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# Oleic acid based heterolipid synthesis, characterization and application in self-microemulsifying drug delivery system

Rahul S. Kalhapure, Krishnacharya G. Akamanchi*

*Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, Matunga (E), Mumbai 400019, India*

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ABSTRACT

There is increasing demand for lipids owing to their use in formulating lipid based drug delivery systems of poorly soluble drugs. The present work discusses the synthesis, characterization of oleic acid based heterolipid and its use as oil in the development of self-microemulsifying drug delivery system (SMEDDS) for parenteral delivery. Synthesis was carried out by Michael addition of *tert*-butyl acrylate to 3-amino-1-propanol to obtain di-*tert*-butyl aminopropanol derivative. Reaction of this di-*tert*-butyl aminopropanol derivative with oleoyl chloride using *p*-dimethylaminopyridine as a coupling agent gave the desired heterolipid. It was characterized by $^{1}$H NMR, $^{13}$C NMR and MS to confirm the structure. It did not exhibit any measurable cytotoxicity, even up to 80 μg/ml concentration. Application in parenteral drug delivery was explored using furosemide (FUR), a BCS class IV drug, as a model. FUR showed three times greater solubility in the heterolipid as compared to oleic acid. SMEDDSs were developed using heterolipid as oily phase, Solutol HS 15® as surfactant and ethanol as a co-surfactant. Developed SMEDDS could form spontaneous microemulsion on addition to various aqueous phases with mean globule size <70 nm without any phase separation or drug precipitation even after 24 h, and exhibited negligible hemolytic potential.


## 1. Introduction

One of the major challenges in parenteral administration of lipophilic drugs is design of appropriate pharmaceutical formulation. Lipid emulsions are attractive systems for improving drug solubility of poorly soluble or practically insoluble drugs due to their ability to incorporate lipophilic drugs. This may result into reduced side effects, increased bioavailability, and prolonged pharmacological effects in comparison to conventional formulations (Youenang Piemi et al., 1999). The oily phase, typically used for pharmaceutical emulsions, consists of digestible wide range of triglycerides, partial glycerides, semi-synthetic oily esters, semi-synthetic non-ionic surfactants esters (Hung et al., 2007; Pouton and Porter, 2008), homolipids and heterolipids (Attama and Nkemnele, 2005). Homolipids and heterolipids have gained renewed interests as oily excipients for different drug delivery systems with an increasing number of lipophilic drugs under development. Heterolipids, such as Phospholipon 90G®, contain other functional groups in addition to fatty acid moiety (Stuchlík and Zák, 2001; Attama and Müller-Goymann, 2007). With increasing number of insoluble drugs and with emphasis on precise performance and alternative route of administration there is a need to expand the tool box of oily excipients with better performance, so as to provide wide choice for development of novel, biocompatible, non-irritating and cost-effective lipidic drug delivery systems.

Lipidic drug delivery systems range from simple oil solutions to complex mixtures of oils, surfactants, co-surfactants and co-solvents. Complex mixtures are typically self-dispersing systems often referred to as self-emulsifying drug delivery systems (SEDDS) or self-micro-emulsifying drug delivery systems (SMEDDS) (Pouton, 2006). Formulations which disperse to form transparent colloidal systems are usually referred to as SMEDDS; though in scientific terms this distinction is somewhat arbitrary. Thermodynamic stability of these microemulsions is usually unknown but the dispersions formed by both SEDDS and SMEDDS are often stable in practice for months (Pouton and Porter, 2008). SMEDDS is an isotropic anhydrous mixture of drug, lipophile and surfactant/s, that forms fine oil-in-water microemulsion (globule size <100 nm) when introduced into aqueous phase under conditions of gentle agitation. SMEDDS are regarded as an attractive approach because of high drug solubilizing capacity, transparency, thermodynamic stability, and ease of manufacture and scale up, improvement in both rate and extent of absorption by the lymphatic uptake (Mandawgade et al., 2008). These properties of SMEDDS suggest their potential use as intravenous (IV) vehicles for hydrophobic drugs (Borhade et al., 2009).

To expand the tool box of oily excipients, the present work was undertaken and oleic acid based heterolipid **6** has been designed, synthesized, characterized with respect to chemical

* Corresponding author. Tel.: +91 22 33611111; fax: +91 22 33611020.
*E-mail addresses:* kgap@rediffmail.com, kg.akamanchi@ictmumbai.edu.in (K.G. Akamanchi).

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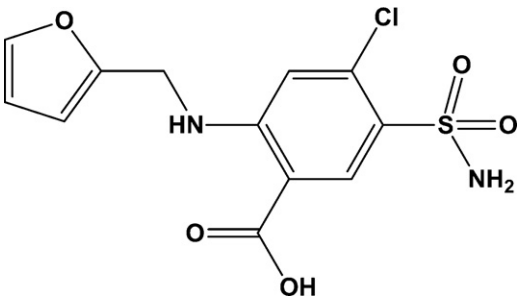



**Fig. 1.** Structure of furosemide.

structure and various properties, and SMEDDS containing heterolipid **6** were evaluated as IV vehicle, taking furosemide (FUR), a BCS Class IV drug, as model. This new heterolipid was an oily liquid. It was evaluated for physical properties like viscosity, density, refractive index and cytotoxicity by sulforhodamine B (SRB) assay. For application in parenteral drug delivery it was incorporated as an oil phase into the formulation of SMEDDS using FUR as a model drug. FUR, 5-(aminosulfonyl)-4-chloro-2-((2-furanylmethyl)amino) benzoic acid (Fig. 1) is a high-loop diuretic widely used for treatment of conditions leading to accumulation of excessive water in the body (edema), normally associated with cardiovascular disorders such as heart failure, infarction and hypertension (Al-Obaid et al., 1989; Patel and Vavia, 2010). It is a weak acid with p*K*a value of 3.8 (carboxylic acid) (Berthod, 1999) and log *P* (*n*-octanol/water) values of 2.29 (Berthod, 1999) and 1.81 (Ruiz-Angel et al., 2004). Calculated log *P* (*n*-octanol/water) for FUR has been reported by Kasim et al. (2004) using two different methods, finding values of 1.9 (CLog P program version 3.0, BioByte Corp.) and 0.74 (ChemDraw Ultra 6.0, CambridgeSoft Corp.). The aqueous solubility of FUR at room temperature has been reported to be 0.01825 mg/ml (Shin and Kim, 2003). Low solubility and permeability are the two critical factors for poor and highly variable human bioavailability of FUR (Zvnor et al., 2010). Improvement of aqueous solubility in such a case is a valuable aim to improve therapeutic efficacy as for any drug molecule to be active pharmacologically, it should have sufficient aqueous solubility. According to the literature data, there is a growing interest in the lipid- and surfactant-based systems, for example, lipid solution, surfactant dispersion emulsion, liposomes, microemulsion, dry emulsion and self-microemulsifying formulations (Shah et al., 1994; Šentjurc et al., 1999; Jumaa et al., 2002; Neslihan and Benita, 2004; Porter et al., 2007; Patil et al., 2007). So, primary goal behind selecting FUR as a model was to improve its aqueous solubility via formulating SMEDDS using novel oleic acid based heterolipid.

The FUR-SMEDDS was developed and evaluated for globule size, drug content by HPLC, dilution potential, freeze thaw cycle, centrifugation potential, stability with respect to sterilization and in vitro hemolysis.

## 2. Materials and methods

### 2.1. Materials

FUR was kindly provided by Ipca Laboratories Ltd., Ratlam, India. Solutol HS 15® and LutrolF68® were kindly provided as gift samples by BASF India Ltd. Oleic acid (technical grade, 90%) was obtained from Sigma, USA. 3-Amino-1-propanol and *tert*-butyl acrylate were obtained from Alfa-Aesar, USA. Thionyl chloride, *p*-dimethylaminopyridine (DMAP), benzyl alcohol, Tween 20, Tween 80, PEG-400, propylene glycol and ethanol were purchased from s d fine Chemicals, India. All the solvents used were of analytical grade and obtained from Merck. For thin layer chromatography, Merck precoated Silica-gel 60$F_{254}$ plates were used.

### 2.2. Instrumentation

FT-IR spectra were obtained using PerkinElmer spectrophotometer. $^{1}$H NMR and $^{13}$C NMR spectra were recorded on Jeol NMR spectrometer at 300 MHz and 75 MHz respectively. An electrospray ionization-mass spectrum (ESI-MS) was recorded on Varian mass spectrometer.

### 2.3. Synthesis of compound 3 (Scheme 1)

Compound **3** was synthesized with slight modification in literature procedure (Krishna and Jayaraman, 2003). Briefly, to a solution of *tert*-butyl acrylate **2** (19.22 g, 150 mmol) in MeOH (500 ml) was added drop wise a solution of 3-amino-1-propanol **1** (3.75 g, 50 mmol) in MeOH (1000 ml) maintaining temperature below 30 °C. The reaction mixture was stirred at room temperature for 8 h and allowed to stand overnight. MeOH and excess *tert*-butyl acrylate were removed in vacuo to get pure compound **3** (16.4 g, 99%), a colorless liquid as residue.

### 2.4. Synthesis of oleic acid based heterolipid 6 (Scheme 2)

#### 2.4.1. Preparation of oleoyl chloride

To a solution of oleic acid **4** (28.25 g, 100 mmol) in $CHCl_3$ (200 ml) contained in 1-liter RBF fitted with a reflux condenser and guard tube was added $SOCl_2$ (17.85 g, 150 mol) drop wise over a period of 2 h under continuous magnetic stirring, and heated at 60 °C for additional 6 h. $CHCl_3$ and excess of $SOCl_2$ were removed under reduced pressure to get oleoyl chloride **5**, a yellow colored viscous liquid, as residue (29.5 g, 98%).

#### 2.4.2. Synthesis of heterolipid *6*

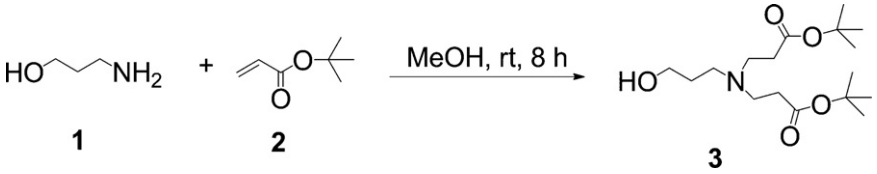
Compound **3** (3.31 g, 10 mmol) and DMAP (1.22 g, 10 mmol) in toluene (50 ml) were charged into a two necked flask fitted with Dean-Stark and reflux condenser and refluxed for 3 h. The reaction mixture was allowed to cool to room temperature and oleoyl chloride (3.01 g, 10 mmol) was added and further refluxed for 8 h. Solvent was removed under reduced pressure and the residue obtained was purified by column chromatography ($SiO_2$, 60–120 mesh) using hexane/EtOAc, 9:1 as eluent to afford heterolipid **6** as colorless to slightly yellow liquid (5.35 g, 90%).

### 2.5. Evaluation of physical properties of heterolipid *6*

Physical properties like density, viscosity, refractive index were determined. Viscosity was determined using Anton Parr MCR101 rheometer and refractive index using Abbe's refractometer ($n = 3$).

### 2.6. In vitro cytotoxicity studies

Cytotoxicity of heterolipid **6** was determined in Human Cervix Cancer Cell Line (HeLa) by SRB assay at concentrations of 10, 20, 40 and 80 μg/ml (Skehn et al., 1990; Vichai and Kirtikara, 2006) using adriamycin as a positive control. The cell lines were grown in Roswell Park Memorial Institute medium, RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. For present screening experiment, cells were inoculated into 96-well microtiter plates in 100 μl at plating densities depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37 °C, 5% $CO_2$, 95% air and 100% relative humidity for 24 h prior to addition of test substance. After 24 h, one 96-well plate containing $5 \times 10^3$ cells/well was fixed in situ with trichloroacetic acid (TCA), to represent a measurement of the cell




**Scheme 1.** Synthesis of compound **3** with primary alcohol as a focal functionality.

population at the time of test compound addition ($T_z$). Heterolipid **6** and adriamycin were initially solubilized in dimethyl sulfoxide at 100 mg/ml and diluted to 1 mg/ml using water and stored frozen prior to use. At the time of addition, an aliquot of frozen concentrate (1 mg/ml) was thawed and diluted to 100 μg/ml, 200 μg/ml, 400 μg/ml and 800 μg/ml with complete medium containing test article. Aliquots of 10 μl of these different dilutions were added to the appropriate microtiter wells already containing 90 μl of medium, resulting in the required final concentrations (10 μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml).

After compound addition, plates were incubated at standard conditions for 48 h and assay was terminated by the addition of cold TCA. Cells were fixed in situ by the gentle addition of 50 μl of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 °C. The supernatant was discarded; the plates were washed five times with tap water and air dried. SRB solution (50 μl) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 min at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1% acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on a plate reader at a wavelength of 540 nm with 690 nm reference wavelength. Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells × 100. Using the six absorbance measurements [time zero ($T_z$), control growth ($C$), and test growth in the presence of drug at the four concentration levels ($T_i$)], the percentage growth was calculated at each of the drug concentration levels. Percentage growth inhibition was calculated as:

$$\left[\frac{T_i - T_z}{C - T_z}\right] \times 100 \quad \text{for concentrations for which } T_i \geq T_z (T_i - T_z)$$

$$\times \text{ positive or zero}$$

$$\left[\frac{T_i - T_z}{T_z}\right] \times 100 \quad \text{for concentrations for which } T_i < T_z (T_i - T_z)$$

$$\times \text{ negative}$$

The dose response parameters were calculated for each test article. Growth inhibition of 50% (GI50) was calculated from $[(T_i - T_z)/(C - T_z)] \times 100 = 50$, which is the concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The concentration resulting in total growth inhibition (TGI) was calculated from $T_i = T_z$. The LC50 (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment is calculated from $[(T_i - T_z)/T_z] \times 100 = -50$. Values were calculated for each of these three parameters if the level of activity was reached; however, if the effect was not reached or was exceeded, the values for that parameter were expressed as greater or less than the maximum or minimum concentration tested.

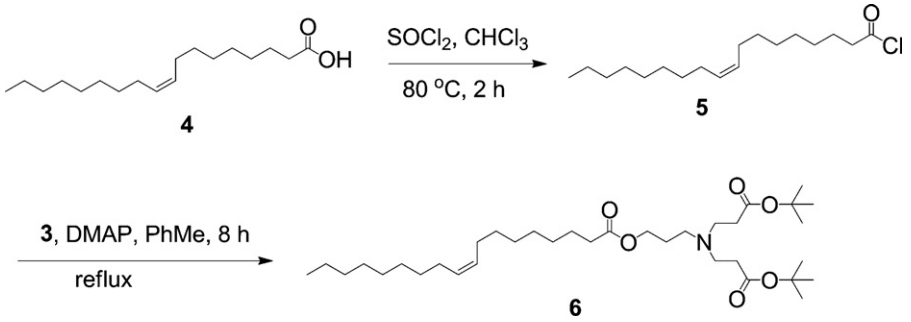
### 2.7. Development and evaluation of SMEDDS of FUR

#### 2.7.1. Solubility studies

The saturation solubility of FUR in oils (heterolipid **6**, oleic acid), 10% (w/w) surfactant solutions and co-surfactants was determined by using shake flask method (Date and Nagarsenker, 2007). Briefly, 1 g of each component was taken in eppendorf tubes and excess of FUR was added. The tubes were sealed, vortexed for 10 min and placed at 25 ± 2 °C for 48 h to achieve equilibrium. After attaining equilibrium each tube was centrifuged at 5000 rpm for 15 min. The supernatant was filtered through 0.45 μm filter (Pall Life Sciences), suitably diluted with methanol and FUR was quantified by a validated HPLC method with UV detection at 229 nm. The components were selected for further studies depending on the maximum drug solubility in oil phase and surfactant.

#### 2.7.2. Screening of surfactants and co-surfactants for emulsifying ability

Emulsification ability of various surfactants and co-surfactants were screened by turbidimetric method (Date and Nagarsenker, 2007). Briefly, 300 mg of surfactant was added to 300 mg of the heterolipid **6**. Mixture was gently heated at 45–60 °C for homogenizing the components. Accurately weighed 50 mg of isotropic mixture was diluted with double distilled water to 50 ml to obtain fine emulsion. The resulting emulsions were observed visually for the relative turbidity. The emulsions were allowed to stand for 2 h and their transmittance was assessed at $\lambda_{max}$ 638.2 nm wavelength. Spectra were recorded on UV-1650 PC, UV–visible spectrophotometer (Shimadzu, Japan) using double distilled water as blank.




**Scheme 2.** Synthesis of heterolipid **6**.

The most suitable co-surfactant was selected based on its ability to microemulsify the surfactant–oil blend as evidenced by the % transmittance value being closer to 100. Briefly, surfactant, 0.2 g was mixed with 0.1 g of co-surfactant. Oil (heterolipid **6**), 0.3 g, was added to this mixture and the mixture was homogenized with the aid of the gentle heat (45–60 °C). The isotropic mixture, 50 mg, was accurately weighed and diluted to 50 ml with double distilled water to yield fine emulsion. The ease of formation of emulsions was noted by noting the number of flask inversions required to give uniform emulsion. The resulting emulsions were observed visually for the relative turbidity. The emulsions were allowed to stand for 2 h and their transmittance was measured at $\lambda_{max}$ 638.2 nm by UV double beam spectrophotometer using double distilled water as blank. The co-surfactant was selected based upon the one that gave maximum % transmittance with the oil, surfactant mixture.

*2.7.3. HPLC analysis of FUR*

The quantity of FUR solubilized in various vehicles was determined by using reported HPLC method (Agyralides et al., 2004). The HPLC system consisted of a high-pressure pump (PU-1580, Jasco) coupled with Jasco UV-1575 UV/vis detector and Rheodyne injector model fitted with 20 μl sample loop. Chromatography was performed on a Purosphere RP 18E (250 mm × 4.6 mm i.d., 5 μm particle size) column at room temperature under isocratic conditions at a flow rate of 1.2 ml/min (Mills et al., 1997) and UV detection was done at a $\lambda_{max}$ 229 nm. The mobile phase comprised of 0.01 M $KH_2PO_4$ (pH5.5):methanol; 70:30 (v/v). Retention time of FUR was found to be 14.2 ± 0.6 min and the method was specific as placebo formulation did not show any interference at the retention time of the drug. Calibration curves, constructed on the basis of peak area versus concentration, were found to be linear over the concentration range studied (10–200 μg/ml) with $r^2 > 0.999$.

*2.7.4. Construction of pseudo-ternary phase diagrams*

The pseudo-ternary phase diagrams were constructed by titration of homogenous liquid mixtures of oil, surfactant and co-surfactant with water at room temperature (Mandawgade et al., 2008). Heterolipid **6** was the oil phase, Solutol HS 15® was the surfactant and ethanol was the co-surfactant. At $K_m$ (surfactant:co-surfactant ratio) values of 2 and 1, mixtures of oil, surfactant and co-surfactant were prepared wherein the ratio of oil to surfactant/co-surfactant blend was varied from 9:1 to 1:9 and vortexed. Each mixture was then slowly titrated with aliquots of distilled water and stirred at room temperature to attain equilibrium. The mixture was visually examined for transparency. After equilibrium was reached, the mixtures were further titrated with aliquots of distilled water until they showed the turbidity. Clear and isotropic samples were deemed to be within the microemulsion region. No attempts were made to completely identify the other regions of the phase diagrams. Based on the results, appropriate percentage of oil, surfactant and co-surfactant was selected, correlated in the phase diagram and were used for preparation of SMEDDS containing FUR.

*2.7.5. Measurement of mean globule size*

The formulations were diluted with various aqueous phases, namely, double-distilled water, 0.9% (w/v) sodium chloride, and 5% dextrose solution. Vehicles used for dilution were filtered thrice through 0.22 μm membrane filter (Pall Life Sciences) before use. Visual observations were made immediately after dilution for self-microemulsification efficiency, transparency, phase separation and precipitation of drug. 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). Microemulsions were diluted to ensure that light scattering intensity (between 6E+004 to 1E+006) was within the instrument's sensitivity range. Samples were placed in transparent polystyrene cuvettes and loaded in thermostatic chamber. Light scattering was monitored at an angle of 90° to the incident beam.

**Table 1**
Composition of placebo SMEDDS with varying oil content.

| Ingredients | Formulation (mg) | | | |
|---|---|---|---|---|
| | D1 | D2 | D3 | D4 |
| Solutol HS 15® + ethanol | 517 | 484 | 450 | 440 |
| Heterolipid **6** | 33 | 66 | 100 | 110 |

*2.7.6. 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 co-surfactant was selected on basis of phase diagrams and maintained at 2:1. Briefly, oil, surfactant, and co-surfactant were weighed in glass vials, mixed by stirring, and heated (40–50 °C) to form homogenous systems. The oil–surfactant mixture (50 mg) was dispersed in 50 ml of various aqueous phases mentioned above with gentle stirring. Globule size and PI were determined immediately after dilution.

*2.7.7. Effect of $K_m$ (surfactant/co-surfactant ratio) on mean globule size*

A series of placebo SMEDDSs were prepared with varying weight ratios of Solutol HS 15® to ethanol ($K_m$ = 1, 2, 3) with 18% of heterolipid **6**. The oil–surfactant mixture (50 mg) was dispersed in 50 ml of aqueous phases with gentle stirring. The globule size and PI were determined immediately. The ability to form spontaneous microemulsions was also observed to judge microemulsification efficiency of oil–surfactant mixtures.

*2.7.8. Effect of drug loading on mean globule size*

Effect of FUR loading on SMEDDS was studied using formulation D3 (Table 1). Accordingly, a series of SMEDDSs were prepared with 2%, 4%, 6%, 8% and 10% (w/w) concentrations of FUR. SMEDDS (50 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 48 h to observe for drug precipitation.

*2.7.9. Optimization of formulae and determination of drug content by HPLC*

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.

In order to determine drug content in SMEDDS, 500 mg of formulation was diluted to 10 ml with methanol, this solution was then injected into the HPLC system.

*2.7.10. Effect of dilution and aqueous phase composition*

Robustness of SMEDDS to the dilution and effect of aqueous phase composition were studied using optimized FUR-SMEDDS composition. Optimized formulation (50 mg) was dispersed in 50 ml of double distilled water, 0.9% sodium chloride solution and 5% dextrose solution 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.

*2.7.11. Accelerated stability tests: centrifugation and freeze thaw cycle*

Optimized SMEDDS was diluted with double distilled water in the ratio of 1:9 (w/w) (one part of 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 physical stability was assessed by measuring globule size before and after centrifugation and freeze–thaw cycle.

*2.7.12. Effect of method of sterilization*

Optimized formulation (3 g) was filled in type I borosilicate glass vials with stoppers, sealed and sterilized by autoclaving at 121 °C for 15 min. After sterilization, SMEDDS was assessed for appearance before dilution, and drug content was determined by the HPLC method. FUR-SMEDDS (50 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. Furthermore, effect of filtration as a sterilization method was evaluated by filtering FUR-SMEDDS through 0.22 μm membrane filters (Pall Life Sciences). Formulations were characterized for the above parameters before and after filtration.

*2.7.13. In vitro hemolysis study*

Hemolysis studies were carried out on heterolipid **6**, placebo and optimized formulation at various concentrations by a reported in vitro method (Quaglia et al., 2009). Freshly collected human blood was washed three times with an isotonic 0.1 M phosphate buffer saline (PBS) solution (pH 7.4) by centrifugation at 2800 rpm for 5 min. Heterolipid **6** and FUR-SMEDDS were diluted with 0.1 M PBS up to a concentration ranging from 0.025 to 0.2 mg/ml for each sample. The RBC suspension (0.2 ml) was added to 1.8 ml of each sample. After incubation at 37 °C for 30 min; the samples were centrifuged at 3000 rpm for 10 min, the supernatant was collected and analyzed for hemoglobin release by spectrophotometric determinations at $\lambda_{max}$ 416 nm. To obtain 0% and 100% hemolysis, 0.2 ml of RBC suspension was added to 1.8 ml of PBS and distilled water, respectively.

The degree of hemolysis was calculated by the following equation:

$$\%\text{Hemolysis} = \frac{\text{ABS} - \text{ABS}_0}{\text{ABS}_{100} - \text{ABS}_0} \times 100$$

where $\text{ABS}_{100}$ and $\text{ABS}_0$ are the absorbances of the solution at 100% and 0% hemolysis, respectively.

*2.7.14. Stability studies*

Stability of FUR-SMEDDS was determined at 40 ± 2 °C/75 ± 5% RH and 5 ± 3 °C as per ICH guidelines by storing them after membrane filtration for a period of three months in type I borosilicate glass vials fitted with rubber stoppers and crimped with aluminum cap. Samples were withdrawn at intervals of 0, 1, 2, and 3 months and evaluated for parameters like physical appearance, dilutability, mean globule size, PI, and drug content.

## 3. Results and discussion

### 3.1. Characterization

Techniques used for characterization of heterolipid **6** were FT-IR, $^1$H NMR, $^{13}$C NMR and ESI-MS.

FT-IR (neat) *ν*: 1731, 1455, 1367, 1255, 1166 cm$^{-1}$. $^1$H NMR ($CDCl_3$) *δ*: 0.88 (t, 3H, —$C_{16}H_{33}C\mathbf{H_3}$), 1.29 (m, 20H, $CH_3(C\mathbf{H_2})_6CH{=}CH(C\mathbf{H_2})_4CH_2CH_2CO$), 1.44 (s, 18H, 2 × $C(C\mathbf{H_3})_3$, 1.63(q, 2H, $C_{15}H_{29}C\mathbf{H_2}CH_2$—CO), 1.75 (m, 2H, —$OCH_2C\mathbf{H_2}CH_2N$—), 2.01 (m, 4H, —$\mathbf{CH_2}CH{=}CHC\mathbf{H_2}$—), 2.33 (m, 6H, —$\mathbf{CH_2}CO$—), 2.47 (t, 2H, —$OCH_2CH_2C\mathbf{H_2}N$—), 2.71 (m, 4H, —$NC\mathbf{H_2}CH_2CO$—), 4.08 (t, 2H, —$OC\mathbf{H_2}CH_2CH_2N$—), 5.34 (m, 2H, —$C\mathbf{H}{=}C\mathbf{H}$—). $^{13}$C NMR ($CDCl_3$) *δ*: 14.12, 22.68, 24.99, 26.69, 27.21, 28.10, 29.18, 29.33, 29.52, 29.71, 29.77, 31.92, 33.81, 34.36, 49.42, 50.15, 62.45, 80.30, 129.7, 129.99, 171.97, 173.85. ESI-MS *m*/*z*: 596 [M$^+$].

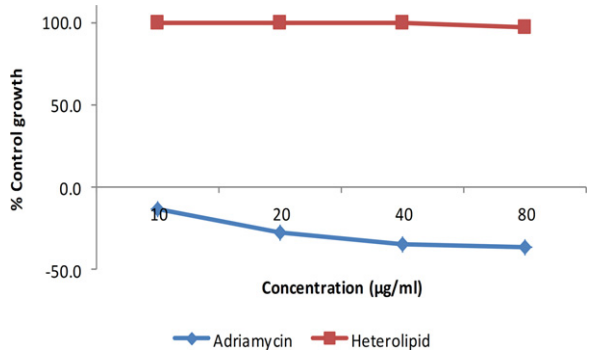



**Fig. 2.** Growth curve of human cervix cancer cell line HeLa.

**Table 2**
LC 50, TGI and GI 50 values derived from Fig. 2.

| | Value (μg/ml) | | |
|---|---|---|---|
| | LC 50 | TGI | GI 50 |
| Heterolipid **6** | >80 | >80 | >80 |

### 3.2. Evaluation of physical properties of heterolipid *6*

Heterolipid **6** was oily in nature with refractive index 1.448, density 0.925 g/ml and viscosity 25.6 cP ($n = 3$).

### 3.3. In vitro cytotoxicity study

SRB study revealed that heterolipid **6** did not exhibit any cytotoxicity even up to 80 μg/ml concentration. Fig. 2 illustrates the growth curves for HeLa cell lines and LC 50, TGI and GI 50 values are given in Table 2.

### 3.4. Development and evaluation of SMEDDS

*3.4.1. Solubility studies*

Identification of oil, surfactant/co-surfactant that has maximum solubilization potential for the drug under investigation is important to determine optimum drug loading (Pouton, 1997). FUR

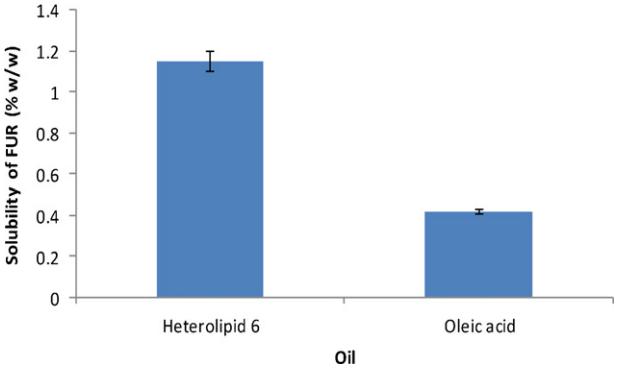



**Fig. 3.** Solubility of FUR in oleic acid and heterolipid **6**. Data are expressed as mean ± SD ($n = 3$).

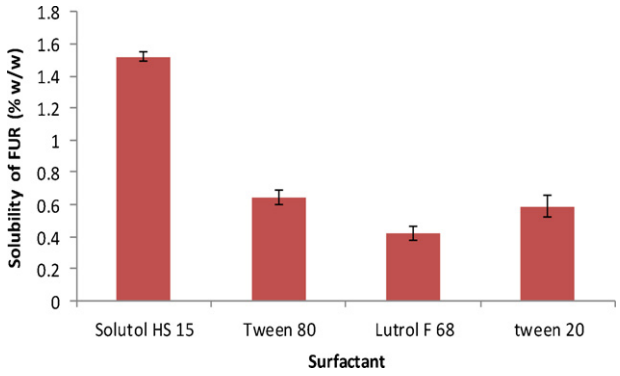



**Fig. 4.** Solubility of FUR in various 10% (w/w) surfactant solutions. Data are expressed as mean ± SD ($n = 3$).

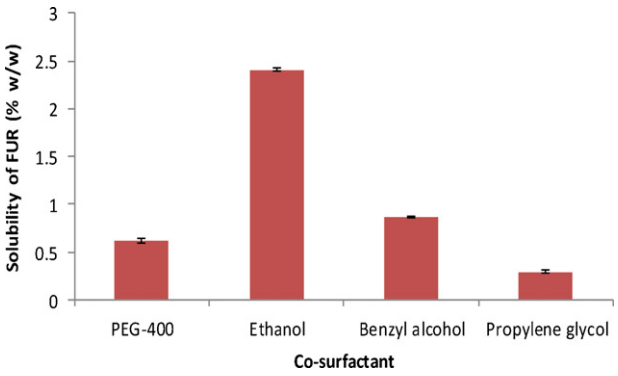



**Fig. 5.** Solubility of FUR in co-surfactants. Data are expressed as mean ± SD ($n = 3$).

showed three times greater solubility (1.15 ± 0.05%, w/w) in heterolipid **6** than in oleic acid (0.41 ± 0.01%, w/w) (Fig. 3). Solubility in 10% (w/w) aqueous solutions of surfactants was determined because drug should be clear and monophasic liquid at ambient temperature when formulation is introduced to aqueous phase and should have good solvent properties to allow presentation of the drug in solution. Amongst the various surfactants evaluated for solubilization of FUR, solubility in Solutol HS 15® was found to be highest (1.52 ± 0.03%, w/w) (Fig. 4). Moreover, Solutol HS 15® is a very safe non-ionic surfactant for injection formulations (Bittner et al., 2003). It has good physiological compatibility upon intravenous administration and is also known to decrease the particle size of o/w emulsions (Buszello et al., 2000). Amongst the co-surfactants ethanol showed maximum solubility of FUR (2.41 ± 0.01%, w/w) (Fig. 5).

### 3.4.2. *Screening of surfactants and co-surfactants for emulsifying ability*

Selection of surfactants and co-surfactants was governed by their emulsification efficiency for the selected oil rather than their ability to solubilize FUR. Good solubility of FUR in the surfactant, co-surfactant was considered as an additional advantage (Borhade et al., 2009).

The % transmittance values for various dispersions of surfactants with heterolipid **6** are presented in Fig. 6. Studies clearly distinguished the ability of various surfactants to emulsify heterolipid **6**. Amongst the surfactants, Solutol HS 15® had a very good ability to emulsify followed by Lutrol F 68® and Tween 80, whereas, Cremophore EL and Tween 20 appeared to be poor. Solutol HS 15® rendered very good emulsion requiring short time for emulsification and was selected for further investigations.

It is very well established that co-surfactants accumulate with surfactants at the interfacial layer and thereby increase the area of existence of microemulsion region. This is because they can further reduce the surface tension and they tend to fluidize the interfacial surfactant film (Gamal and Maghraby, 2008; Narang et al., 2007; Lawrence and Rees, 2000). Fig. 7 shows the emulsification efficiency of various co-surfactants. Larger the chain length or structure (or molecular volume) of the co-surfactant, lesser was the transmittance value. This observation is in line with the investigations reported by Date and Nagarsenker (2007). Studies showed that Solutol HS 15® with ethanol can give microemulsions with highest clarity and hence ethanol was selected as the co-surfactant.

### 3.4.3. *Construction of pseudo-ternary phase diagrams*

In order to determine self-microemulsifying regions and to optimize the concentration of oil, surfactant, and co-surfactant, pseudoternary phase diagrams were constructed (Spernath and Aserin, 2006). The phase diagrams as depicted in Fig. 8 revealed that the existence of microemulsion area is more at $K_m$ of 2 than that at 1. This demonstrates that Solutol HS 15®/ethanol in the ratio of 2:1 are able to microemulsify heterolipid **6** to a greater extent and hence this ratio was followed in the final formulation.

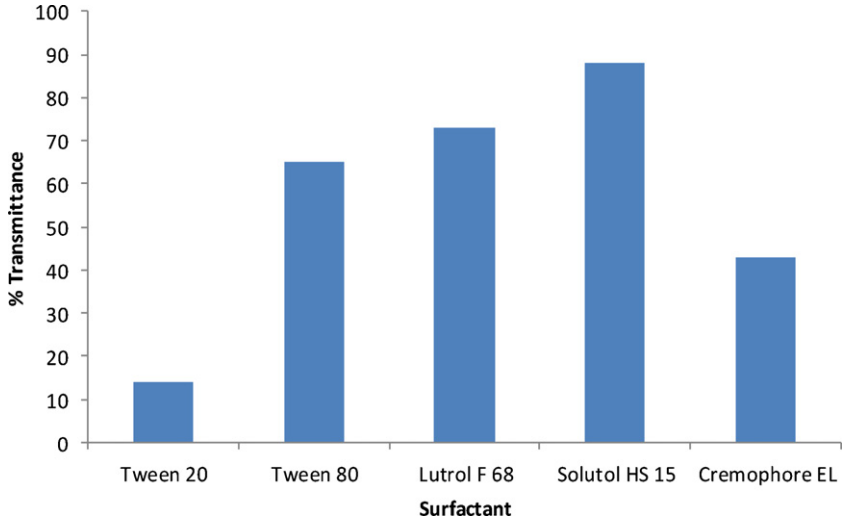



**Fig. 6.** Emulsification efficiency of various surfactants with heterolipid **6**. Data expressed as mean ($n = 2$).

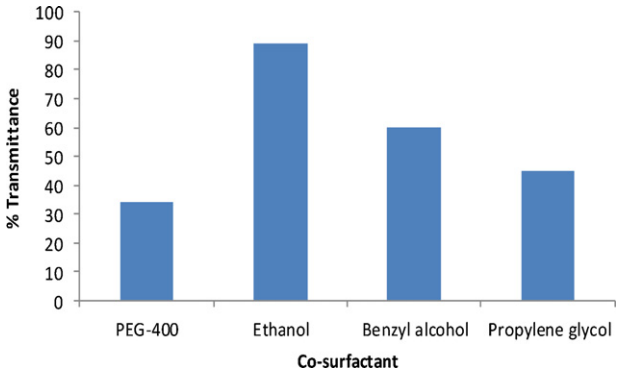



**Fig. 7.** Emulsification studies on Solutol HS 15®, heterolipid **6** and various co-surfactants combination. Data expressed as mean ($n = 2$).

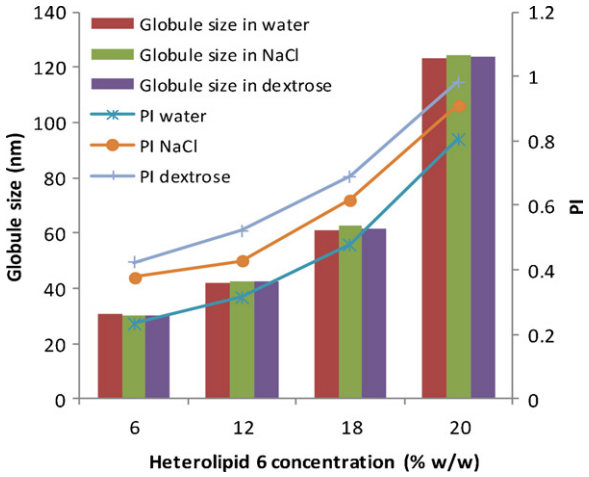



**Fig. 9.** Effect of heterolipid **6** concentration on mean globule size and PI. Data expressed as mean, $n = 3$, where relative SD < 10%).

### 3.4.4. *Effect of oily phase content on mean globule size*

The effect of heterolipid **6** phase content on globule size was carried out at $K_m$ of 2. Oil content was varied from 6% to 20% (w/w) in the oil and surfactant/co-surfactant mixture. It was observed that as the oil content was increased up to 18% (w/w), there was increase in globule size from 30 to 62 nm but PI was maintained below 0.6 (Fig. 9). When the oil concentration reached to a level of 20% (w/w), there was a drastic increase in globule size along with increase in PI. This behavior is consistent with earlier literature data (Borhade et al., 2009). The increase in globule size may be due to less number of surfactant/co-surfactant molecules available to effectively emulsify the oil. This may cause some of the oil droplets to coalesce leading to larger globule size along with a wider size distribution range. Upon further variation of heterolipid **6** concentration, it was found that concentration up to 18% (w/w) displayed globule size less than 70 nm with PI below 0.6 and hence this concentration was taken in the final formula.

### 3.4.5. *Effect of $K_m$ on mean globule size*

It is long known that the quantity of surfactant in SMEDDS plays an influential role on the globule size of SMEDDS. Taking this into consideration, effect of $K_m$ on mean globule size was determined (Fig. 10). It was found that at $K_m$ of 1, oil/surfactant-co-surfactant mixtures when dispersed in aqueous media, gave very high globule size of more than 100 nm along with wider size distribution. At $K_m$ of 2 and 3, the globule size was below 60 nm and size distribution in aqueous media was also found to be small. This may be attributed to stabilization of oil droplets due to surfactant localization at the oil–water interface (Gursoy and Benita, 2004). Moreover, microemulsions were spontaneously formed; this may

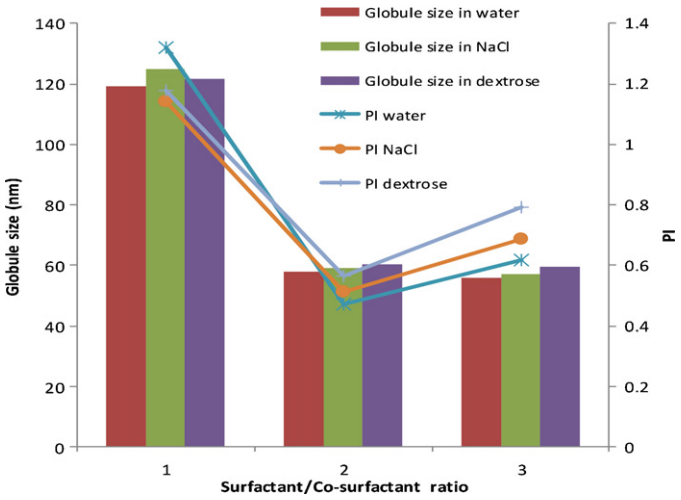



**Fig. 10.** Effect of $K_m$ on mean globule size and PI (data expressed as mean, $n = 3$, where relative SD < 10%).

be due to good hydrophilicity of the surfactant/co-surfactant blend. Surfactant/co-surfactant ratio of 2 was chosen in the final formula as both $K_m$ values of 2 and 3 gave almost the same globule size and size distribution profile when dispersed in aqueous media and to

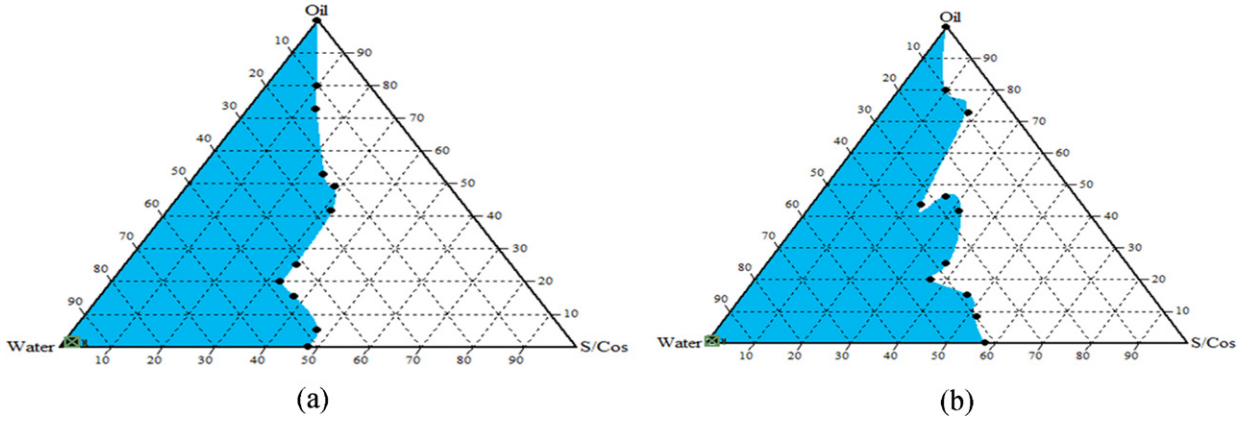



**Fig. 8.** Pseudoternary phase diagrams at (a) $K_m = 1$ and (b) $K_m = 2$. Oily phase = heterolipid **6**, surfactant = Solutol HS 15®, co-surfactant = ethanol and aqueous phase = double distilled water.

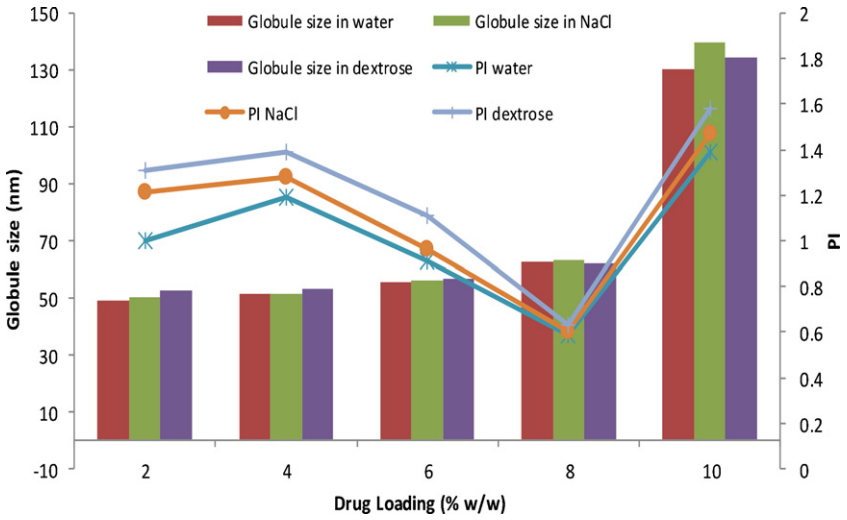



**Fig. 11.** Effect of drug loading on mean globule size and PI (data expressed as mean, $n = 3$, where relative SD < 10%).

**Table 3**
Compositions of formulations attempted to achieve final optimized formulation.

| Formulation | Composition (mg) | | |
|---|---|---|---|
| | FUR | Surfactant/co-surfactant (2:1) | Heterolipid **6** |
| F1 | 11 | 450 | 100 |
| F2 | 23 | 450 | 100 |
| F3 | 35 | 450 | 100 |
| F4 | 48 | 450 | 100 |
| F5 | 50 | 450 | 100 |
| F6 | 61 | 450 | 100 |

keep surfactant concentration at minimum possible level to avoid the risk of hemolysis.

### 3.4.6. *Effect of drug loading on mean globule size*

Drug incorporation, especially of highly hydrophobic drugs like FUR, needs prime consideration when it is related to its influence on globule size and size distribution. Therefore, effect of drug incorporation on mean globule size was determined at concentrations 2%, 4%, 6%, 8% and 10% (w/w) of FUR (Fig. 11). Formulations containing 2%, 4%, 6% and 8% (w/w) of FUR did not exhibit any precipitation of the drug when observed for a period of 24 h. At 4% (w/w) drug concentration, although the globule size was below 60 nm, PI was more than 1. At 8% (w/w) globule size was below 70 nm and moreover, this showed a very narrow size distribution range of less than 0.6 irrespective of composition of the aqueous phases. 10% (w/w) concentration led to an increase in globule size where microemulsion became progressively turbid with precipitation of the drug.

### 3.4.7. *Optimization of formulae and determination of drug content by HPLC*

Different compositions attempted to achieve final optimized formulation are shown in Table 3. Composition, given in Table 4, was selected as the final optimized SMEDDS formulation because it spontaneously formed microemulsions upon dilution, displayed a globule size of <70 nm with a PI of 0.603. Drug content, by HPLC, was found to be 99.60 ± 1.2%.

**Table 4**
Composition of optimized FUR-SMEDDS.

| Ingredient | Quantity (mg) |
|---|---|
| FUR | 50 |
| Heterolipid **6** | 100 |
| Solutol HS 15® | 300 |
| Ethanol | 150 |

### 3.4.8. *Effect of dilution and aqueous phase composition*

In order to administer intravenously, SMEDDS has to be diluted with isotonic solutions like 0.9% NaCl, 5% dextrose. These dilutions may cause drug precipitation, phase separation and it may also bring about changes in globule size and PI. Hence, it is necessary to determine robustness of SMEDDS to dilution in various aqueous media. SMEDDS upon dilution were observed for these parameters immediately and at the end of 48 h. SMEDDS upon dilution gave microemulsions that were clear and transparent. It was found that even upon 500-fold dilution; there was no drug precipitation or phase separation at the end of 48 h indicating good robustness of SMEDDS to dilution. Moreover, there was no significant change in globule size and PI at the end of 48 h period (Fig. 12).

### 3.4.9. *Accelerated stability tests: centrifugation and freeze–thaw cycle*

These parameters were investigated to establish the stability of SMEDDS under stress conditions. SMEDDS upon centrifugation showed no evidence of drug precipitation or phase separation and globule size also did not change significantly indicating good stability of SMEDDS. Freeze thaw cycle indicates stability of SMEDDS when subjected to extremes of temperature. SMEDDS survived

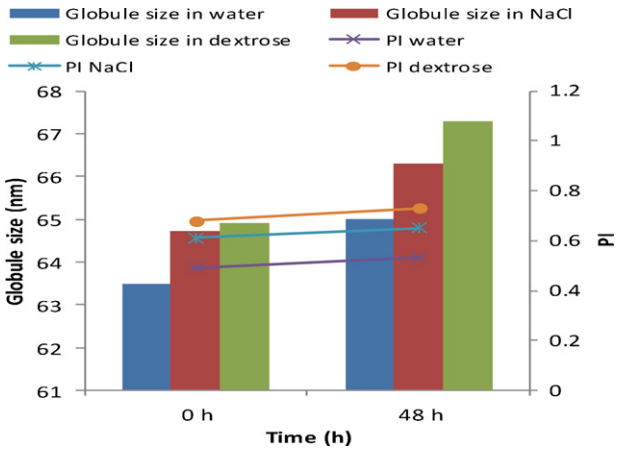



**Fig. 12.** Effect of dilution and aqueous phase composition on mean globule size and PI (data expressed as mean, $n = 3$, where relative SD < 10%).

**Table 5**
Effect of accelerated stability tests on mean globule size and PI of microemulsion.

| Formulation | Centrifugation | | | | Freeze–thaw cycle | | | |
|---|---|---|---|---|---|---|---|---|
| Accelerated stability tests | Placebo SMEDDS | | FUR-SMEDDS | | Placebo SMEDDS | | FUR-SMEDDS | |
| Parameters | Before | After | Before | After | Before | After | Before | After |
| Globule size (nm) | 62.9 | 60.1 | 66.43 | 64.6 | 63.8 | 62.4 | 68.46 | 68.40 |
| PI | 0.603 | 0.654 | 0.518 | 0.607 | 0.598 | 0.610 | 0.773 | 0.76 |

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

**Table 6**
Effect of method of sterilization on stability of SMEDDS.

| Formulation | Autoclaving | | | | Membrane filtration | | | |
|---|---|---|---|---|---|---|---|---|
| Method of sterilization | Placebo SMEDDS | | FUR-SMEDDS | | Placebo SMEDDS | | FUR-SMEDDS | |
| Parameters | Before | After | Before | After | Before | After | Before | After |
| Globule size (nm) | 64.7 | 96.3 | 65.2 | 102.06 | 62.1 | 64.3 | 65.46 | 67.83 |
| PI | 0.696 | 1.021 | 0.773 | 1.45 | 0.598 | 0.621 | 0.627 | 0.674 |
| Drug content (%, w/w) | NA | NA | 99.96 ± 1.71 | 76.11 ± 3.7 | NA | NA | 100.01 ± 1.26 | 99.96 ± 1.92 |

NA = not applicable. Drug content expressed as mean ± SD, $n = 3$; mean globule size and PI expressed as mean, $n = 3$, where SD < 10%.

**Table 7**
Effect of storage condition and time on mean globule size, PI and drug content.

| Storage condition | Globule size (nm) | | PI | | PI | |
|---|---|---|---|---|---|---|
| Time (months) | 5 °C | 40 °C/75% RH | 5 °C | 40 °C/75% RH | 5 °C | 40 °C/75% RH |
| 0 | 63.23 | 63.17 | 0.632 | 0.566 | 99.60 | 99.95 |
| 1 | 65.23 | 52.77 | 0.596 | 0.585 | 99.62 | 100.12 |
| 2 | 62.6 | 64.43 | 0.577 | 0.597 | 100.44 | 100.1 |
| 3 | 62.9 | 63.1 | 0.601 | 0.574 | 100.14 | 99.79 |

Mean globule size and PI expressed as mean, $n = 3$, where relative SD < 10%; drug content expressed as mean ± SD, $n = 3$.

efficiently the freeze–thaw cycling test as there was no phase separation, drug precipitation or change in globule size after the cycle (Table 5).

### 3.4.10. *Effect of method of sterilization*

The formulation when administered intravenously has to be sterile. When SMEDDS are subjected to sterilization methods, it may cause physical changes which is undesirable and needs to be investigated. Table 6 shows the effect of method of sterilization on stability of SMEDDS.

It is observed that upon autoclaving, the globule size of microemulsion increased drastically along with reduction in drug content. This may be due to degradation of the formulation upon autoclaving. Membrane filtration was the other method that was attempted to achieve sterilization. After membrane filtration, globule size of SMEDDS did not change significantly and drug content was found to be 99.96%. Moreover, upon dilution with various aqueous media, microemulsions were formed spontaneously without showing any sign of drug precipitation or phase separation. This suggests that membrane filtration is suitable as a sterilization method for the developed FUR-SMEEDS.

### 3.4.11. *In vitro hemolysis study*

At 0.2 mg/ml concentration heterolipid **6** showed less than 1% hemolysis indicating its nontoxicity to RBCs. Any formulation when administered intravenously has potential to cause hemolysis of RBCs. This untoward reaction can be fatal and hence it is essential to determine the safety of formulation in this regard. At 0.2 mg/ml concentration placebo and FUR-SMEDDS showed 1.35 and 1.61% hemolysis respectively. Clinically FUR is administered at maximum concentration of 40 mg (0.096 mg/ml formulation:blood ratio), at this ratio less than 1% hemolysis was observed. The developed FUR-SMEDDS thus exhibited negligible hemolytic potential and appeared to be nontoxic to RBCs.

### 3.4.12. *Stability studies*

SMEDDSs were subjected to stability studies for a period of three months. At specific time points, parameters like ability to form clear microemulsions, globule size, size distribution and drug content were estimated (Table 7). It was found that there was no significant difference in these parameters even at the end of three months. Hence, it can be concluded that FUR-SMEDDS exhibited good stability at both the storage conditions even at the end of three months.

## 4. Conclusion

The novel oleic acid based heterolipid **6** was successfully synthesized and well characterized. The cytotoxicity studies revealed that it is exceptionally safe. It significantly increased the solubility of FUR as compared to that of the parent oil, oleic acid. This paves way in exploring its potential to improve solubility of other poorly soluble drugs as well. Furthermore, the heterolipid **6** was incorporated as an oil phase in the formulation of SMEDDS using FUR as a model drug. SMEDDS displayed clear and transparent microemulsions with globule size in nanometer range and narrow size distribution. FUR-SMEDDS was stable to dilution in various aqueous media and in accelerated stability studies like centrifugation and freeze–thaw cycle. Sterilization method by membrane filtration was found to be suitable. In vitro hemolysis study proved the safety of both heterolipid **6** as well as the developed FUR-SMEDDS and hence can be conveniently used for intravenous administration. In addition, FUR-SMEDDS exhibited good stability even at the end of three months. In conclusion it can be said that heterolipid **6** can be used as an

oil phase for SMEDDS for intravenous delivery. It has potential to increase solubility of Class IV drugs. It can be further concluded that, semi-synthetic derivatives of natural lipophiles hold promise as biocompatible, safe and cost-effective lipidic excipients.

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