

Enhanced Oral Bioavailability and Anticoagulant Activity of Dabigatran Etexilate by Self-Micro Emulsifying Drug Delivery System: Systematic Development, *In vitro*, *Ex vivo* and *In vivo* Evaluation

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Abstract

Dabigatran etexilate (DE), a prodrug of Dabigatran, is a potent, oral, reversible and direct thrombin inhibitor with low oral bioavailability due to active efflux by intestinal P-glycoprotein receptors. No lipid based oral formulation is marketed for DE till date. Hence, we formulated and evaluated self-micro emulsifying drug delivery system (SMEDDS), by using P-gp modulator excipients to tackle this issue and elevate the systemic availability of DE. The SMEDDS were developed using Capmul MCM C8, Cremophor EL and Transcutol HP as oil, surfactant and co-surfactant respectively. The formulation was optimized using statistical D-optimal design. The globule size of 73.24 nm with 0.085 PDI was achieved upon spontaneous emulsification with >99% Transmittance at 250 times dilution and discrete globules were observed under TEM. The *In vitro* and *Ex vivo* drug release from DE-SMEDDS was found to be significantly higher in comparison to that from plain drug suspension. The DE-SMEDDS was observed to be non-cytotoxic and safe when assessed by MTT assay on Caco-2 cells. Moreover, a deeper penetration in the Caco-2 cells was observed with DE-SMEDDS when assessed using confocal laser scanning microscopy. Flow-cytometric studies also revealed greater uptake of fluorescent probe in Caco-2 cell-lines from the DE-SMEDDS when compared with drug suspension. Furthermore, the AUC_{0-t} of DE from the optimized DE-SMEDDS formulation was found to be 2.5 times higher and relative bioavailability was enhanced by 3.36 folds than that from drug suspension on oral administration to rats. Moreover, DE SMEDDS exhibited higher anticoagulant activity than drug suspension, further indicating better bioavailability.

Keywords: Bioavailability; Nanoemulsion; Nanosized

Introduction

Oral administration is the most convenient and preferred mode of drug administration in conscious and co-operating patients, due to convenience, possibility of self-administration, improved patient safety and better compliance. However, more than 40% new chemical entities exhibit poor oral bioavailability due to undesired physicochemical and pharmacokinetic properties [1].

P-glycoprotein (P-gp) efflux and first-pass metabolism by cytochrome P450 (CYP3A) play critical roles in limiting the absorption and bioavailability of orally administered drugs [2]. P-gp is a transmembrane receptor protein which gets over expressed naturally in many organs like blood brain barrier [3], small intestine [4] etc. for homeostasis. Intestinal P-gp decreases intracellular drug accumulation by actively extruding them from the enterocytes [2]. Inhibition of P-gp in the intestinal lumen can be a good strategy for improving bioavailability of such drugs.

There are several pharmaceutical approaches to deal with the poor solubility, dissolution rate and bioavailability of insoluble drugs viz., micronization, use of salt form, lipid based system, alteration of micro environment pH, use of metastable polymorphs, solute-solvent complex formation, solvent deposition, solid dispersion, molecular encapsulation with cyclodextrins etc. One of the promising techniques is lipid based system which comprises Self-Micro Emulsifying Drug Delivery Systems (SMEDDS), Nanoemulsion, Microemulsion etc. The lipid component enhances the extent of lymphatic transport and increases bioavailability directly or indirectly via reduction of first pass metabolism [5]. Presence of lipid in the gastro intestinal tract (GIT) stimulates an increase in Bile Salts (BS) and endogenous biliary lipids like Phospholipid (PL) and cholesterol (CL) leading to formation of BS/PL/CL intestinal mixed micelles and increases solubilization capacity of the GIT. Some surfactants, which are generally a part of these systems

like Polysorbates, Cremophore etc., have the ability to minimize the activity of intestinal efflux transporters like P-gp efflux pump [6-8]. Incorporation of P-gp inhibitor (surfactant like cremophor EL) could reduce the drug efflux by altering the membrane fluidity and lead to inhibition of transport activity. The change in secondary and tertiary structure is found to be the reason for loss of P-gp function due to interruption in hydrophobic environment by the surfactant molecules, [1] thereby increasing the bioavailability.

SMEDDS are mixtures of oils and surfactants, ideally isotropic, sometimes including co-solvents, which emulsify under conditions of gentle agitation, similar to those which would be encountered in the GIT. Typical size of a droplet lies in the range of 10-200 nm. Hydrophobic drugs can be formulated into SMEDDS allowing them to be encapsulated as unit dosage forms for peroral administration [9]. When such a formulation is released into the GI lumen, it disperses there to form a fine emulsion containing solubilised drug thereby avoiding the dissolution step which frequently limits the rate of absorption of hydrophobic drugs [10]. Generally this can lead to improved bioavailability and a more consistent temporal profile of

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Received: January 03, 2018; **Accepted:** January 25, 2018; **Published:** January 29, 2018

Citation: Mundada VP, Sawant KK (2018) Enhanced Oral Bioavailability and Anticoagulant Activity of Dabigatran Etexilate by Self-Micro Emulsifying Drug Delivery System: Systematic Development, *In vitro*, *Ex vivo* and *In vivo* Evaluation. J Nanomed Nanotechnol 9: 480. doi: [10.4172/2157-7439.1000480](https://doi.org/10.4172/2157-7439.1000480)

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absorption from the gut enabling reduction in dose, selective targeting of drug toward specific absorption window in GIT and protection of drug from the unreceptive environment in gut.

Dabigatran (DAB) is a potent, synthetic, non-peptide competitive thrombin inhibitor belonging to BCS class II. It is poorly absorbed following oral dosing; hence it is administered in the form of prodrug, Dabigatran etexilate (DE) which does not possess anticoagulant activity [11]. This prodrug is activated after oral administration by nonspecific esterases to DAB in plasma and in the liver. Dabigatran etexilate is low molecular weight, reversible, rapidly acting direct thrombin inhibitor [12] and is the first direct thrombin inhibitor to be approved by the US Food and Drug Administration (FDA) for stroke and systemic embolism in patients with atrial fibrillation [13,14], as well as for the prevention of venous thromboembolism (VTE) after hip and knee surgery [15]. Solubility is strongly pH dependent with increased solubility at acidic pH. The low bioavailability of DE (7.2%) after oral administration is attributed to its low solubility and P-gp efflux [11]. Various researchers have reported different formulation approaches to improve the oral bioavailability of dabigatran etexilate like solid self-nanoemulsifying drug delivery system [16], Soluplus®-TPGS binary mixed micelles system [17] and drug-phospholipid complex nanoemulsion [18].

The aim of present study was to develop a self-micro emulsifying drug delivery system for DE to prevent P-gp efflux from small intestine and to improve its oral bioavailability. Formulations were optimized using D-optimal statistical design. D-optimal design is alluded as a computer-aided design, where the determinate data lattice is amplified and the summed up difference is minimized [19]. Cell viability (Caco2-cell line) studies were performed to investigate the safety aspects of the surfactants and co-surfactants used to formulate DE-SMEDDS. The optimized formulation was characterized for particle size, morphology, intestinal permeability and *in vivo* performance in rats.

Materials and Methods

Materials

Dabigatran etexilate was received as a gift sample from Alembic Research Centre, Vadodara, India. Capmul MCM C8 was obtained as gift sample from Abitec Corporation, USA. Cremophor EL and Cremophor RH 40 were received as gift sample from BASF, India. Peceol, Labrasol and Transcutol HP were obtained as gift sample from Gattefosse India Ltd, India. Tween 80 was received from Merck Ltd, Germany. PEG 200 was obtained as gift sample from Croda, India. PEG 400, propylene glycol, and Tween 20 were purchased from S.D. Fine chemicals (India). Methanol, Acetonitrile (ACN) and other analytical reagents were purchased from Spectrochem Mumbai, India. Nylon filter paper 0.45 µm pore size (Pall Life sciences, India). Dialysis membrane-70, Fetal Bovine Serum (FBS), Trypan Blue, Trypsin-EDTA 1X Solution, MTT, MEM and Antibiotic Antimycotic solution 100X liquid were procured from Himedia Lab. Pvt. Ltd. India. 0.22 µm membrane syringe filters were obtained from Merck Millipore, India. Caco-2 cell line was obtained from National Centre for Cell Science (NCCS), India. All other chemicals used were of analytical reagent grade.

Fabrication of DE-SMEDDS

Based on screening studies, Capmul MCM C8, Cremophor EL and Transcutol HP were selected as Oil, surfactant and cosurfactant. Various drug loaded batches of DE-SMEDDS were prepared. The drug (20 mg) was dissolved in Capmul MCM C8 under continuous

stirring followed by sonication for 3 min. S_{mix} of Cremophor EL and Transcutol HP was added to the drug mixture and stirred for 10 min using a magnetic stirrer (Remi, India). Clear, transparent SMEDDS formulation thus prepared was further characterized for globule size (GS) and %transmittance (T).

Optimization of SMEDDS formulation

Optimization of SMEDDS was carried out using D-optimal design to study the influence of effect of independent variables [concentration of oil (X1), concentration of surfactant (X2) and concentration of cosurfactant (X3)] on critical dependent variables [Globule Size (Y1) and %Transmittance (Y2)]. The different levels of the formulation variables were selected based on preliminary experiments to get desirable constraints for the two responses Globule size (GS) and %Transmittance (T) to judge the formation of a self-micro emulsifying system. The Design Expert® software (Version 8.0.3, Suite, Minneapolis, USA) was used to generate the design. The design layout is as shown in Table 1.

The D-optimal design comprised of 16 runs, 3-factors at 2-levels with five centre point trials for reproducibility. The design allowed the fitting of cubic model on two responses for process optimization in preparation of DE-SMEDDS with minimum Globule size and maximum %T. Contour Plots and Response surface Plots were also generated.

Check point analysis/desirability function: After the fitting of mathematical model, the desirability function was used for optimization. The desirability function consolidates all the responses into one variable and leaves the possibility to anticipate the ideal levels for the independent variable [20]. The established contour plots and response surface plots were confirmed by performing check point analysis. Difference in the predicted and actual values of experimentally obtained responses (Y1 and Y2) were checked using student's 't' test along with desirability function. The criteria of desired GS and %T was set to predict optimum conditions [21].

Evaluation of DE-SMEDDS

Robustness to dilution test: Robustness to dilution was studied by diluting the optimized SMEDDS formulation to 50, 100, 250 and 1000 times with distilled water, 0.01 N HCl (pH 2.0) and Phosphate buffer (pH 6.8). The diluted SMEDDS were stored for 12 h at room temperature and observed for any signs of phase separation or drug precipitation. Globule size and %Transmittance was measured for all the samples [22].

Thermodynamic stability studies: Thermodynamic stability studies were performed to assess the stability of microemulsion formed after diluting SMEDDS formulation at following accelerated stress conditions [23].

Heating cooling cycle: Six cycles between refrigerator temperatures

Variables (Factors)	Levels	
	-1	1
X1: concentration of oil (%)	10	20
X2: concentration of surfactant (%)	40	60
X3: concentration of co-surfactant (%)	20	40
Responses	Constraints	
Y1=Globule size (nm)	Minimize	
Y2=Transmittance (%)	Maximize	

Table 1: Layout of D-optimal design for DE SMEDDS.

4°C and 45°C with storage at each temperature for not less than 48 h was studied. Stable formulation was subjected to centrifugation test.

Centrifugation test: Formulation was centrifuged at 3500 rpm for 30 min using cooling centrifuge (Remi equipments, India) and observed for phase separation visually and uniform formulation was taken for freeze thaw stress test.

Freeze thaw cycle: Three freeze thaw cycles between -21°C and +25°C with storage at each temperature for not less than 48 h were performed and observed for any phase separation, cracking, creaming and turbidity visually.

Globule size, poly dispersibility index (PDI) and zeta-potential: Globule Size, PDI and zeta potential of the diluted SMEDDS formulation (1:250) were determined using dynamic light scattering (Malvern, Nano ZS, UK). All studies were performed in triplicate [22].

%Transmittance: The optimized SMEDDS formulation was freshly diluted to 50, 100, 250 and 1000 times with distilled water and dilutions were kept undisturbed for 10 min and were observed visually for any turbidity. Thereafter, %transmittance was measured at 650 nm using UV-Visible spectrophotometer against distilled water as the blank [23].

Cloud point measurement: The optimized SMEDDS formulation was diluted with distilled water (250 times), placed in a temperature regulated water bath and temperature was increased gradually. Cloud point was determined as the temperature at which there was a sudden appearance of cloudiness both visually and by determining %transmittance at 650 nm by using UV-vis spectrophotometer [24]. All studies were repeated in triplicates.

Viscosity: The viscosity of the optimized batch of SMEDDS was determined employing Brookfield viscometer (DV-III+Rheometer, Brookfield, USA) using cone and plate at 10 rpm speed and 25°C temperature in triplicate.

Self-emulsification time and precipitation assessment: The emulsification time of optimized batch of SMEDDS was assessed by USP type II (paddle type) dissolution apparatus (DS 8000, M/s Lab India Instruments, India). Formulation equivalent to 20 mg of DE was added to 250 mL of 0.01 N HCl (pH 2) at 37.5°C. Mild agitation was provided by the paddle rotating at 50 rpm. The time required for complete dispersion of the formulation in aqueous phase to form microemulsion was recorded as self-emulsification time. Precipitation was evaluated by visual inspection of the resultant emulsion after 24 h storage at 37°C. The formulations were then categorized as clear (transparent or transparent with bluish tinge) or non-clear (turbid), stable (no precipitation at the end of 24 h) or unstable (showing precipitation within 24 h) [25,26]. All studies were performed in triplicate.

Morphological examination using transmission electron microscopy (TEM): The morphology of the oil globules of SMEDDS were visualized using TEM. Drop of microemulsion formed after dilution (100-fold dilution in distilled water) of optimized SMEDDS was placed on a piece of parafilm. A carbon coated grid (3 mm, 300#) was placed on top of the drop and left for 1 min. Excess fluid was removed by using filter paper. Negative staining was then performed by placing the grid on a drop of 2% phosphotungsten acid (PTA) for 1 min. The grid was examined under a transmission electron microscope (model Tecnai 20, 200 KV, Phillips, Netherland) [22].

Drug content: Optimized batch of DE-SMEDDS equivalent to 20 mg of DE was dispersed into appropriate quantity of methanol,

stirred sufficiently to dissolve the drug, and centrifuged at 3000 rpm for 10 min [27]. The supernatant was duly diluted and analyzed spectrophotometrically (UV-1800 Shimadzu, Japan) at 315 nm.

Drug release studies

In vitro dissolution study: The dissolution study was performed using USP type II (paddle) dissolution apparatus (DS 8000, M/s Labindia Instruments, India), using 900 mL of 0.01N HCl and pH 6.8 phosphate buffer at 37 ± 0.5°C and 50 rpm. The optimized SMEDDS formulation and drug suspension each equivalent to 20 mg of DE were filled into hard gelatin capsules shells (Size: 00). A sample aliquot of 3 mL was removed at each time interval followed by replacement with an equivalent amount of fresh dissolution medium in order to maintain the sink condition and analyzed spectrophotometrically (UV-1800 Shimadzu, Japan) at 325 nm and 316 nm for 0.01N HCL and pH 6.8 phosphate buffer respectively. All the experiments were performed in triplicate.

In vitro diffusion study: The diffusion study was performed using activated dialysis membrane with a molecular weight cut-off of 12000 Daltons and pore size 2.4 nm (Hi-media, India). The optimized SMEDDS formulation and drug suspension (1 mL) equivalent to 20 mg of DE were filled in dialysis membrane bag which was sealed and suspended in glass beaker with help of clips and immersed in 250 mL of 0.01N HCl and pH 6.8 phosphate buffers under continuous stirring at 37 ± 0.5°C. A sample aliquot of 3 mL was removed at each time interval followed by replacement with an equivalent amount of fresh dissolution medium in order to maintain the sink condition [25] and analyzed spectrophotometrically (UV-1800 Shimadzu, Japan) at 325 nm and 316 nm for 0.01N HCL and 6.8 phosphate buffer respectively [18]. All the experiments were performed in triplicate. *In vitro* diffusion study data was further fitted to various release models viz zero order, first order, Hixon Crowell, Korsemeyer Peppas and Higuchi model to identify the mechanism and kinetics of drug release from optimized SMEDDS formulation. Regression coefficient (r^2) was calculated to identify the best-fit model [21].

Ex vivo release study: All experiments and protocols described in this study were approved by the Institutional Animal Ethics Committee (IAEC) of Faculty of Pharmacy, The M. S. University of Baroda, Gujarat, India., and were conducted as per the norms of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Male Sprague-Dawley rats (250-300 g) were humanitarily sacrificed and the stomach and small intestine were isolated and washed thoroughly with PBS to remove the mucous and lumen contents. The optimized SMEDDS formulation equivalent to 20 mg of DE was filled in both the stomach and intestine tissues. Both the ends of the tissue were tied properly to avoid any leakage and were placed in an organ bath with continuous aeration at 37 ± 0.5°C. The receptor compartment (organ tube) was filled with 50 mL of 0.01N HCl and pH 6.8 phosphate buffer respectively. At predetermined time intervals, samples were withdrawn from the receptor compartments. Fresh buffer was used to replenish the receptor compartment. Similarly, equivalent amount of plain DE suspensions in 0.01N HCl and pH 6.8 phosphate buffer respectively were also studied [27]. Samples were analyzed spectrophotometrically at 325 nm and 316 nm for 0.01N HCL and pH 6.8 phosphate buffer, respectively for the content of DE [16]. All the experiments were performed in triplicate.

In vitro cell line studies: Caco-2 cell lines are well known for the over-expression of P-gp efflux transporters and additionally a build

up model for *in vitro* oral drug absorption. Caco-2 cell lines were obtained from NCCS, Pune, India and the cell passages between 35 and 40 were used in the experiment. Caco-2 cells were cultured in 50 cm² tissue culture flasks. MEM medium with Earle's salts, 2 mM L-Glutamine, 1 mM Sodium pyruvate, NEAA and 1.5 g/L sodium bicarbonate, supplemented with 10% Fetal Bovine Serum (Origin: Brazil, EU Approved, Gamma irradiated), and 1% Antibiotic Antimycotic Solution with 10,000 U Penicillin, 10 mg Streptomycin and 25 µg Amphotericin B per mL in 0.9% normal saline was used as culture medium. Caco-2 cell line was incubated at 37°C in humidified atmosphere containing 5% CO₂ in Jouan IGO150 incubator (Thermo-Fisher, Waltham, USA). Media was changed after every 2-3 days and sub-culturing was done when cell confluence became more than 70-80%. Trypsin-EDTA solution containing 0.25% trypsin, 0.038% EDTA in Hanks' Balanced Salt Solution w/o Calcium and Magnesium was used to detach the cells.

Cell viability study: Cell viability study was carried out by 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoleum bromide (MTT) assay on the previously grown Caco-2 cell monolayer. Tissue culture flasks containing Caco-2 cells were trypsinized and cell suspension was suitably diluted to fixed volume. The cells were then counted using hemocytometer using trypan blue as staining dye. Successively, cells were first cultured in a 96-well plate at a seeding density of 1.0 × 10⁴ Caco-2 cells/well and incubated for 24 h. Preparations were diluted with MEM culture medium to different concentrations. After incubation, cells were treated separately with 150 µL of sample preparations viz. optimized SMEDDS formulation, drug suspension, SMEDDS-placebo in the concentration of 200 µg/mL, 100 µg/mL, 80 µg/mL and 60 µg/mL and incubated at 37°C in CO₂ incubator for 4 h. The cells were also treated with Triton-X100 which acted as positive control and phosphate buffer pH 7.4 as negative control. After 4 h of incubation, the medium was removed and 150 µL of MTT reagent (1 mg/mL) in serum free medium was added to each well. The plates were then incubated at 37°C for another 4 h. At the end of the incubation period, the medium was removed and the intracellular formazan was solubilized with 150 µL DMSO and quantified by reading the absorbance at 590 nm with a reference filter of 620 nm using Micro plate multi detection instrument (680-XR, Bio-Rad Laboratories, France). Percentage cell viability was calculated based on the absorbance measured relative to that of cells exposed to the negative control (Phosphate buffer saline) [28,29].

Cell uptake study by FACS: Caco-2 cell line was used to determine cell uptake of 6-Coumarin dye (lipophilic) using FACS. For the study, dye loaded formulation was prepared similar to DE-SMEDDS where drug was replaced with 6-Coumarin dye. The cell uptake study was carried out by seeding 1.0×10⁶ Caco-2 cells/well in 6 well plates for 48 h. For time-dependent uptake, cells were treated with 100 µL each of dye loaded optimized formulations (equivalent to 1 mg/mL) and plain dye solution at predetermined time intervals of 1 and 4 h and incubated at 37°C in Jouan IGO150 CO₂ incubator (Thermo-Fisher, Waltham, USA). At the end of time points, the culture medium was washed twice with PBS (pH 7.4). Further, the cells were trypsinized with Trypsin-EDTA solution and centrifuged at 6000 rpm for 1 min to get cell pellet. The cells were then resuspended in FACS buffer (9.8 mL PBS+0.1 mL FBS+100 mg BSA), passed through strainer (0.20 µ) and were analysed using FACS (FACS Canto-II, BD Biosciences, San Jose, USA) using software provided with the instrument (BD FACS Diva 6.1.3 software, BD Biosciences, USA) [30].

Qualitative cell uptake study by confocal microscopy: The qualitative cell uptake study was carried out by seeding 1.0×10⁵ Caco-2

cells on rounded glass cover slips at bottom of 6 well plates for 24 h. On reaching 80% confluence, the culture medium was replaced with HBSS. After 30 min of incubation at 37°C, cell monolayers were washed three times with HBSS for 5 min. The cells were then incubated with 100 µL of 100 µg/mL of 6-Coumarin dye solution and dye loaded formulations. To investigate time dependent uptake, the cells were then incubated for 60 and 120 min. After the specified incubation period, the cells were removed from the medium and washed twice with PBS (pH 7.4) for visual observation using optical microscope (Nikon Digital Sight DS-Fi2, Japan) for evaluating fluorescence intensity as a function of cellular uptake. The cell monolayers were then fixed with 70% ethanol solution for 20 min and rinsed with HBSS. After rinsing, the nuclei were counter stained with DAPI for 3 min and rinsed again with HBSS, mounted in glycerol and localization of dye loaded formulations were observed using confocal laser scanning microscope (LSM 710, Carl-Zeiss Inc., San Diego, USA) [24,30].

Cell permeability study using Transwell insert: The cell permeability study was carried out by seeding 5x10³ Caco-2 cells/insert in Transwell® inserts-3470-clear (6.5 mm diameter inserts, 0.4 µm pore size, Corning, NY14831) for 21 days. Media was changed every second day for first 7 days followed by every alternate day thereafter. The integrity of the monolayers was checked by monitoring the permeability of the paracellular leakage marker, Lucifer yellow across the monolayers combined with transepithelial electrical resistance (TEER) measurement using EVOM-Epithelial Volt-ohmmeter fitted with planar electrodes (World Precision Instruments, Sarasota, FL). All transport studies were conducted at 37°C. The transport buffer containing 150 µL of drug suspension and SMEDDS formulation were added on the apical (0.5 mL) side while the basolateral side of the inserts contained 1.5 mL of the transport buffer. After 30, 60, 120, 180 and 240 min of incubation, 100 µL aliquot was withdrawn from the receiver chamber and was immediately replenished with an equal volume of pre-warmed HBSS [31]. The concentration of the test compounds in the transport medium was immediately analyzed by HPLC technique using reported method but with slight modification [17]. The apical-to-basolateral permeability coefficient (Papp in cm/s) was calculated according to following equation:

$$P_{app} = \frac{dQ/dt}{A * C_0 * 60} \quad (1)$$

where dQ/dt is the amount of DE-SMEDDS in basolateral compartment as a function of time (mg/min), A is the monolayer area (cm²), and C₀ is the initial concentration of drug in apical compartment (mg/ml) [30].

***In vivo* studies**

The studies involving animal experiments were carried out in accordance with the experimental protocols approved by the Institutional Animal Ethics Committee of Faculty of Pharmacy, The M. S. University of Baroda, Gujarat, India. Female Sprague-Dawley rats, weighing 300 ± 30 g were used for all the studies. Animals were housed in Polypropylene cages (38 cm × 23 cm × 10 cm) under standard laboratory conditions at 25 ± 2°C and 60 ± 5% RH. The animals were housed, three rats per cage and had free access to standard diet and water *ad libitum*. Animals were fasted overnight on the day of study. Proper care and maintenance of the animals was undertaken following the guidelines of Committee for Prevention, Control and Supervision of Experimental Animals, Govt. of India.

Pharmacokinetic study: The optimized DE-SMEDDS formulation and drug suspension were evaluated for *in vivo* pharmacokinetic study

upon oral administration. The dose calculation of the rats was based on body weight and was calculated as DE equivalent to 7.7 mg/Kg. The rats were fasted for 24 h prior to the beginning of experiments while having free access to drinking water. The animals were divided into two groups (n=6) and were orally administered with drug suspension to group I and optimized DE-SMEDDS formulation to group II using standard feeding tube. Each animal was anaesthetized with diethyl ether at the time of blood sampling and blood samples (0.2 mL) were carefully withdrawn from the retro orbital venous plexus with the aid of capillary tubes at predetermined time points (0.5, 1, 2, 4, 6, 8 and 10 h) post administration. The withdrawn blood samples were transferred to a series of graduated centrifuge tubes containing 0.1 mL of 100 IU heparin solution. The heparinized blood samples were centrifuged at 3600 rpm and 4°C for 10 min (Remi Centrifuge, India) and the supernatant plasma was collected in Eppendorf tubes. 200 µL of acetonitrile was added to it. The tube was vortexed for 5 min followed by centrifugation at 4000 rpm for 10 min [24,28]. The supernatant plasma (0.1 mL) was then filtered through 0.22 µm membrane syringe filter and 20 µL of supernatant was injected manually using syringe through rheodyne injector into the HPLC column and analyzed by previously reported method but with slight modifications using isocratic HPLC (LC 20 AT, Shimadzu Japan) with a SPD-20 A UV-visible detector [16]. Mobile phase consisted of acetonitrile and phosphate buffer (pH 5.8) 60:40 (% v/v), pumped at a flow rate of 1 mL/min at room temperature.. The retention time of DE was found to be 4.60 min at 230 nm of detection for a run time of 10 min.

Intestinal transport of DE SMEDDS in rats: To investigate the intestinal transport of DE SMEDDS in rats, a chylomicron flow blocking approach was employed [32]. Cycloheximide (CHM) solution (3 mg/Kg) and normal saline were injected intraperitoneally into the rats to inhibit intestinal lymphatic transport pathway and considered as negative control. One hour after the injection, rats were orally administered with DE-SMEDDS (equivalent to 7.7 mg/Kg of DE). Blood samples (0.2 mL) were carefully withdrawn from the retro orbital venous plexus with the aid of capillary tubes at predetermined time points (15, 30, 45, 60, 90, 120 and 180 min) post administration in heparinised Eppendorf tubes. Plasma was immediately centrifuged at 4°C and 4000 rpm for 10 min [17]. Drug was extracted with ACN and analyzed by HPLC [16].

Pharmacodynamic study-cutaneous bleeding time: Cutaneous Bleeding Time method was used to study the pharmacodynamic activity of DE in rats. Animals were divided in three groups (n=6). Group I was considered as control group, group II was administered drug suspension while group III was administered optimized DE-SMEDDS formulation. The rats (weighing 250-350 g) were placed in plastic rat holder with several openings from one of which animal tail was emerged. Tail was cleaned properly with water wetted cotton. Then incision (10 mm long and 1.5 mm deep) was made with a scalpel between 8 and 9 cm from the tip of the tail. The bleeding time was assessed at intervals of 15 s [33,34].

Statistical analysis: Each value was expressed as mean ± standard deviation (SD). Statistical significance was assessed with a ANOVA test in *in vivo* studies. A 'p' value of less than 0.05 was considered significant.

Results and Discussion

Formulation optimization by experimental design

A three-factor, two-level D-optimal statistical experimental design was used to optimize the formulation variables. The observed responses

are shown in Table 2. The globule size (GS) and %Transmittance (T) were chosen as responses because these are considered as critical factors for development of SMEDDS formulation. A small droplet size allows better drug absorption since it provides an increased surface area and allows faster drug release [35] while transparency confirms the microemulsion formation. All responses were simultaneously fitted to linear, quadratic, special cubic, and cubic models by using the Design-Expert software version 7.0. The cubic model showed maximum R-squared value among all fitted models and was suggested as the fitting mathematical model for both Y1 and Y2. Several statistical parameters, such as sequential p-value, lack of fit p-value, standard deviation, R-squared (R^2), adjusted and predicted R-squared (R^2) values (Table 3) also justified the model. Model F-value for both the responses implied that the models were significant. The Pred R-Squared for both the responses were in reasonable agreement with the Adj R-Squared indicating that the selection and model fitting were acceptable.

The GS and %T values for the 16 batches showed a wide variation from 70.2 to 772.100 nm and 30.200 to 99.600%, respectively. This variation can be seen in cubic polynomial equations in terms of U_Pseudo Components in eq.2 and eq. 3 for GS and %T respectively. The p-value and t-stat demonstrated the significance of each coefficient [21].

$$Y_1 = 502.47X_1 + 583.77X_2 + 324.25X_3 - 12867.86X_1 X_2 - 15300.01X_1 X_3 - 802.58X_2 X_3 + 14040.69X_1 X_2 X_3 - 4635.34X_1 X_2 (X_1 - X_2) - 8911.96X_1 X_3 (X_1 - X_3) + 836.89X_2 X_3 (X_2 - X_3) \quad (2)$$

$$Y_2 = -251.89 X_1 + 44.14 X_2 + 60.16 X_3 + 529.40 X_1 X_2 + 777.85 X_1 X_3 + 48.73 X_2 X_3 - 319.49 X_1 X_2 X_3 + 139.44 X_1 X_2 (X_1 - X_2) + 378.80 X_1 X_3 (X_1 - X_3) + 72.50 X_2 X_3 (X_2 - X_3) \quad (3)$$

Influence of independent variables on droplet size: The magnitude of coefficient indicates its contribution to the respective response. The coefficients of X1 and its interaction terms had high magnitude, indicating that X1 was a critical factor for determining droplet size. Coefficient X1 was positive, indicating that X1 directly influenced the response Y1. In other words, globule size decreased with decrease in oil content. Together X2 and X3 gave positive response on Y1 Response Y1 was inversely proportional to interactive effect of surfactant and cosurfactant in combination. This suggested that a smaller amount of oil and higher amount of surfactant and cosurfactant in the SMEDDS

S. No.	Conc. of oil (X ₁)	Conc. of surfactant (X ₂)	Conc. of co-surfactant (X ₃)	GS (nm) (Y ₁)	T (%) (Y ₂)
1	10.135	60.000	29.865	70.2 ± 2.1	99.1 ± 2.4
2	17.204	46.936	35.860	217.3 ± 5.6	75.2 ± 3.6
3	10.251	49.749	40.000	768.6 ± 4.3	32.3 ± 3.3
4	13.313	53.453	33.234	101.3 ± 3.6	90.2 ± 2.9
5	20.000	40.010	39.990	585.5 ± 5.2	45.8 ± 4.2
6	19.994	60.000	20.006	320.8 ± 3.4	60.1 ± 3.5
7	20.000	51.183	28.817	265.5 ± 2.9	63.8 ± 4.1
8	10.135	60.000	29.865	71.6 ± 2.2	99.6 ± 3.7
9	14.763	45.237	40.000	336.6 ± 3.1	55.8 ± 3.4
10	19.994	60.000	20.006	330.1 ± 2.8	59.9 ± 2.7
11	10.251	49.749	40.000	772.1 ± 2.7	30.2 ± 4.4
12	20.000	55.810	24.190	321.1 ± 3.4	58.9 ± 3.1
13	15.121	60.000	24.879	93.3 ± 4.4	92.1 ± 2.9
14	20.000	40.010	39.990	580.2 ± 2.8	42.6 ± 3.1
15	20.000	51.183	28.817	268.5 ± 4.9	61.5 ± 3.2
16	16.007	55.461	28.532	171.2 ± 4.2	84.8 ± 2.6

Table 2: Design matrix for D-Optimal design for DE-SMEDDS.

formulation obtained using the D-optimal mixture design resulted eventually in decreasing the droplet size.

Influence of independent variables on %transmittance: Coefficient of X1 was negative while X2 and X3 were positive hence giving positive response for transmittance. On decreasing the concentration of oil and increasing surfactant and cosurfactant concentrations, transmittance increased. Since the design selected is mixture design, the interactions among different variables had significant effect on their respective responses.

Contour plots and response surface analysis: The relationship among variables and responses was exemplified by contour plots and response plots for both the responses. For each response, globule size and %Transmittance, contour plots were generated between X1, X2 and X3 as shown in Figure 1. Globule size decreased on decreasing oil concentration and sharp increase was observed on increasing surfactant concentration. Similar observations were observed for

response Y2. Response surface plots show the relationship between these variables even more clearly when plotted between X1, X2 and X3 (Figure 2). Minimum globule size and maximum transmittance were observed when X1 was in mid of A(0) and A (20), X2 was near to B (60) and X3 was in middle of C (20) and C (40).

Check point analysis/desirability function: The independent variables were concurrently optimized for both the responses using desirability function. The optimum formulation was selected based on the criteria of attaining the minimum value of GS and the maximum value of %T. Response Y₁ was set to be minimum and Y2 to be maximum. The desirability function is a transformation of the response variable to 0 to 1 scale. Response of 0 represents a completely undesirable response and 1 represents the most desirable response [21]. Based on this, 7 different solutions were predicted with the desirability of 1. Figure 3 shows the overlay plot for the effect of different variables on the two responses.

Response	Model	Model F-value	R-Squared	Adjusted R-Squared	Predicted R-Squared	Sequential p-value	Lack of Fit p-value	Std. Dev.
Y1	Cubic	1147.29	0.999	0.999	0.990	<0.0001	0.0707	4.85
Y2	Cubic	45.14	0.998	0.995	0.959	<0.0001	0.2965	2.75

Table 3: Model Statistics for Y1 and Y2 responses.

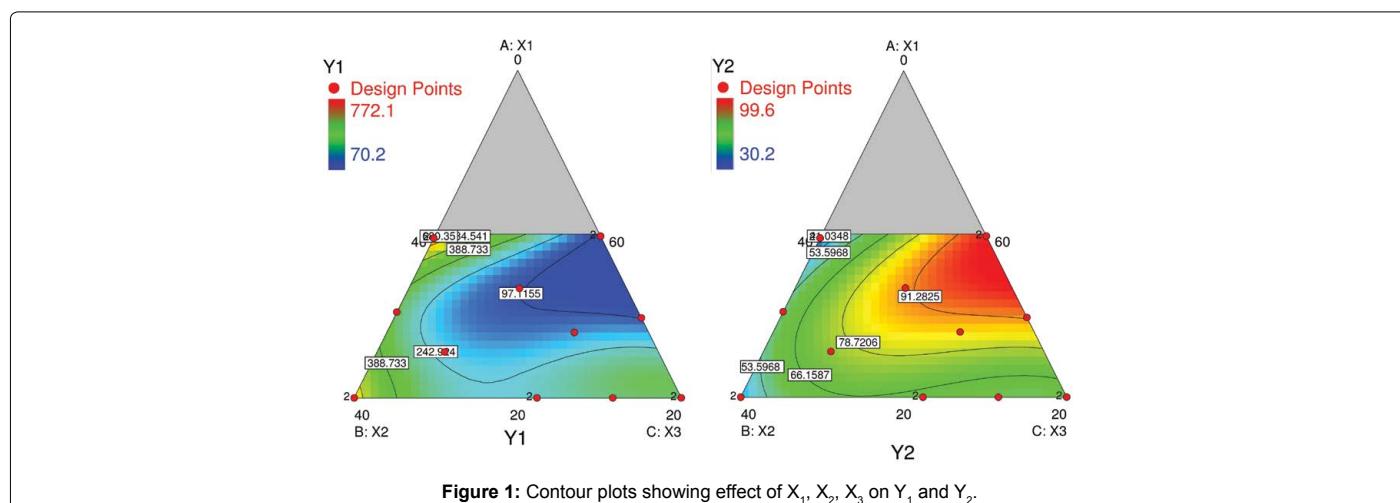


Figure 1: Contour plots showing effect of X₁, X₂, X₃ on Y₁ and Y₂.

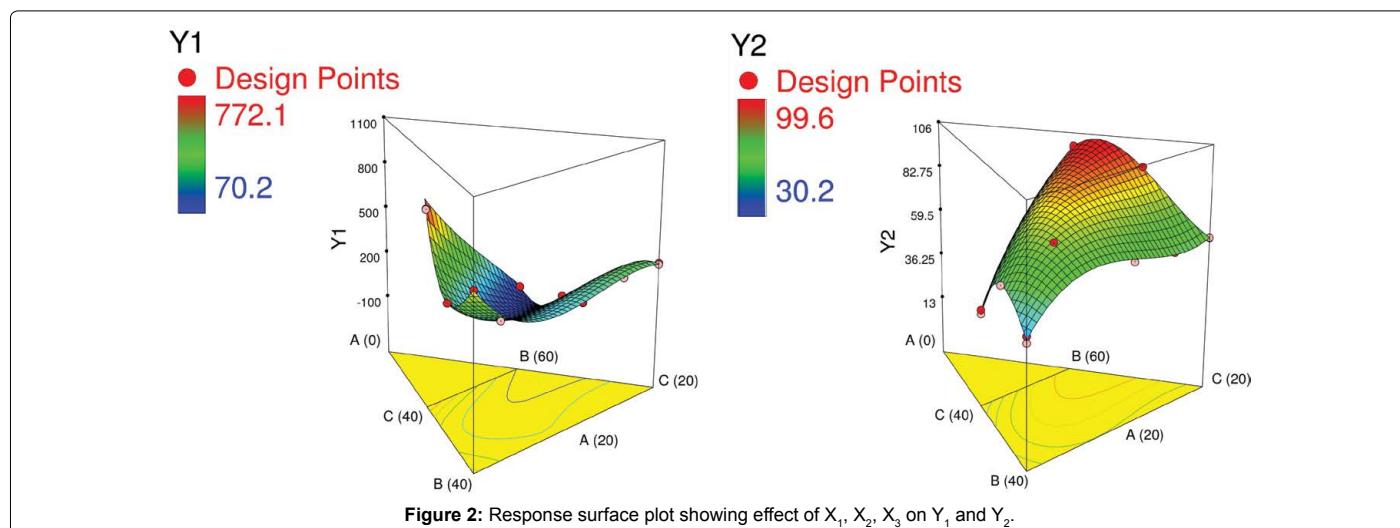


Figure 2: Response surface plot showing effect of X₁, X₂, X₃ on Y₁ and Y₂.

The experimental and predicted results were compared for the three check point formulations. Data analysis using student's t-test showed that there was no statistically significant difference ($p < 0.05$) between experimentally obtained values and predicted values (Table 4) indicating that the measured responses can be accurately predicted by the contour and response plots.

Evaluation of DE-SMEDDS

Robustness to dilution test: Robustness to dilution is important for SMEDDS to ensure that the emulsion formed have similar properties at different dilutions to achieve uniform globule size, drug release profile and to ensure that the drug will not get precipitated at higher dilutions *in vivo* which may significantly retard the absorption of the drug from the prepared formulation [23]. Uniform microemulsions should form upon self-emulsification of SMEDDS at different dilution conditions. Dilutions may affect globule size, transparency, drug release etc. if drug gets precipitated or any phase separation is observed [22]. The effect of extent of dilution on globule size of optimized batch of DE-SMEDDS was evaluated (Table 5). All the diluted batches exhibited a globule

size of <100 nm and transmittance above 90% irrespective of type and volume of dilution medium. Therefore, the optimized SMEDDS was considered to be robust against dilution as neither precipitation of the drug nor any phase separation was observed even after 24 h [25].

Thermodynamic stability studies: SMEDDS should be thermodynamically stable with no phase separation, cracking and creaming [36]. Hence, the optimized DE-SMEDDS was subjected to heating cooling cycle, centrifugation and freeze thaw cycle stress tests to evaluate its thermodynamic stability. Neither phase separation nor any precipitation was observed upon centrifugation, indicating the stability of the microemulsion thus formed after self-emulsification of the formulated SMEDDS.

Globule size, PDI and zeta-potential: Globule size is a key factor in determining self-emulsification performance as it determines the rate and extent of drug release, absorption as well as the stability of the emulsion [37]. The globule size of optimized batch of DE-SMEDDS was found to be 73.24 ± 1.1 nm with 0.085 PDI. Small PDI revealed narrow size distribution of microemulsion. The smaller globule size was particularly observed at low concentrations of lipid, high concentration of surfactant and intermediate concentrations of co solvent in this formulation. Zeta potential measurement was done to identify the charge on the surface of droplets. Zeta potential of optimized batch of DE-SMEDDS was found to be -24.20 mV. Zeta potential should usually reach an absolute value ± 30 mV to obtain stable emulsion by preventing flocculation and coalescence of nanosized droplets [23]. Such results inferred towards better synergism of components used in development of SMEDDS for obtaining desired globule size.

%Transmittance: The optimized batch of DE-SMEDDS was visually found to be transparent and without any turbidity. %Transmittance of diluted DE-SMEDDS was as shown in Table 5. A transmittance value of $>80\%$ indicates good microemulsification [38]. Thus, the prepared formulation showed desired transmittance for fabrication of good product.

Cloud point measurement: The cloud point is a crucial parameter in SMEDDS to decide integrity of the emulsion at elevated temperature particularly in formulations consisting of non-ionic surfactants. The temperature above which a clear formulation turns cloudy is known as cloud point. At temperatures higher than the cloud point, an irreversible phase separation occurs due to dehydration of its ingredients, which may affect the formulation adversely. Hence, to avoid this phenomenon,

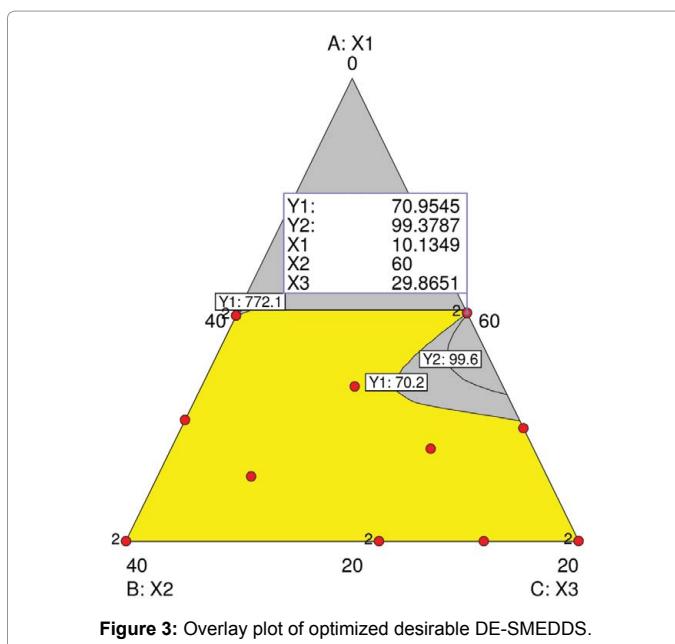


Figure 3: Overlay plot of optimized desirable DE-SMEDDS.

Sr. No.	Conc. of oil (X ₁)	Conc. of surfactant (X ₂)	Conc. of co-surfactant (X ₃)	Globule size (nm) (Y ₁)		Transmittance (%) (Y ₂)	
				Experimental (Mean)	Predicted	Experimental (Mean)	Predicted
1	10.425	60.251	29.962	72.254	70.684	96.124	99.392
2	11.579	57.366	31.056	71.634	72.193	98.732	96.465
3	12.307	56.203	31.490	70.912	71.837	97.314	95.139
$t_{calculated}$				0.995		0.859	
$t_{tabulated}$				4.302		4.302	

Table 4: Check point analysis with 't' test.

Dilution Factor	Distilled water		0.01 N HCl (pH 2.0)		Phosphate buffer pH 6.8	
	Globule Size (nm)	Transmittance (%)	Globule Size (nm)	Transmittance (%)	Globule Size (nm)	Transmittance (%)
50 Times	51.54 ± 1.36	96.6 ± 0.35	65.32 ± 1.23	96.21 ± 0.10	85.69 ± 2.10	96.8 ± 0.11
100 Times	49.85 ± 1.74	96.8 ± 0.11	56.35 ± 0.65	99.1 ± 0.16	77.78 ± 1.23	99.5 ± 0.08
250 Times	55.54 ± 1.03	99.2 ± 0.20	52.14 ± 0.74	99.4 ± 0.03	81.01 ± 0.98	98.1 ± 0.16
1000 Times	58.34 ± 0.98	99.1 ± 0.16	61.32 ± 1.41	97.8 ± 0.11	98.65 ± 1.31	91.25 ± 0.11

The values are mean of n=3.

Table 5: Effect of dilution and media on globule size of optimized batch of DE-SMEDDS.

the cloud point for SMEDDS should be above body temperature (37°C) [24]. The cloud point for DE-SMEDDS was observed to be much higher ($77.5 \pm 2.9^{\circ}\text{C}$) than body temperature, indicating that it will form stable microemulsion at physiological temperature i.e. *in vivo* and during storage without any phase separation. No turbidity was observed visually and this was confirmed by high %Transmittance ($99.2 \pm 0.3\%$).

Viscosity: SMEDDS can be formulated either into tablet or capsule dosage forms. However, challenges like leaching of oil onto the surface are encountered in the tablet dosage form. A much simpler way to overcome this problem is filling of SMEDDS into hard or soft gelatin/HPMC capsule shells. In such case, viscosity is crucial in determining its ability to be filled in hard or soft gelatin capsules [22]. If the system has very low viscosity, there may be probability of leakage from the capsule while the system with very high viscosity may create problem in pourability [39]. The viscosity of the DE-SMEDDS at 25°C was found to be 124.8 ± 4.01 cps. As the value was less than 10,000 cps, it implied that the developed SMEDDS can be filled in capsule shells by commercial liquid filling equipments [22].

Self-emulsification time and precipitation assessment: The time of emulsification is an important parameter to assess efficiency of self-emulsification since it is indicative of faster solubilization of the drug in the gastrointestinal fluid [29] and a prerequisite for SMEDDS to disperse quickly and completely when subjected to dilution under mild agitation. Two min have been considered as an index for evaluating the emulsification process [16]. The time of emulsification in 0.01 N HCl (pH 2) at 37.5°C was found to be 26.0 ± 3.0 sec which indicated the spontaneity of emulsification of the prepared SMEDDS. Moreover, the resultant microemulsion was appeared to be clear (transparent or isotropically clear) which indicated desirable emulsification efficiency owing to complete miscibility of lipids in the aqueous phase by micellar solubilization [29].

Morphological examination using transmission electron microscopy (TEM): The morphology of the 100 fold diluted DE-SMEDDS when examined using TEM (Figure 4) revealed discrete, spherical oil globules of less than 100 nm size. As the oil globules were discrete and non-aggregated, we can state that the microemulsion formed spontaneously and was physically stable.

Drug content: The drug content of optimized batch was found to be $98.78 \pm 2.02\%$, indicating uniform dispersion of drug in formulation.

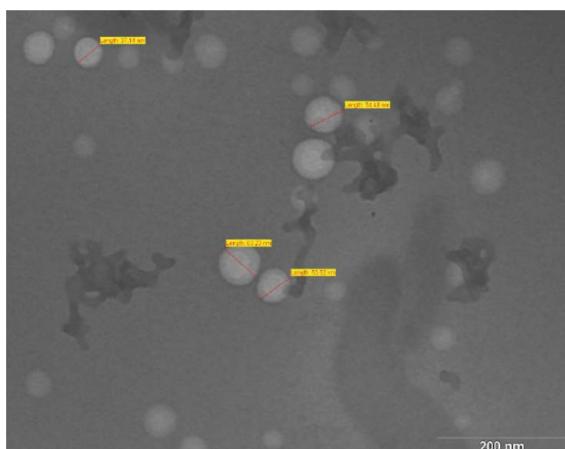


Figure 4: TEM of DE-SMEDDS.

Drug release studies

In vitro dissolution study: *In vitro* dissolution studies of DE-SMEDDS and pure drug suspension were performed in 0.01N HCl and pH 6.8 phosphate buffer (Figure 5). In 0.01 N HCl, almost 100% ($98.74 \pm 3.72\%$) drug was released after 60 min from DE-SMEDDS while only $39.86 \pm 2.41\%$ drug was released from pure drug suspension. This suggests that the SMEDDS led to enhancement in solubility due to reduction in particle size by micro emulsification [40]. Thus, this greater availability of dissolved DE from the SMEDDS formulation could lead to higher absorption and higher oral bioavailability.

In case of pH 6.8 phosphate buffer, cumulative drug release was $70.42 \pm 2.93\%$ in 60 min from DE-SMEDDS and only $7.47 \pm 2.84\%$ from drug suspension (Figure 5). Almost complete drug release was achieved in 0.01 N HCl than pH 6.8 phosphate buffer. This slower rate of dissolution in pH 6.8 phosphate buffer was due to lower solubility of DE at higher pH. Reason for higher dissolution rate in 0.01N HCl is the high solubility of drug in acidic conditions as compared to neutral and alkaline conditions. Overall, results of *in vitro* dissolution studies indicated that the formulation of DE in the form of SMEDDS enhanced its dissolution properties.

In vitro diffusion study: The *in vitro* release pattern was also studied through dialysis bag in which drug diffused through a semi permeable membrane of 12000 Daltons and pore size of 2.4 nm. In case of 0.01 N HCl, the cumulative percent drug released was $97.89 \pm 2.09\%$ after 300 min from DE-SMEDDS and $26.68 \pm 2.24\%$ from drug suspension. Similar results were observed for pH 6.8 phosphate buffer with $68.84 \pm 2.71\%$ and $5.33 \pm 2.28\%$ drug diffused from DE-SMEDDS and drug suspension respectively (Figure 6). *In vitro* drug diffusion

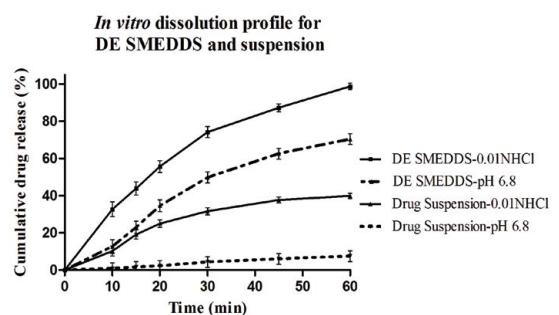


Figure 5: *In vitro* dissolution profile of DE-SMEDDS and drug suspension in 0.01 N HCl and pH 6.8 phosphate buffer.

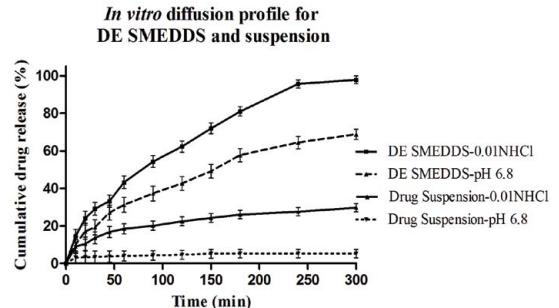


Figure 6: *In vitro* diffusion study of DE-SMEDDS and drug suspension in 0.01 N HCl and pH 6.8 phosphate buffer.

profiles are strong indicators of bioavailability. The higher amount of drug diffused from SMEDDS as compared to plain drug suspension can be attributed to the increased solubility and dissolution rate [27]. The data from *in vitro* diffusion study (for both 0.01 N HCl and pH 6.8 phosphate buffer) was fitted to various mathematical models to determine the best-fit model (Table 6). The r^2 values were found to be highest for Korsmeyer-Peppas ($r^2=0.994$) in 0.01N HCl and in Higuchi model ($r^2=0.994$) for pH 6.8 phosphate buffer. Since the drug release is from oil globules, so its geometry was considered to be sphere and the type of drug release mechanism was defined. The value for release component 'n' was between 0.43 and 0.85, indicating Non-Fickian diffusional release kinetics [41].

Ex vivo release study: The cumulative% release drug from rat stomach and intestine are shown in Figure 7. It was observed that $98.63 \pm 3.21\%$ drug diffused from the SMEDDS formulation in 0.01N

HCl in 300 min while from plain drug suspension, the diffusion was found to be only $31.38 \pm 2.74\%$. Thus, the amount of the drug diffused through the biological membrane increased when it was given in the form of a SMEDDS. In pH 6.8 phosphate buffer, the drug diffusion was relatively slower than in 0.01N HCl i.e. $69.34 \pm 2.76\%$ drug was diffused from the SMEDDS formulation and $10.65 \pm 1.87\%$ through plain drug suspension which could be attributed to the higher solubility of DE (weak base) in acidic conditions. Altogether such a pattern is desirable for our purpose as significant amount of drug will be carried to the intestinal portion inside the microemulsion droplets. Since P-gp efflux pumps are present in the intestinal site, presence of modulator excipients in the microemulsion droplets might lead to effective absorption of the drug [24].

***In vitro* cell line studies**

Cell viability study: Safety of DE-SMEDDS in Caco-2 cells was assessed by MTT assay. Since Caco-2 cell lines were used as *in vitro* absorption barrier, safety/toxicity of formulation on this absorption barrier was checked before performing transport studies. The viability studies on Caco-2 cells were performed for 4 h. Figure 8 represents the concentration versus percent viability data of cells incubated with

Release models	0.01N HCl	pH 6.8 Phosphate Buffer	n' Values for sphere geometry	Drug release mechanism
	R ²			
Zero order	0.926	0.923	0.43	Fickian diffusion
First order	0.950	0.980		
Hixson-Crowell	0.958	0.967	0.43<n<0.85	
Higuchi	0.992	0.994		Non-fickian (anomalous) diffusion
Korsmeyer-Peppas (Release component - 'n')	0.994 (0.565)	0.991 (0.552)	0.85	Case-II transport

Table 6: Regression coefficient of various *in vitro* release models applied and interpretation of release mechanism based on release exponent value.

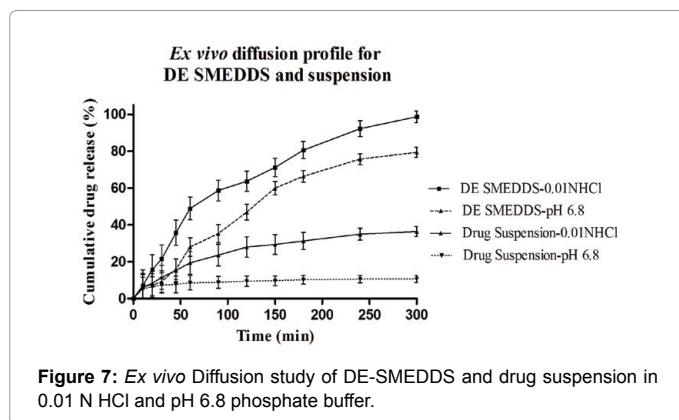


Figure 7: Ex vivo Diffusion study of DE-SMEDDS and drug suspension in 0.01 N HCl and pH 6.8 phosphate buffer.

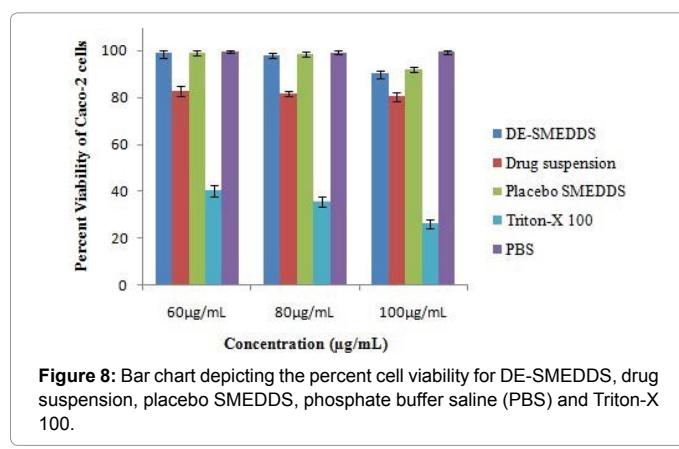


Figure 8: Bar chart depicting the percent cell viability for DE-SMEDDS, drug suspension, placebo SMEDDS, phosphate buffer saline (PBS) and Triton-X 100.

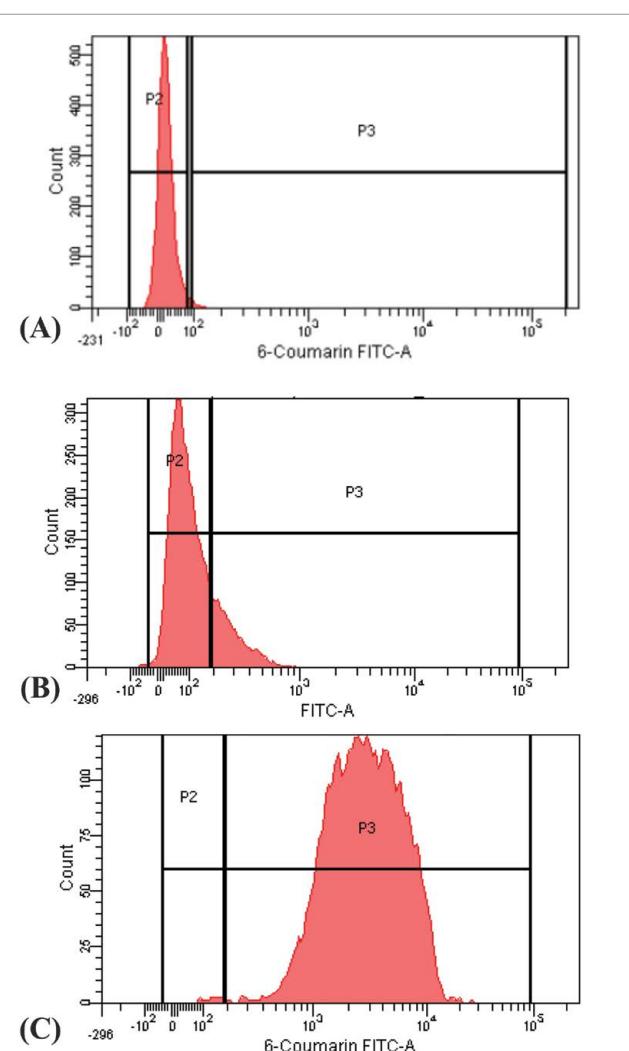


Figure 9: Cell uptake studies of coumarin-loaded SMEDDS by FACS for (A) Dye solution (B) 1 h and (C) 4 h.

DE-SMEDDS, Placebo SMEDDS, drug suspension, Triton-X 100 and Phosphate buffer saline (PBS) respectively. Reduction in the percent viability was observed for cells incubated with Triton-X 100 (Positive control). The percent cell viability data for Caco-2 cells was found to be >80% for DE-SMEDDS, Placebo SMEDDS formulation and drug suspension, at all the studied concentrations. Placebo SMEDDS and drug loaded SMEDDS showed almost similar cell viability at all three concentrations. In addition it was observed that the cell viability was higher in case of SMEDDS than the drug suspension. These results suggested that the drug when encapsulated inside the oil carriers becomes safer for the intestinal tissues than direct drug suspension. All the values were compared with negative control (PBS). These results also indicate that the surfactant and co surfactant did not cause any cytotoxicity at the levels studied and hence could be termed safe and non-toxic. Hence, we can say the developed formulation is safer than drug suspension.

Cell uptake study by FACS: Relative extent of uptake of 6-coumarin-loaded SMEDDS in comparison to plain dye solution was analyzed by FACS in Caco-2 cells. Caco-2 cells are reported to over express P-gp. FACS uptake studies showed that the fluorescence intensity inside the cells increased with SMEDDS formulation than plain dye solution. The shift of peak for fluorescent intensity clearly showed a significantly higher uptake and internalization for DE-SMEDDS as compared to plain dye solution (Figure 9). The enhancement in uptake of fluorescence by SMEDDS was doubled from 1 to 4 h, which supported the superiority of developed formulation than plain drug

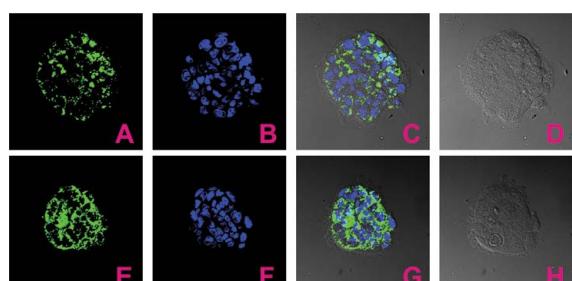


Figure 10: Qualitative cellular uptake of DE SMEDDS in Caco-2 cells; at 1 h (A-D) and 4 h (E-H); Green fluorescence spots represents dye-loaded SMEDDS, blue fluorescence represents DAPI-stained nuclei, (C and G) represents overlapped images and (D and H) represents Differential Interference Contrast (DIC) images showing cells.

solution in cell uptake. Thus, enhanced uptake of SMEDDS by Caco-2 cells implies greater intestinal absorption and can be correlated with enhanced therapeutic activity of the drug [30].

Qualitative cell uptake study by confocal microscopy: Cellular uptake and distribution of 6-coumarin loaded SMEDDS and 6-coumarin solution was examined by confocal microscopy using Caco-2 cells. 6-Coumarin was chosen as hydrophobic model dye to mimic hydrophobic nature of drug. The confocal micrograph images, as depicted in Figure 10, showed enhanced fluorescent intensity inside cells for SMEDDS as compared 6-coumarin solution after 4 h incubation which implies the enhanced absorption through the M cells of Peyer's patches of intestine. The uptake of SMEDDS by Caco-2 cells was time dependent and increased with incubation time.

Cell permeability study using transwell insert: The Caco-2 cell model has been the most extensively characterized and useful cell model in the field of drug permeability study. As the permeation characteristics of drugs across Caco-2 cell monolayers correlates with that of human intestinal mucosa, it has been suggested that Caco-2 cells can be used to predict the oral absorption of drugs in humans. Transepithelial permeability of DE-SMEDDS was measured at concentration of 100 µg/ml, as negligible toxicity towards Caco-2 cells was found at this concentration during MTT assay of the same. The average P_{app} for Lucifer yellow with Caco-2 cells was found $0.66 \pm 0.08 \times 10^{-5}$ cm/sec, which confirmed the integrity and suitability of monolayers for further experiment. Also the TEER value for Caco-2 cells grown on filters after 21 days was found to be $768 \Omega\text{cm}^2$, indicating the presence of tight junctions and good integrity of the monolayer. The permeability coefficient of DE-SMEDDS across the Caco-2 cells was found to be $40.15 \pm 0.68 \times 10^{-6}$ cm/sec while for drug solution it was found to be $2.19 \pm 0.57 \times 10^{-6}$ cm/sec, which showed 3.79 times increase in permeability that was attributed to the higher uptake of SMEDDS by endocytosis in Caco-2 cells [42,43] (If the P_{app} value of a compound is less than 1×10^{-6} cm/sec, in between $1-10 \times 10^{-6}$ cm/sec, and more than 10×10^{-6} cm/sec can be classified as poorly (0-20%), moderately (20-70%) and well (70-100%) absorbed compounds, respectively). This all might be because of small particle size of SMEDDS formulation, combined with amphiphilic nature of non-ionic surfactants present in the formulation. Additionally, Cremophor EL inhibits P-gp function and thereby enhances the intestinal absorption of various drugs [44].

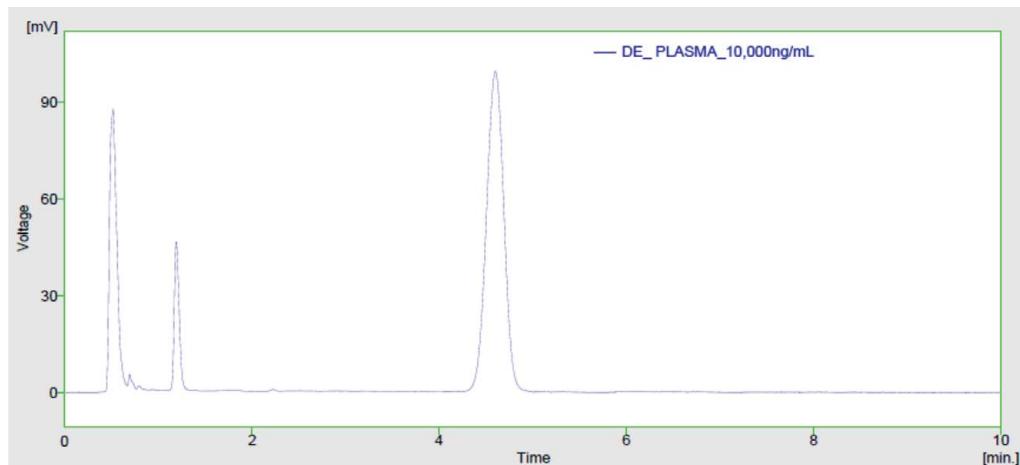


Figure 11: The HPLC chromatogram of DE in Plasma.

In vivo studies

Pharmacokinetic study: The plasma concentration of Dabigatran was estimated by HPLC using a validated method and chromatogram is as shown in Figure 11. The plasma drug concentration versus time profile for plain drug suspension and DE-SMEDDS following oral delivery in rats ($n=6$) is illustrated in Figure 12. The pharmacokinetic parameters including C_{max} (ng/mL) and T_{max} (h), $AUC_{0-\infty}$ (ng·ml⁻¹·h), $T_{1/2}$ (h) and relative bioavailability were analyzed by Kinética software 5.0 version by non-compartmental (model independent) method. The comparative pharmacokinetic parameters are summarized in Table 7. C_{max} for plain drug suspension was 440.0 ± 88.24 ng/mL while SMEDDS exhibited increased peak plasma concentration ($C_{max}=1102.0 \pm 80.65$ ng/mL). Compared with the control group (DE suspension), the SMEDDS resulted in 2.5 times increase in AUC. Extent of absorption of drug was significantly increased as evident from the AUC and C_{max} . Relative bioavailability of DE SMEDDS was enhanced to 3.36 folds than drug suspension.

Intestinal transport of DE SMEDDS in rats: Cycloheximide (CHM) is reported to inhibit the synthesis of apoproteins and block chylomicron flow to inhibit the intestinal lymphatic transport pathway without nonspecific damage to other active and passive absorption

pathways [45]. In this study, chylomicron flow blocking approach was employed to measure the lymphatic transport of DE SMEDDS. The results showed that the plasma concentration of DE in rats treated with CHM was lower than that of control i.e. saline treated (Figure 13). Blood or lymphatic pathway could be involved in intestinal transport when DE SMEDDS entered into the systemic circulation. The peak plasma concentration remarkably decreased by 173.17% and the AUC_{last} value of DE SMEDDS was significantly reduced by 209.13% ($P<0.05$) in rats after CHM treatment which could block intestinal lymphatic transport. The comparative pharmacokinetic parameters with CHM and saline treated are summarized in Table 8.

Pharmacodynamic study-bleeding time: Bleeding time is the time from the moment the tail is incised to first arrest of bleeding (stop of bleeding for a minimum of 30s) [33]. The bleeding time was assessed after 2 h of treatments (DE SMEDDS and plain drug suspension). Compared with the control group, all treatments prolonged the bleeding time (Table 9). Untreated animals (control group) had bleeding times less than 60 sec while the animals treated with drug suspension showed comparatively increased bleeding time of 1.5 to 2 min. However, DE SMEDDS exhibited sharply increased bleeding time upto 2.5 to 3 min which correlated well with pharmacokinetic data. Hence, we could conclude that the developed SMEDDS formulation exhibited better anticoagulation activity than plain drug suspension by improving the oral bioavailability of DE.

Conclusions

The gist of current study suggested that the optimized SMEDDS formulation exhibited globule size in nano sized range, with significant improvement in the drug release rate and many-folds enhancement in permeability and absorption of the drug as suggested by *in vitro*, *ex vivo* and *in vivo* assessments when compared to drug suspension. The nano-sized oil droplets from the optimized SMEDDS were capable of enhancing the oral bioavailability of DE which is a BCS Class II drug exhibiting low solubility. P-gp efflux of DE was successfully obstructed by employing P-gp inhibitor (Cremophor-EL). The developed SMEDDS was also found to be safe on enterocytes (Caco2 cells) with no significant

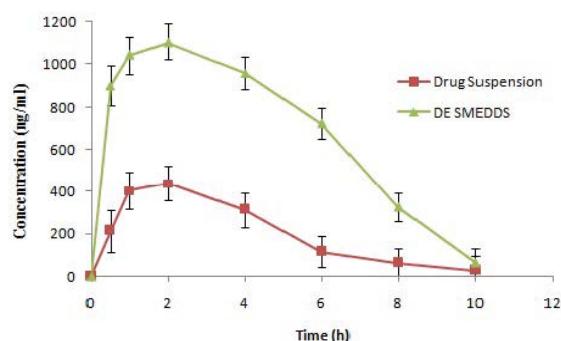


Figure 12: Plasma concentration-time profiles of DE in rats after oral administration of drug suspension and DE-SMEDDS. Each data is given as mean \pm standard deviation ($n=6$).

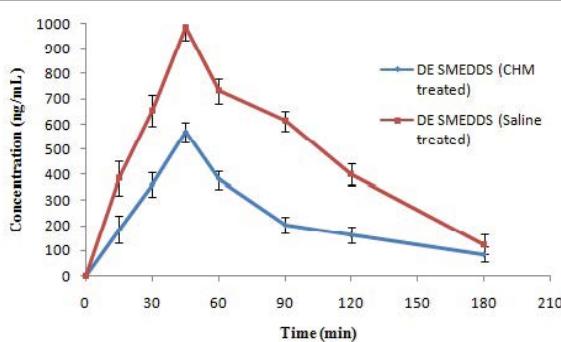


Figure 13: The plasma concentration after IP administration of DE SMEDDS to rats treated with (CHM) or aline ($P<0.05$) ($n=6$).

Pharmacokinetic parameters	Drug Suspension	DE SMEDDS
C_{max} (ng/mL)	440.0 ± 88.24	$1102.0 \pm 80.65^*$
T_{max} (h)	2 ± 0.32	2 ± 0.24
AUC_{Last} (ng·mL ⁻¹ ·h)	2029.31 ± 269.86	$6822.129 \pm 542.21^*$
MRT (h)	3.728 ± 0.214	3.948 ± 0.368

* $p<0.05$, compared with Drug suspension by the ANOVA test.

Table 7: Pharmacokinetic parameters of drug suspension and DE SMEDDS ($n=6$).

Pharmacokinetic parameters	DE SMEDDS (CHM treated)	DE SMEDDS (Saline treated)
C_{max} (ng/mL)	$569.6 \pm 39.9^*$	986.4 ± 52.2
T_{max} (min)	45 ± 0.96	45 ± 0.84
AUC_{Last} (ng·mL ⁻¹ ·min)	$40688.189 \pm 326.3^*$	85091.39 ± 419.8
MRT (min)	$111.97 \pm 10.68^*$	88.419 ± 8.24

* $p<0.05$, compared with DE SMEDDS (Saline treated) by the ANOVA test.

Table 8: Pharmacokinetic parameters of DE SMEDDS (CHM treated) and DE SMEDDS (Saline treated) ($n=6$).

S. No.	Bleeding Time (Seconds)		
	Control group (untreated)	Drug Suspension treated	DE SMEDDS treated
1	48	104	168
2	52	118	172
3	45	97	162
4	41	93	158
5	47	101	164
6	55	121	179
Average	48	106	167*
%RSD	10.37	10.77	4.52

* $p<0.05$, compared with control group by the ANOVA test.

%RSD: Percent Relative Standard Deviation.

Table 9: Bleeding time of untreated and treated animals.

toxicity. The cellular uptake of DE from SMEDDS in Caco-2 cells was dramatically increased than drug suspension. Pharmacodynamic study displayed better *in vivo* anticoagulation effect. These observations lead us to the conclusion that SMEDDS seems to be a promising drug delivery system, which can provide an effective and practical solution to the problem of formulating drugs with low aqueous solubility and poor systemic bioavailability. In a nutshell, the current studies proved the effectiveness of SMEDDS for enhancing the oral biopharmaceutical performance of DE along with improved anticoagulant activity.

Acknowledgement

The authors would like to acknowledge University Grant Commission, India for providing the Research Fellowship in Science for Meritorious Student (UGC Letter No.4-1/2006(BSR)7-129/2007(BSR) Dated 22.10.2013) to Veenu Mundada; Technology Information, Forecasting and Assessment Council (TIFAC) - Centre of Relevance & Excellence (CORE) in NDDS for providing research facilities; Alembic Research Centre, India for providing Dabigatran etexilate; Abitec Corporation, USA, BASF, India, and Gattefosse India Ltd, India for providing gift sample of Capmul MCM C8, Cremophor EL and Transcutol HP respectively; Dr. Vikram Sarabhai Institute of Cell and Molecular Biology, The M.S. University of Baroda, India for providing facilities for cell uptake studies.

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