. E-mail: ddhegde2003@yahoo.co.in

causing inconvenience to medical staff and pain to patients (Gelderblom et al., 2001; He, Wang, & Zhang, 2003; Lee, 1995). Thus, in light of aforementioned issues, it is apparent that there is a need for an alternative formulation of FK 506 with improved solubility, and an IV formulation of it without castor oil derivative could be of great value.

Microemulsion-based systems (microemulsions and self-microemulsifying drug delivery system, SMEDDS) have attracted considerable interest in recent years. Part of this interest is due to their versatility and advantages such as high drug solubilization, transparency, thermodynamic stability, and ease of manufacture and scale-up (Lawrence & Rees, 2000; Prince, 1977). These properties suggest the potential of microemulsion-based drug delivery systems as an IV vehicle for hydrophobic drugs. It was therefore thought worthwhile to extend the concept of microemulsion-based drug delivery systems to overcome the problems of poor aqueous solubility and toxicity associated with currently available formulation of FK 506.

MATERIALS AND METHODS

Materials

Tacrolimus was a gift from Glenmark Pharmaceuticals Ltd. (Nashik, India). Lutrol F 68, Lutrol F 127, Solutol HS 15 (BASF India Ltd., Mumbai, India), Lipoid MCT, Miglyol 810, Miglyol 812, MYS-40, and Soybean oil (S. Zaveri & Co. Mumbai, India) were obtained as gift samples. Glycofurol (E. Merck, Mumbai, India), benzyl alcohol, ethyl oleate, isopropyl Myristate, Polyethelene Glycol 200, Polyethelene Glycol 300, Polyethelene Glycol 400, propylene glycol, Tween 20, and Tween 80 (SD fine chemicals, Mumbai, India) were purchased. All other reagents were AR grade and used as received. Double-distilled water was prepared freshly whenever required.

Analysis of FK 506

A high-performance thin layer chromatography (HPTLC) method with derivitization was developed for analysis of FK 506. HPTLC system (Camag, Switzerland) comprising of Limomat 5 sample applicator, Hamilton syringe (100 μL), twin trough chamber (10 × 10 cm), dipping chamber, and TLC scanner 3 with winCATS software V1.1.3.0 was used. Silica gel 60 F254 TLC plates (E. Merck, Darmstadt, Germany) were used as stationary phase. Samples were dissolved in methanol and spotted under drying stream of nitrogen. Mobile phase composed of toluene : acetonitrile : glacial acetic acid (6:4:0.1, by volume) was used. Prior to development, chamber was saturated with mobile phase for 20 min. FK 506 was visualized and quantified (R_f = 0.4 ± 0.03) at 675 nm using anisaldehyde–sulfuric acid reagent. Minimum detectable amount was 28.90 ng/spot with limit of quantitation being 97.04 ng/spot. Assay was linear (r^2 = .998) in the range of 100–800 ng/spot. The method was accurate, precise, and robust, and the relative standard deviation was consistently <5%.

Solubility Studies

Solubility of FK 506 in various oils, surfactants, and cosurfactants was determined by shake flask method. Briefly, an excess amount of FK 506 was added to each vial containing 1 g of the selected vehicle. After sealing, mixtures were vortexed for 10 min and shaken for 48 h in reciprocating water bath shaker (Remi Equipments, Mumbai, India) at 25 ± 2°C. After 48 h, vials were centrifuged at 500 × g for 5 min, and the undissolved drug was discarded by filtration using membrane filter (0.45 μm; Pall Life Sciences, Mumbai, India). The filtrate was suitably diluted with methanol, and FK 506 was quantified by a validated HPTLC method developed in house.

Further, solubility of FK 506 in various buffers ranging from pH 1.2 to 10.6 (pH solubility profile) was determined by shake flask method as described above.

Construction of Pseudoternary Phase Diagrams

The pseudoternary phase diagrams of quaternary system consisting of ethyl oleate, Solutol HS 15, glycofurol, and water were constructed using titration method at 25 ± 2°C (Corswant, Engstrom, & Soderman, 1997). The effect of glycofurol as a cosurfactant on phase behavior of aforementioned system was studied by mixing Solutol HS 15 with glycofurol in various weight ratios, namely, 0.5:1, 1:1, 3:2, and 2:1. The ratio of Solutol HS 15 to glycofurol was regarded as K_m. At specific K_m, Solutol HS 15 and glycofurol were mixed with double-distilled water in weight ratios of 10:0 to 0:10 in glass vials. A small amount of oily phase, that is, ethyl oleate, was added in 0.5% (wt/wt) increment, vortexed, and allowed to equilibrate. Resulting mixtures were examined visually for transparency, flow properties, and observed through cross polarizer for optical isotropy. Endpoint of titration was the point where mixture became turbid or phase separation was observed. Transparent, single-phase, low viscous, and nonbirefringent mixtures were designated as microemulsions and represented in phase diagrams as ME area.

Globule Size Analysis

Mean globule size and polydispersity index (PI) of microemulsions were determined by photon correlation spectroscopy (PCS) at 25°C, using Beckman Coulter N5 plus Submicron Particle Size Analyzer (Coulter Corporation, Janesville, WI, USA). Various aqueous phases, namely, double-distilled water, 0.9% (wt/vol) sodium chloride, and 5% dextrose solution, used for dilution were filtered twice through 0.22-μm membrane filter (Pall Life Sciences). Microemulsions were diluted to ensure that light scattering intensity was within the instrument's sensitivity range. Samples were placed in transparent polystyrene cuvettes and loaded in thermostated chamber. Light scattering was monitored at an angle of 90° to the incident beam.

TABLE 1
Composition of Placebo SMEDDS with Varying Oil Content at K_m = 1.5

	Formulation (mg)			
Ingredients	S1	S2	S3	S4
Solutol HS 15 + glycofurol	470	460	450	440
Ethyl oleate	30	40	50	60

Effect of Oily Phase Content on Mean Globule Size

A series of placebo SMEDDSs were prepared with varying oil content (Table 1) to study the effects of oil content on mean globule size. Ratio of surfactant to cosurfactant was selected on basis of phase diagrams and maintained at 3:2. Briefly, oil, surfactant, and cosurfactant were weighed in glass vials, mixed by stirring, and heated (40–50°C) to form homogenous systems. The oil–surfactant mixture (500 mg) was dispersed in 50 mL of aqueous phases with gentle stirring. Globule size and PI were determined immediately after dilution.

Effect of Weight Ratios of Solutol HS 15 to Glycofurol (K_m) on Mean Globule Size

A series of placebo SMEDDSs were prepared with varying weight ratios of Solutol HS 15 to glycofurol (K_m = 0.5, 1.0, 1.5, 2.0, 3.0) with 12% of ethyl oleate. The oil–surfactant mixture (500 mg) was dispersed in 50 mL of aqueous phases with gentle stirring. The globule size and its distribution of resulting aqueous dispersions were determined immediately. Self-microemulsification efficiency of oil–surfactant mixtures was judged by observing their tendency to form spontaneous microemulsion.

Effect of Drug Incorporation on Mean Globule Size

Effect of FK 506 loading on physical stability of microemulsions was studied using formulation S4. Accordingly, a series of SMEDDSs were prepared with 0.4, 0.6 0.8, and 1% (wt/wt) concentrations of FK 506. SMEDDS (500 mg) was dispersed in 50 mL of different aqueous phases mentioned above. Globule size and its distribution were determined immediately by PCS, and microemulsions were stored at 25 ± 2°C for 24 h to assess drug precipitation.

Preparation of FK 506 SMEDDS

FK 506 was dissolved in ethyl oleate–glycofurol mixture in glass vials and then accurately weighed Solutol HS 15 was added to it. Components were mixed and heated (40–50°C) to form a homogenous mixture and stored at 25°C until used.

Effect of Dilution and Aqueous Phase Composition on FK 506 SMEDDS

Robustness of SMEDDS to the dilution and effect of aqueous phase composition were studied using optimized FK 506 SMEDDS composition. FK 506 SMEDDS (500 mg) was dispersed in 500 mL of various aqueous phases mentioned above with gentle stirring. Resulting microemulsions were kept at 25 ± 2°C and evaluated for drug precipitation, phase separation, and changes in size over the period of 24 h.

Accelerated Stability Tests: Centrifugation and Freeze–Thaw Cycle

Optimized FK 506 SMEDDS was diluted with double-distilled water in the ratio 1:9 (wt/wt) (one part of FK 506 SMEDDS and nine parts of double-distilled water) and centrifuged at 500 × g for 30 min. In addition, it was subjected to freeze–thaw cycle by storing it at –20°C for 24 h and then for another 24 h at 40°C. Microemulsions were observed visually for phase separation and drug precipitation, whereas its physical stability was assessed by measuring globule size before and after centrifugation and freeze–thaw cycle.

Effect of Method of Sterilization of FK 506 SMEDDS

Optimized FK 506 SMEDDS (3 g) was filled in type I borosilicate glass vials with teflon-coated butyl stoppers, sealed and sterilized by autoclaving at 121°C for 15 min. After sterilization, FK 506 SMEDDS was assessed for appearance before dilution, and drug content was determined by developed HPTLC method. FK 506 SMEDDS (500 mg) was diluted with 50 mL of double-distilled water, and the globule size of microemulsion was determined. In addition, microemulsion was observed for 24 h for phase separation or drug precipitation. Effect of filtration as a sterilization method was evaluated by filtering FK 506 SMEDDS through 0.22-μm membrane filters. Formulations were characterized for aforementioned parameters before and after filtration.

Osmotic Pressure of FK 506 SMEDDS

Osmolarity of FK 506 SMEDDS was determined by freezing point depression method using Osmomat 030 osmometer (Gonastec, Berlin, Germany) at 25 ± 2°C. The instrument was calibrated using 0.9% sodium chloride solution and pure water. FK 506 SMEDDS was reconstituted with 0.9% sodium chloride solution to 5, 10, 15, and 20 μg/mL of FK 506, and 50 μL was filled in osmometer vials to determine osmolarity.

In Vitro Hemolytic Potential of FK 506 SMEDDS

Hemolytic potential of FK 506 SMEDDS with and without FK 506 was investigated by a reported in vitro method (Soderlind, Wollbratt, & Corswant, 2003) using heparinized fresh human whole blood. FK 506 SMEDDS was diluted with 0.9% sodium

chloride solution to obtain 10, 20, and 40 μg/mL of FK 506. Formed microemulsion (1.8 mL) was mixed gently with 0.2 mL blood for 5–10 s and incubated at 37°C for 2 h. After incubation, mixtures were placed in ice-cold water for 2 min to quench hemolysis. Intact red blood cells were separated from supernatant by centrifugation at 500 × *g* for 5 min at 5°C. Supernatant was analyzed for hemoglobin content at 576 nm. Sample corresponding to basal hemolysis, that is, negative control, was prepared by incubating 0.2 mL blood with 1.8 mL 0.9% sodium chloride solution for 2 h. A completely hemolysed sample was prepared by incubating 0.2 mL blood with 1.8 mL distilled water. Controls were analyzed for hemoglobin in a similar way. Percent extent of hemolysis (% H) was determined by using the equation $\% H = (Abs - Abs_{control})/(Abs_{100} - Abs_{control}) \times 100$, where *Abs* is absorbance of sample, $Abs_{control}$ is absorbance of negative control sample, and Abs_{100} is absorbance of sample with 100% hemolysis. Effect of contact time of formulation on hemolysis was determined by incubating samples containing 20 μg/mL FK 506 for 2, 4, 6, and 8 h.

Stability Studies of FK 506 SMEDDS

Chemical and physical stability of FK 506 SMEDDS was assessed at 40 ± 2°C/75 ± 5% RH and 5 ± 3°C as per ICH guidelines. FK 506 SMEDDS sterilized by membrane filtration was filled into 5 mL type I borosilicate glass vials with teflon-coated butyl stoppers, crimped with aluminum caps, and were stored upright for 3 months. Samples were withdrawn at 0, 1, 2, and 3 months and assessed for physical appearance, pH, dilutability, mean globule size, PI, and FK 506 content.

Toxicity Studies of FK 506 SMEDDS

Single-dose acute toxicity studies of FK 506 SMEDDS with and without FK 506 were performed using OECD 423 guidelines (http://www.oecd.org/dataoecd/17/51/1948378.pdf). Female Swiss albino mice weighing 25–30 g were divided into groups of three animals for each dose level. Sterility of FK 506 SMEDDS was achieved by membrane filtration. FK 506 SMEDDS was diluted with 0.9% sterile sodium chloride solution and administered as slow IV bolus dose through tail vein at four dose levels (10, 25, 50, and 75 mg/kg) in a stepwise manner. A saline control group was also included. Absence or presence of FK 506 SMEDDS-related mortality of animals dosed at one step determined whether further testing was needed, that is, dosing of three additional animals with same dose is required or dosing of three additional animals at next higher dose is required. Time interval between treatment groups was determined by onset, duration and severity of toxicity signs. Animals were observed individually after dosing, with special attention given during the first 4 h, periodically during first 24 h, and daily thereafter for 30 days. Individual weights of animals were recorded shortly before study and at least weekly thereafter.

RESULTS AND DISCUSSION

Solubility Studies

Solubility of FK 506 in various excipients is shown in Table 2. As it is important to achieve optimum drug loading (Pouton, 2000), solubility study was aimed to identify suitable SMEDDS components that possess good solubilizing capacity for FK 506. Therefore, its solubility was determined in lipophilic solvents that are used in commercially available injectable products.

Oils are nonpolar solvents possessing low dielectric constants. Nonpolar solvents can dissolve nonpolar solutes with similar internal pressures through induced dipole interactions. However, the solubility of FK 506 was very less in most of the oils examined; no significant difference in its solubility was observed irrespective of the nature of oil, that is, whether the oily phase was a medium-chain triglyceride such as Miglyols and Lipoid MCT or long-chain triglyceride such as soybean oil. Among several oily phases examined, the only exception was ethyl oleate, which exhibited a solubility of 8.79 mg/g and therefore was selected as oily phase for further study. The solubility of FK 506 in the oils confirmed its poor lipophilicity although log partition coefficient of FK 506 is reported as 4.6, which prevents it from being formulated as an emulsion or microemulsion.

As shown in Table 2, various surfactants exhibited good solubilizing potential for FK 506. Even though surfactants with different chemical nature were screened, all of them appeared to be equally effective for improving the solubility of FK 506.

FK 506 exhibits very good solubility in various glycols that are proposed as cosurfactants. The solubility of FK 506 in the PEGs was higher than that in the oils. This shows that the affinity of FK 506 to the polyethylene oxide (PEO) group was higher than that in the oils. In addition, the higher affinity may be due to the ability of FK 506 to form a hydrogen bond with the PEO groups in PEGs. Further, the solubility study clearly indicated that FK 506 has poor aqueous solubility and it does not depend on pH of the medium (Table 2). This behavior may be attributed to the neutral nature of FK 506.

Selection of Excipients

Based on the data obtained from the solubility study and preliminary studies carried out in the research laboratory (Borhade, 2006), ethyl oleate, Solutol HS 15, and glycofurol were selected as SMEDDS components. The criteria for the selection of oily phase were its potential to solubilize maximum amount of FK 506 and the ability to yield systems with higher microemulsion existence region. Oily phases such as Miglyol 810, Miglyol 812, isopropyl myristate, and soybean oil were assessed for their ability to form microemulsion. However, microemulsion regions obtained with these oily phases were much smaller than that obtained with ethyl oleate (Borhade, 2006). Furthermore, with respect to its safety, ethyl

TABLE 2
Solubility of FK 506 in Various Excipients and Aqueous Phases

Excipients	Chemical Composition	Solubility[a]
Oily phases		
Ethyl oleate	Ethyl 9-octadecenoate	8.51 ± 1.69
Isopropyl myristate	1-Methylethyl tetradecanoate	0.75 ± 0.86
Lipoid MCT	Medium-chain triglycerides	2.16 ± 1.11
Miglyol 810	Caprylic/capric triglyceride	1.65 ± 1.29
Miglyol 812	Caprylic/capric triglyceride	2.1 ± 0.56
Soybean oil	Long-chain triglycerides	1.39 ± 0.59
Surfactants		
Lutrol F 68[b]	Polyethylene–propylene glycol copolymer	13.45 ± 1.21
Lutrol F 127[b]	Polyethylene–propylene glycol copolymer	9.41 ± 0.41
MYS-40[b]	Polyoxyl 40 stearate	11.35 ± 0.96
Solutol HS 15	PEG-660-12-hydroxystearate	9.60 ± 0.89
Tween 20	POE-20-sorbitan monolaurate	12.76 ± 1.04
Tween 80	POE-20-sorbitan monooleate	14.45 ± 2.19
Cosurfactants		
Glycofurol	Tetrahydrofurfuryl PEG ether	22.48 ± 1.02
Labrasol	Caprylocaproyl macrogol-8 glycerides	9.63 ± 0.82
Polyethelene Glycol 200	Polyoxyethylene glycol	35.66 ± 3.79
Polyethelene Glycol 300	Polyoxyethylene glycol	32.89 ± 4.39
Polyethelene Glycol 400	Polyoxyethylene glycol	39.52 ± 4.64
Propylene glycol	1,2-Dihydroxypropane	37.88 ± 4. 21
Aqueous phases[c]		
0.9% NaCl Solution	—	5.2 ± 4.46
5% Dextrose Solution	—	5.9 ± 2.53
Buffer pH 1.2	Hydrochloric acid–potassium chloride buffer	6.87 ± 3.26
Buffer pH 3.5	Glycine–hydrochloric acid buffer	6.02 ± 3.17
Buffer pH 4.5	Acetate buffer	5.71 ± 2.11
Buffer pH 6.8	Citrate–phosphate buffer	6.83 ± 3.14
Buffer pH 7.4	Phosphate buffer	7.17 ± 2.32
Buffer pH 9.0	Glycine–sodium hydroxide buffer	7.02 ± 2.23
Buffer pH 10.6	Carbonate–bicarbonate buffer	6.29 ± 1.69
Purified water	—	7.32 ± 2.59

[a]Solubility expressed as mg/g, data expressed as mean ± *SD*, $n = 3$.
[b]10% wt/wt surfactant solutions.
[c]Solubility expressed as μg/mL, data expressed as mean ± *SD*, $n = 3$.

oleate is included in the *FDA Inactive Ingredients Guide*, parenteral medicines licensed in the UK, and *Canadian List of Acceptable Nonmedicinal Ingredients*. It is used as a vehicle in parenteral preparations and considered of low toxicity with minimal tissue irritation potential. It is also reported as a suitable solvent for steroids and other lipophilic drugs (Strickley, 2004). Because of these properties of ethyl oleate, in addition to its solubilizing potential for FK 506, it was selected as oily phase.

Selection of surfactants and cosurfactants was governed by their emulsification efficiency for selected oily phases rather than their ability to solubilize FK 506. Good solubilization potential was considered as an added advantage for their selection. Preliminary studies indicated that compared with Lutrol F 68, Lutrol F 127, MYS-40, Tween 20, and Tween 80, Solutol HS 15 exhibited better emulsification properties for ethyl oleate with larger microemulsion existence region and hence was selected as a primary surfactant (Borhade, 2006). Further, Solutol HS 15 has emerged as an alternative to Cremophor EL and has been generally regarded as a relatively nontoxic and nonirritant excipient. It has been used in preclinical injectable formulations of water-insoluble molecules. Further, it is reported to be used in parenteral pharmaceutical preparations in concentrations up to 50% to solubilize diclofenac, propanidid, and

vitamin K1 (Brendan O'Leary, personal communication. BASF Corporation, 2003; Strickley, 2004).

The preliminary study also indicated the superiority of glycofurol for improving emulsification efficiency of various surfactants, especially Solutol HS 15 (Borhade, 2006). In addition, glycofurol is miscible with water and ethyl oleate. It was therefore anticipated that it can act as a good solubilizer for both water and oily phases. This amphiphilic nature of glycofurol would enable itself to distribute between aqueous and oily phase thereby altering the relative hydro/lipophilicity and could remain at interface after dilution. So, it was selected as a cosurfactant for further studies. Furthermore, with respect to its parenteral acceptability, glycofurol is reported to be used as a solvent in parenteral products for IV or intramuscular injection in concentrations up to 50% (vol/vol). Glycofurol is generally regarded as a relatively nontoxic and nonirritant material at the levels used as a pharmaceutical excipient, and its tolerability is approximately the same as propylene glycol (Weller, 2006).

Construction of Pseudoternary Phase Diagrams

Boundaries of microemulsion domains were determined with the aid of ternary phase diagrams that makes it easy to find out concentration range of components for the existence of microemulsion region. Figure 1A shows the pseudoternary phase diagram of ethyl oleate : Solutol HS 15 : water without cosurfactant (glycofurol). Figure 1B–F shows the effect of cosurfactant (at various K_m) on phase behavior of the system. It is evident that addition of cosurfactant significantly increased the area of ME formation at all K_m values than that with Solutol HS 15 alone. Clear isotropic regions appeared at all K_m studied; however, it was found that ME region was increased gradually with increase in K_m. Largest area was obtained at K_m value of 3.0, which allowed incorporation of highest amount of ethyl oleate, whereas the smallest ME region was obtained at K_m = 0.5. An increase in microemulsion region was more toward oil–water axis. This indicates that with increase in Solutol HS 15 concentration, amount of water and ethyl oleate that could be incorporated into microemulsion could be increased. However, considering the extent of ME area and anticipitated solubility of FK 506 in oil–surfactant mixture, K_m = 1.5 was selected for further studies.

Similar results have been reported for microemulsion containing diazepam and triptolide wherein Tween 80 was used as surfactant and propylene glycol and ethanol as cosurfactants (Chen et al., 2004; Li, Nandi, & Kim et al., 2002). These results suggested a significant contribution of Solutol HS 15 to microemulsions formation. However, Solutol HS 15, alone, at high ratios with ethyl oleate could produce very small ME area, and it was necessary to add cosurfactants such as glycofurol

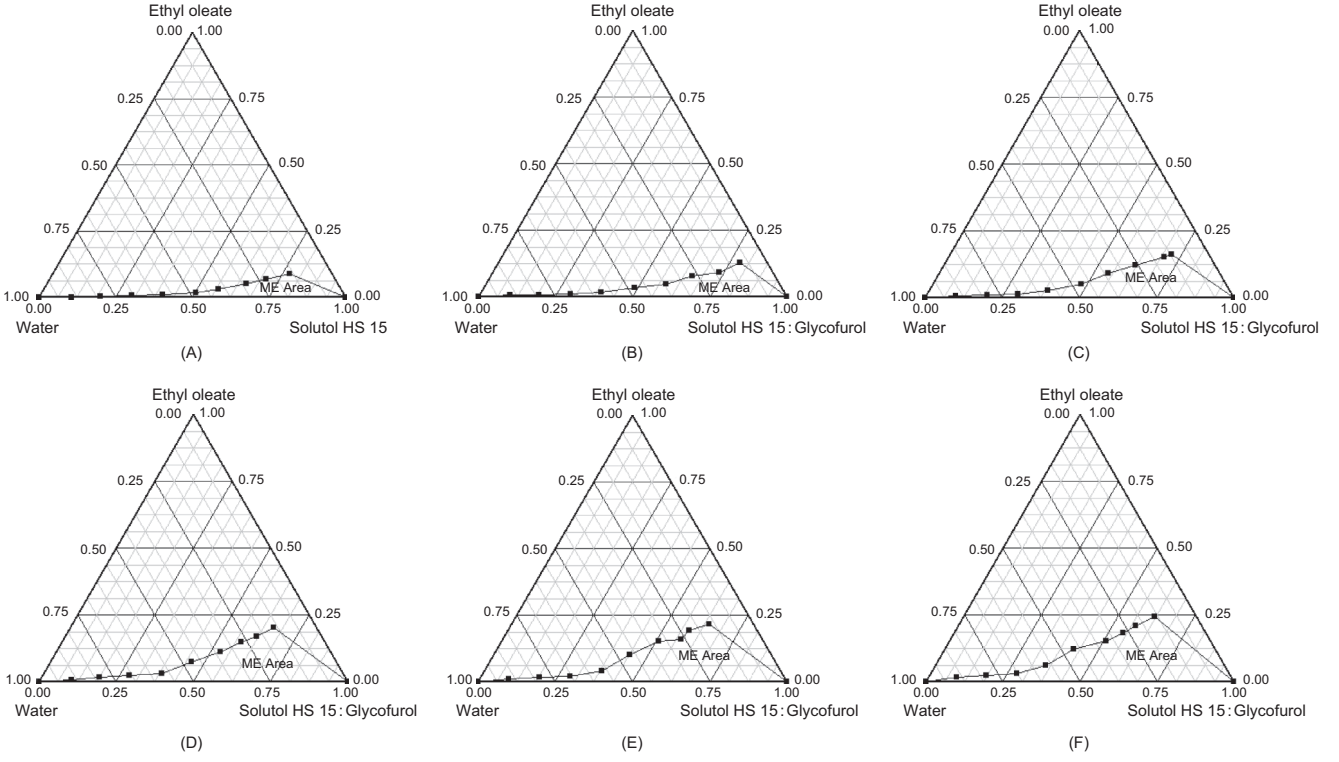

FIGURE 1. Pseudoternary phase diagrams indicating o/w microemulsion existence region (ME) of ethyl oleate : Solutol HS 15 : water system in the absence of glycofurol (A) and in the presence of glycofurol at various k_m values 0.5 (B), 1.0 (C), 1.5 (D), 2.0 (E), and 3.0 (F).

(Borhade, 2006). An increase in the ME region may be because of an increased fluidity at interface caused by the addition of glycofurol resulting in decrease in the interfacial tension. Further, because of its amphiphilic nature, such as short-chain alcohols, it might be increasing the mutual solubility of aqueous and oily phases due to its partitioning between these phases, thereby altering relative hydro/lipophilicity. This observation to some extent confirms the cosurfactant-like activity of glycofurol. Moreover, physicochemical properties of glycofurol were quite in line with most of the physicochemical characteristics of a classical cosurfactant [C No. 4–6, C (%) 60–65, O (%) 20–30, log *P* 0.2–0.9 and log 1/*S* close to zero] as stated by Alany, Rades, Davies, and Tucker (2000). High and low total surfactant content parts of diagram led to the formation of gel-like structure and emulsion-type disperse systems, respectively, regardless of K_m. Liquid crystalline phases were not observed when mixtures were examined through polarizing microscope. Microemulsion types could not be determined as distinct conversion from oil-in-water (o/w) to water-in-oil (w/o) microemulsions was not observed. However, systems exhibited continuity between water-rich and water-poor regions, and thus it may be predicted that microemulsion type varies progressively from o/w to w/o, as composition varies.

Effect of Ethyl Oleate Concentration on Mean Globule Size

Effect of ethyl oleate concentration on mean globule size was studied at K_m = 1.5 by increasing its concentration from 6 to 15% (wt/wt) of oil–surfactant mixture (Figure 2). The mean globule size of microemulsion was increased from 6 to 25 nm with PI < 0.5 up to 12% oil incorporation. This suggests that oil constitutes the inner structure of microemulsion droplets, which is consistent with o/w type of structure. Maximum concentration of ethyl oleate that can be incorporated was about 12% (wt/wt) of oil–surfactant mixture. Further increase in oily phase concentration to 15% resulted in the formation of turbid systems, which eventually led to phase separation. These observations are in line with the inferences drawn from phase behavior studies described in the section "Construction of Pseudoternary Phase Diagrams." A similar trend of increase in mean globule size with increase in oil content has been reported in the literature for phospholipid-based microemulsions (Moreno, Ballesteros, & Frutos, 2003; Sadurni, Solans, Azemar, & Jose, 2005).

Effect of Weight Ratios of Solutol HS 15 to Glycofurol (K_m) on Mean Globule Size

Figure 3 indicates the influence of K_m on mean globule size and size distribution of resulting aqueous dispersions. An increase in K_m, that is, concentration of Solutol HS 15, led to a reduction in globule size and its distribution. Mean globule size remained <30 nm at K_m 1.5, 2, and 3 with PI < 0.5. Oil–surfactant mixtures were found to disperse in a minute with immediate formation of o/w microemulsions on addition to aqueous phase, thus exhibiting good self-microemulsifying properties. This could be attributed to high hydrophilicity of surfactant–cosurfactant.

However, at K_m 0.5 and 1, aqueous dispersions were not sufficiently stable as indicated by significant increase in globule size. This confirmed the important contribution of Solutol HS 15 as a primary surfactant on reduction of globule size and physical stability of microemulsion. Nevertheless, glycofurol has a positive effect on the reduction of globule size and stability of microemulsion; as emulsification efficiency of Solutol HS 15 was improved compared with its use without addition of glycofurol, smaller ME area was obtained with Solutol HS 15 alone. Results are in line with the phase studies discussed in the earlier section.

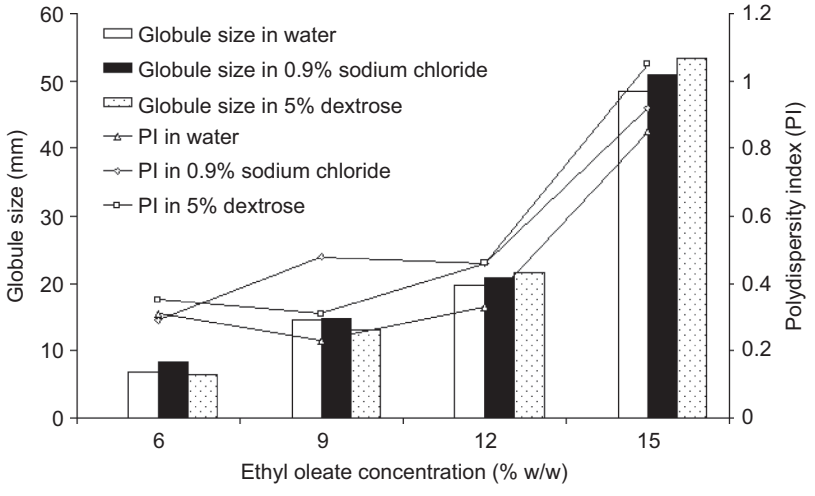

FIGURE 2. Effect of ethyl oleate concentration on mean globule size and polydispersity index (data expressed as mean, n = 3, where relative SD < 10%).

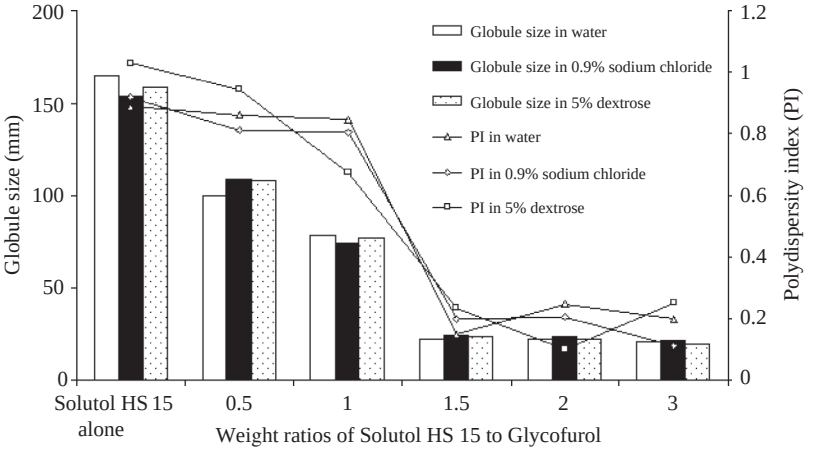

FIGURE 3. Effect of K_m on mean globule size and polydispersity index (data expressed as mean, $n = 3$, where relative $SD < 10\%$).

Therefore, considering the physical stability of microemulsions formed and anticipated solubility of drug in the SMEEDS components, K_m 1.5 was selected for further study.

Effect of Drug Incorporation on Mean Globule Size

Effect of FK 506 loading on mean globule size is shown in Figure 4. Drug incorporation can have significant influence on mean globule size and needs to be investigated. Further, because of hydrophobic nature of FK 506, it may precipitate on dilution. Formulations containing 0.4, 0.6, and 0.8% (wt/wt) of FK 506 did not exhibit any precipitation of drug when observed for a period of 24 h. The self-microemulsification efficiency, globule size, and PI of microemulsions remained unchanged irrespective of the composition of the aqueous phases. However, 1% (wt/wt) incorporation of drug led to an increase in globule size where microemulsion became progressively turbid with the precipitation of the drug.

Selection of Optimum FK 506 SMEDDS Composition

Optimized composition was selected based on larger microemulsification area, fast dispersion, drug loading efficiency, physical stability of formed microemulsions, and minimum influence of aqueous phase composition and dilution on mean globule size. Accordingly, formulation S4 was selected for further studies and its composition is shown in Table 3.

Robustness to Dilution and Effect of Aqueous Phase Composition

Robustness to dilution and effect of aqueous phase composition on globule size is shown in Figure 5. Ability of microemulsion to be diluted without any phase separation and drug precipitation is essential for its use as a drug delivery system. The results indicated that SMEDDS can be diluted up to 1,000-fold without any phase separation or drug precipitation and remained stable over a period of 24 h without any significant increase in globule size and PI. Aqueous phase composition

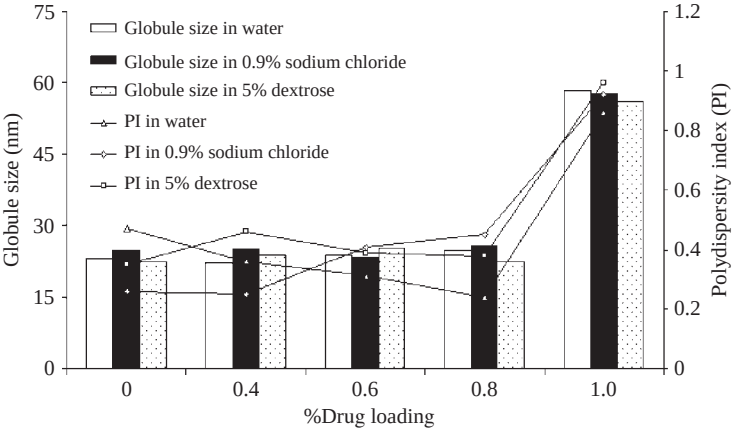

FIGURE 4. Effect of drug incorporation on mean globule size and polydispersity index (data expressed as mean, $n = 3$, where relative $SD < 10\%$).

TABLE 3
Composition of Optimized FK 506 SMEDDS

Ingredients	Quantity (mg)
FK 506	5.00
Ethyl oleate	77.06
Solutol HS 15	325.69
Glycofurol	217.13

also did not affect the physical stability of the resulting microemulsion. These results were in contrast with the phospholipid-based microemulsion systems described in the literature (Ruth, Attwood, Ktistis, & Taylor, 1995; Trotta, Pattarino, & Grosa, 1998) that became turbid leading to phase separation after dilution. This suggested that Solutol HS 15–glycofurol system could reside at interface for sufficiently longer period despite larger dilutions, producing stable microemulsion. In addition, drug was not precipitated even after large dilution up to 24 h, thus confirming the solvent capacity of microemulsion. Dilutability of developed SMEDDS was an important criteria, as FK 506 is clinically administered as a continuous 24 h IV infusion diluted to a final concentration of 4–20 μg/mL with 0.9% sodium chloride or 5% dextrose solution (Christine & Susan, 2004). Thus, developed SMEDDS could be designed as a preconcentrate to be reconstituted or diluted as microemulsions suitable for IV administration.

Accelerated Stability Tests: Centrifugation and Freeze–Thaw Cycle

The effect of centrifugation and freeze–thaw cycling on globule size and its distribution is shown in Table 4. Both accelerated tests are carried out to ascertain stability of microemulsions under stress conditions. Resulting microemulsion did not exhibit any drug precipitation, phase separation, or marked changes in globule size after centrifugation confirming its stable nature. Similarly, microemulsion survived the freeze–thaw cycling as it was found to be reconstituted without any phase separation, drug precipitation, and increase in globule size and its distribution after exposure to freeze–thaw cycling.

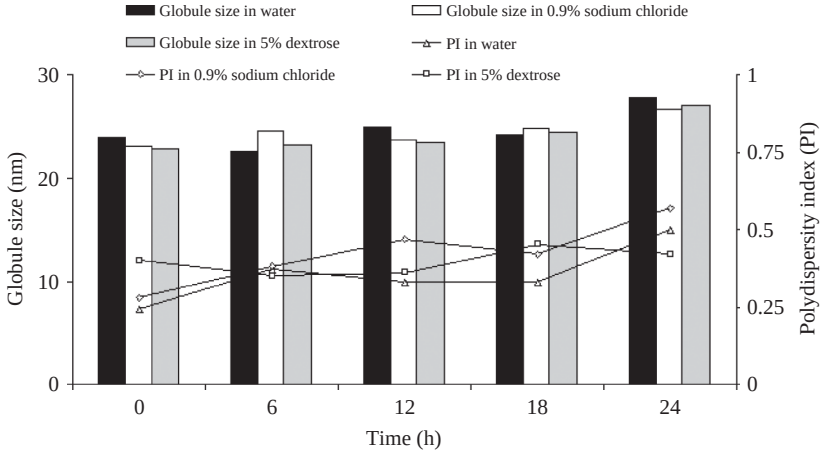

FIGURE 5. Effect of aqueous phase composition and duration after dilution on mean globule size and polydispersity index (data expressed as mean, $n = 3$, where relative $SD < 10\%$).

TABLE 4
Effect of Accelerated Stability Tests on Mean Globule Size and Polydispersity Index of Microemulsion

Formulation	Centrifugation				Freeze–Thaw Cycle			
Accelerated Stability Tests	Placebo SMEDDS		FK 506 SMEDDS		Placebo SMEDDS		FK 506 SMEDDS	
Parameters	Before	After	Before	After	Before	After	Before	After
Globule size (nm)	23.7	23.1	23.5	22.6	24.4	23.8	23.8	22.9
Polydispersity index	0.42	0.29	0.34	0.37	0.42	0.36	0.36	0.31

Data expressed as mean, $n = 3$, where relative $SD < 10\%$.

Effect of Method of Sterilization of FK 506 SMEDDS

Effect of autoclaving and membrane filtration as sterilization methods on FK 506 SMEDDS is shown in Table 5. FK 506 SMEDDS displayed a high physical stability as it remained clear, monophasic liquids without any precipitation of FK 506 on autoclaving. On addition to 0.9% sodium chloride solution, FK 506 SMEDDS formed microemulsion within a minute indicating that its self-microemulsification efficiency was retained. Formulations did not show any phase separation after dilution. Globule size and PI of formulation also remained unaffected. However, drug content was reduced to 69.42 ± 3.98 and pH of formulation was found to decrease slightly. FK 506 SMEDDS exhibited reduced drug content on autoclaving and it may possibly be due to degradation of FK 506. Therefore, it can be concluded that placebo SMEDDS exhibited sufficient physical stability toward autoclaving process and can be used as a vehicle for the incorporation of drugs that can withstand autoclaving. FK 506 SMEDDS when sterilized by membrane filtration exhibited a similar behavior with respect to physical stability. In addition, drug content of the formulations remained unchanged indicating that no component of the formulation was adsorbed on membrane filter. Hence, membrane filtration could be a suitable method for the sterilization of developed FK 506 SMEDDS.

Osmotic Pressure of FK 506 SMEDDS

Hypo- or hyperosmolarity of parenteral formulation is known to cause morphological changes of red blood cells, pain, and tissue damage at the injection site. The ideal osmotic pressure of injectable formulation ranges from 250 to 350 mOsm/kg, a value difficult to achieve in case of formulations containing water-insoluble drugs (Lee, Park, Lim, & Kim, 2002). Osmotic pressure of drug incorporated microemulsion, that is, reconstituted FK 506 SMEDDS was measured with sequential dilutions at concentrations of 5, 10, 15, and 20 μg/mL of FK 506 and was found to be 299 ± 1.2, 301 ± 2.2, 305 ± 1.8, and 307 ± 1.3 mOsm/kg, respectively. Thus, the osmotic pressure remained constant and close to physiological values at concentrations of FK 506 used clinically.

In Vitro Hemolysis of FK 506 SMEDDS

There is always a risk of lysis of red blood cells when microemulsions are intended to be used for IV administration because of the presence of surfactants and cosurfactants. This may result in release of hemoglobin and other cellular components into the bloodstream. An increase in free hemoglobin in the plasma is associated with many undesirable medical conditions, including renal dysfunction, splenomegaly, jaundice, and kernicterus. Therefore, potential of microemulsions to induce hemolysis is determined prior to its IV administration. Effect of drug concentration and contact time of formulation with blood on hemolysis is shown in Table 6. Increase in concentration of drug resulted in an increase in hemolysis. Clinically, FK 506 is administered at maximum concentration (20 μg/mL). At this concentration, formulation was found to cause only 0.5% hemolysis. It was also observed that, as contact time of formulation with blood was increased, hemolysis was increased. This could be because of a static method applied in this study, which is in contrast with dynamic behavior observed in vivo (Krzyaniak & Raymond, 1997). The developed FK 506 SMEDDS thus exhibited negligible hemolytic potential and appeared to be nontoxic to RBCs.

TABLE 5
Effect of Method of Sterilization on Stability of SMEDDS

Formulation	Autoclaving				Membrane Filtration			
Method of Sterilization	Placebo SMEDDS		FK 506 SMEDDS		Placebo SMEDDS		FK 506 SMEDDS	
Parameters	Before	After	Before	After	Before	After	Before	After
pH	4.58 ± 0.12	4.32 ± 0.11	4.54 ± 0.09	4.23 ± 0.08	4.55 ± 0.1	4.60 ± 0.06	4.56 ± 0.1	4.51 ± 0.13
Drug content (% wt/wt)	NA	NA	98.11 ± 1.71	69.42 ± 3.98	NA	NA	98.21 ± 1.23	98.91 ± 2.1
Globule size (nm)	23.5	23.3	23.8	23.5	23.6	22.9	24.1	23.1
Polydispersity index	0.32	0.37	0.5	0.25	0.32	0.41	0.37	0.38

NA, Not applicable.

pH and drug content expressed as mean ± *SD*, $n = 3$; mean globule size and polydispersity index expressed as mean, $n = 3$, where relative *SD* <10%.

TABLE 6
Effect of Drug Concentration and Contact Time on Hemolysis

Concentration (μg/mL)	% Hemolysis	Time (h)	% Hemolysis
10	0.35 ± 0.21	1	0.17 ± 0.19
20	0.50 ± 0.14	2	0.51 ± 0.22
40	1.66 ± 0.21	4	0.69 ± 0.15
		6	1.03 ± 0.29
		8	1.89 ± 0.36

Samples contain 20 μg/mL of FK 506, data expressed as mean ± *SD*, *n* = 3.

Stability Studies of FK 506 SMEDDS

No change in the physical parameters such as homogeneity and clarity of FK 506 SMEDDS was observed during stability studies. The stability data of FK 506 SMEDDS at stated storage conditions is summarized in Table 7. Interestingly, no decline in the FK 506 content was observed at the end of 3 months indicating that FK 506 remained chemically stable in SMEDDS. Furthermore, other parameters such as self-microemulsion efficiency, globule size, and size distribution remained unchanged at all storage conditions during the entire period of study.

Single-Dose Acute Toxicity Studies

Single-dose acute toxicity study was performed to evaluate the safety of developed FK 506 SMEDDS from four independent single-dose experiments (Table 8). The test was based on a stepwise procedure with the use of a minimum number of animals per step. Animals administered with dose of 10 and 25 mg/kg did not show any signs of toxicity. All three mice administered with a dose of 50 mg/kg showed signs of acute toxicity in the form of a transient high respiratory pattern followed by very low respiratory pattern. They regained the respiratory rhythm and normal body movements after 4–5 h. However, three animals administered with dose 75 mg/kg of FK 506 SMEDDS showed signs of severe acute toxicity and died within few minutes. The toxicity of the vehicle was evaluated by repeating the study using placebo formulations at four different dose levels and similar results were obtained as that of drug-incorporated SMEDDS. All surviving animals were in a good physical condition and were found to gain more than 10% weight within 30 days. Data obtained indicated that the toxicity could be attributed to quantity and viscosity of formulation as it was highly viscous at a dose of 75 mg/kg. The volume of mice's blood is approximately 72 mL/kg, which would not be sufficient for dilution of vehicle in the body. The formulation was developed as SMEEDS and is supposed to be diluted to 250-fold or more as per dose requirements before clinical use. Therefore, results clearly demonstrate that FK 506 SMEDDS was well tolerated and could be administered up to 25 mg/kg safely in mice.

CONCLUSION

The potential of SMEDDS was explored successfully for parenteral delivery of poorly water-soluble immunosuppressant FK 506 for the first time. Developed SMEDDS improved the solubility of FK 506 without the use of hydrogenated castor oil derivative. FK 506 SMEDDS, designed as preconcentrate, could be reconstituted as microemulsion suitable for IV administration. It could be diluted up to 1,000-fold without any phase separation or drug precipitation with effective surfactant concentration of less than 1% for administration. Dilutability, low toxicity, and high safety exhibited by the developed system makes it suitable for parenteral administration and can be employed to deliver drugs of different lipophilicities. Furthermore, to our knowledge, this is the first report of using glycofurol successfully as a cosurfactant for producing IV microemulsions. Overall, the results obtained highlight the potential of ethyl oleate–Solutol HS 15–glycofurol-based SMEDDS as a novel and commercially viable formulation for the IV delivery of FK 506.

TABLE 7
Effect of Storage Condition and Time on Mean Globule Size and Polydispersity Index (Mean, *n* = 3, Where Relative *SD* < 10%) and Drug Content (Mean ± *SD*, *n* = 3)

Storage Condition	Globule Size (nm)		Polydispersity Index		Drug Content (% wt/wt)	
Time (days)	5°C	40°C/75% RH	5°C	40°C/75% RH	5°C	40°C/75% RH
0	24.6	22.6	0.38	0.26	99.7 ± 1.66	99.72 ± 1.66
15	23.8	23.9	0.22	0.42	98.2 ± 0.76	99.33 ± 0.81
30	24.4	23.1	0.23	0.24	99.15 ± 2.47	99.02 ± 2.27
60	23.8	24.2	0.39	0.47	99.49 ± 1.52	98.91 ± 1.95
90	23.1	23.6	0.12	0.22	98.58 ± 2.89	99.49 ± 1.33

TABLE 8
Acute Toxicity Studies of FK 506 SMEDDS

Group	% Survival after 30 days	Mortality
Saline control	100	None
10 mg/kg	100	None
25 mg/kg	100	None
50 mg/kg	100	None
75 mg/kg	0	Immediate

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