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Cyclosporine A Loaded Self-nanoemulsifying Drug Delivery System (SNEDDS): Implication of Functional Excipient Based Co-encapsulation Strategy on Oral Bioavailability and Nephrotoxicity†

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The present work focusses on the formulation development and evaluation of functional excipient (surfactant stabilizer), Vitamin E TPGS loaded self-nanoemulsifying drug delivery system (SNEDDS) for improving deliverability and safety profile of Cyclosporine A (CyA). Saturation solubility of individual bioactives were evaluated in series of oils and surfactants. Further, the ternary phase diagram based exhaustive optimization was performed to identify the nanoemulsification region that yields desired quality attributes and maximum loading capacity of CyA and Vitamin E TPGS. The optimized formulation exhibited excellent stability in simulated gastrointestinal fluids. In vitro drug release studies revealed significantly higher rapid release of CyA from CyA-TPGS SNEDDS as compared to that of clinically available counterpart Bioral® or in-house CyA-SNEDDS. In vivo pharmacokinetics further demonstrated 4.48-fold increase in oral bioavailability in case of developed formulation as compared to Bioral®. CyA induced reactive oxygen species (ROS) generation in HEK cell lines was significantly diminished in case of CyA-TPGS SNEDDS in contrast to that of CyA SNEDDS, Bioral® and CyA+Vitamin E TPGS physical mixture. The results were further corroborated by in vivo nephrotoxicity studies wherein the levels of biochemical markers of nephrotoxicity, blood urea nitrogen and serum creatinine levels were comparable to that of negative control in case of developed formulation in contrast to that of Bioral®. In nutshell, the employed strategy of functional excipient loaded SNEDDS pose viable strategy for developing value-added nanoformulations of CyA.

1. Introduction

Cyclosporine A (CyA), a lipophilic cyclic polypeptide, was firstly isolated from crude extract of the fungus *Tolypocladium inflatum gams* in 1972. It acts as an immunosuppressant agent and is clinically utilized for immunomodulation in the different surgical process and prevents physiological rejection mechanism following the kidney, liver, bone marrow and pancreas transplantation. CyA is also used in rheumatoid arthritis, psoriasis, aplastic anemia, atopic dermatitis, ulcerative colitis and severe corticosteroid dependent asthma. FDA has approved CyA for the prevention of transplant rejection and various dermatological diseases such as psoriasis, alopecia areata, pyoderma gangrenosum etc. [1].

Noteworthy, it is one of few peptide drug which is insensitive to enzymatic degradation in gastrointestinal fluids [2]. CyA possess very low aqueous solubility (6.6 µg/ml) and low intestinal permeability due to the presence of cyclic undecapeptide chain and high molecular weight. Extensive pre-systemic metabolism in the gut wall, liver and P-glycoprotein (P-gp) efflux in the enterocytes further reduces the oral bioavailability of CyA. [3]. The low oral bioavailability of CyA led to the development of Sandimmune®, which is an oily concentrate consisting of a lipidic ingredient (olive oil) and solubility enhancers intended for the oral administration after the dilution with different fluids like fruit juice or cola. This nonstandard method resulted into the formation of larger droplet size of emulsion which after oral administration leads to the variable oral bioavailability varies form 10-60% along with patient noncompliance due to the unpleasant taste of the formulation [4]. Subsequently, Neoral® (microemulsion formulation of CyA) was developed which could overcome the problems of low and variable absorption exhibited by Sandimmune®. Neoral® showed rapid drug absorption which was revealed by 70% and 40% increase in the peak concentration and area under curve (AUC) respectively as compared to Sandimmune® [5]. However, the commercially available formulations of CyA exhibit nephrotoxicity during different surgical procedures [6]. Sandimmune® has shown the nephrotoxicity in 25 % of cases of renal transplantation, 38% of cases of cardiac transplantation, and 37% of cases of liver transplantation. The nephrotoxicity of CyA is associated with the elevated levels of blood urea nitrogen (BUN) and creatinine at a range of 35-45 mg/dl and 2.0-2.5 mg/dl, respectively. Of note, relatively newer formulation of CyA, Neoral®, is also associated with nephrotoxicity measured as a function of reduction in the renal plasma flow and glomerular filtration rate [7]. Besides the nephrotoxicity, hepatotoxicity, hypertension, hyperglycaemia and other malignancy are also

associated with the free CyA as well as with the marketed formulations [6].

Furthermore, in order to overcome the problem of low oral bioavailability and nephrotoxicity associated with free CyA and marketed formulations, various nanoformulation based approaches have also been investigated for oral bioavailability enhancement and reducing the nephrotoxicity of CyA. In this regard, PLGA nanoparticles [8-10], solid lipid nanoparticles [11], pH sensitive nanoparticles [12], charged nanoparticles [13], cubic nanoparticles [14], hydroxypropyl methylcellulose phthalate nanoparticles[15], polycaprolactone nanoparticles [16], stearic acid nanoparticles [17] etc. have been mainly formulated for oral bioavailability enhancement and some of the formulations have been investigated for reducing the nephrotoxicity of CyA.

Additionally, co-administration of different antioxidants with CyA has also been investigated to reduce the side effects associated with CyA. These include different exogenous antioxidants like vitamin E, vitamin C, epigallocatechin gallate, and resveratrol, curcumin, melatonin etc. [18-20]. Among the various reported antioxidants, Vitamin E TPGS (D-alpha tocopherol polyethylene glycol 1000 succinate) is a water soluble derivative of Vitamin E and employed to enhance bioavailability of many poorly soluble drugs including the CyA by inhibiting the P-gp efflux [21]. Vitamin E TPGS is converted into the vitamin E which acts as an antioxidant [22] and scavenge the free radicals thereby reducing the free radical toxicities. Nevertheless the oral bioavailability of most of the antioxidants is limited by their poor aqueous solubility and instability in the GI tract, which ultimately impairs their antioxidant potential.

In this regard, different nanoformulations containing the antioxidant have been investigated to reduce the CyA induced nephrotoxicity [9, 23]. Quercetin loaded self-nanoemulsifying drug delivery system has been recently developed by our group which revealed significantly higher antioxidant potential as compared to free drug suspension against CyA induced nephrotoxicity [24].

The present research work encompasses co-loading of CyA and functional antioxidant, Vitamin E TPGS into self-emulsifying formulation comprising of an oil, surfactant and co-surfactant with intention to increase the oral bioavailability of CyA vis-à-vis reduction in CyA induced nephrotoxicity. Further the problems of poor loading of antioxidant, poor industrial scalability and stability have also been addressed. The said potential of the developed formulation has been established in comparison to the free drug and Bioral™ (commercially available formulation of CyA in India, manufactured by Panacea Biotec, bioequivalent to Neoral®).

2. Materials and Methods

2.1 Materials

Cyclosporine A and Vitamin E TPGS were obtained as generous gift from Panacea Biotec Limited, New Delhi, India and Isochem, Vert-le-Petit, France, respectively. Plurol Oleique, Peceol, Gelucire, Labrafil M 2130 CS, Labrafil 1944 CS were obtained as gift samples from Gattefosse, France. Captex 355, Capmul MCM, Capmul MCM-C8 were procured as generous gift from Abitec Corporation, Janesville, USA. Cremophor RH 40, Tween 20, Tween 80, Triton X-100 and Coumarin-6 (C-6) were purchased form Sigma, St. Louis, MO, USA. All other oils were procured from the local suppliers. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics (Antibiotic-antimycotic solution) and Hanks's balanced salt solution (HBSS) were purchased from PAA Laboratories GmbH, Pasching, Austria. Ethyl acetate (LR grade), acetonitrile (HPLC grade), methanol (LR grade) and methanol (HPLC grade) were purchased from Fischer Scientific, Pittsburgh, PA, USA. Creatinine Assay Kit and Urea Assay Kit were purchased from Accurex Biomedical Pvt Ltd, Mumbai, India. Ultra-pure deionized water (LaboStar™ ultrapure water Systems, Erlangen, Germany) was used for all the experiments. All other reagents used were of analytical grade.

2.2 Screening of oily phase and compatibility testing of CyA and Vitamin E TPGS

The oily phase for formulation of self-nanoemulsifying drug delivery system was screened on the basis of their solubilization potential. Saturation solubility of both CyA and Vitamin E TPGS in series of oils were estimated using shake flask method [25]. Briefly, excess amounts of CyA and Vitamin E TPGS were added to vials containing 0.5 g of the oils and vortexed for 2 min. The obtained homogenous mixture was then incubated in shaker water bath (Lab Tech, Korea) operated at 50 strokes/min for 72 h at 37°C. Subsequently, mixtures were centrifuged at 500 g for 10 min to separate excess drug and supernatant diluted with ethyl acetate and methanol. The amounts of CyA and vitamin E TPGS solubilized in various oils were quantified by a validated HPLC method at 210 nm for CyA and at 285 nm for vitamin E TPGS using methanol as a blank (Supplementary information). In separate set of experiments, the compatibility testing of CyA and vitamin E TPGS with the selected oil was also carried out incubating the mixture for stipulated period of time. Bioactives were then extracted in methanol and estimated quantitatively using validated HPLC method.[10]

2.3 Screening of surfactants and co-surfactants

Different surfactants were screened for emulsification ability of the selected oil phase. Briefly, 1:1 ratio of surfactant and oil were mixed and gently heated at 50°C for homogenization of the components. On similar line of action co-surfactants were also screened by mixing oil, surfactant and co-surfactant in the ratio of 3:2:1, respectively. Each mixture, 250 mg, was then diluted with distilled water to 50mL in a stoppered volumetric flask. The screening parameters were set as droplet size, PDI, % transparency and ease of emulsification, estimated as per standard protocols [24].

2.4 Construction of pseudo ternary phase diagram

Ternary diagrams with different concentration of surfactant, co-surfactant and oil were plotted, each of them, representing an apex of the triangle. In the ternary mixtures the concentration of selected oil, surfactant and co-surfactant was varied from 30-70% (w/w), 10-70% (w/w) and 0-30% (w/w), respectively. Various compositions were prepared by incrementing the co-surfactant concentration by the order of 5% while keeping the oil concentration constant. Surfactant concentration was adjusted appropriately so as to make resulting mixture 100 % [26]. Forty-seven such mixtures with varying compositions of surfactant, co-surfactant and oil were prepared and evaluated for droplet size, PDI and % transparency. The compositions which formed submicron emulsion <200 nm were plotted on the pseudo ternary phase diagram.

2.5 Optimization of CyA and Vitamin E TPGA loading into SNEDDS

The effect of CyA on the phase behavior and area of Nano emulsion formation was studied independently and in combination with Vitamin E TPGS. Briefly, varying concentrations of CyA was dissolved in the representative formulations of Nano emulsion region

obtained previously with blank formulation and results of droplet size and PDI were plotted in the ternary diagram. Similarly, effect of Vitamin E TPGS on the resultant nanoemulsification region was studied. Special attention was given to consider the surfactant properties of the Vitamin E TPGS.

2.6 Stability studies

Stability in simulated gastrointestinal fluids: The representative formulations from the nanoemulsification region was evaluated for its ability to form the nanoemulsion in simulated gastric fluids (SGF, pH 1.2) and simulated intestinal fluids (SIF, pH 6.8). Briefly, 250 mg of selected SNEDDS formulation was diluted with 50 ml of SGF and SIF and allowed to stand for 2 h. Subsequently, droplet size, PDI and self-emulsification time of the resultant nanoemulsion was determined.

Robustness to dilution: Optimized SNEDDS composition was subjected to dilution effect with various vehicles like water, buffer pH-1.2, and pH-6.8. Briefly, 50 mg SNEDDS was diluted with above vehicles around 200, 400, 600 and 800 times dilution. Diluted Nano emulsion was studied at 0, 2 and 6 h for droplet size analysis and visual observation for precipitation of CyA and Vitamin E TPGS.

Freeze thaw stability: Optimized SNEDDS composition was further exposed to freeze thaw cycles to evaluate the stability of formulation at extreme temperatures for short period of time. The formulation was subjected to 3 freeze-thaw cycles, which included freezing below 0 ºC for 24 h followed by thawing at 40 ºC for 24 h. The formulation was then evaluated for the critical quality attributes such as droplet size, PDI and self-emulsification time.

Long term stability: Room temperature stability of optimized formulation was evaluated for six months. The formulation was checked for the spontaneity of Nano emulsion formation, droplet size, PDI and drug content at regular intervals as per aforementioned protocol.

2.7 Physicochemical evaluation and characterization of CyA-TPGS-SNEDDS

Transmission electron microscopy (TEM) of the reconstituted Nano emulsion: The morphology of submicron oil droplets after dilution of CyA-TPGS-SNEDDS was evaluated by transmission electron microscope (FEI Tecnai G2). Diluted samples (1:1000) were negatively stained with 1% aqueous solution of phosphotungstic acid and visualized after placing on 200-mesh carbon coated grids under the electron microscope at 10–100 k-fold enlargements at an accelerating voltage of 60.0 kV [27].

2.8 In vitro drug release

CyA SNEDDS and CyA-TPGS-SNEDDS equivalent to 25 mg of CyA dispersed in simulated gastric (pH 1.2 for 2 h) and intestinal fluid (pH 6.8 for 8 h and pH 7.4 up to 24 h) were filled in the dialysis bag (MWC 12000 Dalton, Sigma, USA). Formulation filled dialysis bag was dispensed in 20 ml of 0.1N HCl and phosphate buffer containing 0.25% w/v SLS and subjected to shaking over shaker water bath operated at 37°C and 100 rpm. Aliquots of 1 ml were withdrawn at the predetermined time intervals (0.5, 1, 2, 4, 8, 12, 18 and 24 h) and replenished with fresh media each time to maintain the sink condition. The cumulative amount of CyA released was analyzed by validated HPLC method.

2.9 *In vivo* pharmacokinetic study

All the animal experiments were carried out under the guidelines compiled by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Culture, Govt. of India) and the study protocols were duly approved by Institutional Animal Ethics Committee of NIPER, Mohali, India. In vivo pharmacokinetics studies of CyA-TPGS-SNEDDS were performed using female Sprague Dawley Rats. Animals were acclimatized at standard housing conditions for one week before experiments. Rats were divided into five groups each containing five animals. Group I received marketed formulation of CyA (BioralTM) and group II received free CyA. Group III received the free CyA and vitamin E TPGS. Group IV and V received CyA-SNEDDS and CyA-TPGS-SNEDDS, respectively. All the formulations were orally administered in the dose of 25 mg/Kg and 80 mg/Kg equivalent to CyA and free TPGS respectively. Considering poor aqueous solubility of CyA, it was administered as suspension with 1% xanthum gum as suspending agent. The blood samples (~0.2 ml) were collected at 1, 2, 4, 8, 12, 24, 48, 72 h from the retro-orbital plexus under mild ether anesthesia into heparinized microcentrifuge tubes. Plasma was separated by centrifuging the blood samples at 500 g for 10 min at 15 ºC and estimated for CyA content using validated HPLC method. The obtained plasma concentration profile was then subjected to pharmacokinetic modeling using Kinetica Software (Version 5.0, Thermo) and various pharmacokinetic parameters were assessed[28, 29]. The limit of quantification for CyA was 1.76 µg/ml and that of Vitamin E TPGS was 1.89 µg/ml with injection volume of 20 µl (Supplementary information).

2.10 CyA induced intracellular reactive oxygen species (ROS) in HEK cell lines

Intracellular ROS generation by CyA was evaluated employing standard 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) fluorescence assay protocol [24]. Briefly, Human Embryonic Kidney (HEK) cells were maintained in Minimum Eagle's Medium supplemented with 2.2% sodium bicarbonate, 1 mM sodium pyruvate, 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µM streptomycin (Sigma, USA) at 37°C/5% CO2. The confluent cells were harvested and seeded at cell density of 1.0 lac cells/well in 6-well tissue culture plate. The seeded cells were allowed to adhere overnight. The cells were then exposed to CyA, CyA + Vitamin E TPGS, CyA SNEDDS and CyA-TPGS SNEDDS for 12 h. The doses of CyA 10 µg/ml and Vitamin E TPGS 32 µg/ml, proportional to that loaded in the developed formulation were employed for the studies. After 12 h, cells were then washed with PBS, pH 7.4 and incubated with 50 µM H2DCFDA for 30 min. The cells were then fixed using glutaraldehyde 2.5% v/v and visualized under a confocal laser scanning microscope (CLSM) (Olympus FV1000, USA) with Ex/Em: ~492–495/517–527 nm. In separate set of experiments, cell cytotoxicity assay for exposed time period was assessed using standard MTT cell viability assay.

2.11 *In vivo* nephrotoxicity

Nephrotoxicity was investigated in healthy mice (Swiss strain) of uniform body weight (25 ± 2 g) with no prior drug treatment. Mice were divided into five groups, wherein group I received marketed formulation of CyA (BioralTM), group II received suspension of CyA containing 1% xanthum gum as a suspending agent, group III received suspension of CyA and Vitamin E TPGS solution, group IV received CyA-SNEDDS and group V was orally administered CyA-TPGS-SNEDDS. All the formulations were orally administered daily for 1 week at the dose of 25 mg/Kg equivalent to free CyA and 80 mg/Kg equivalent to Vitamin E TPGS. Following the treatment

blood was collected and evaluated for Creatinine and blood urea nitrogen (BUN) levels using commercially available kits [30].

2.12 Statistical analysis

All the data are expressed as mean ± standard deviation (SD) for all in vitro results and as mean ± standard error of mean (SEM) for all in vivo results. Statistical analysis was performed using Sigma Stat (version 3.5) utilizing one-way ANOVA followed by a Tukey−Kramer multiple comparison test. $P < 0.05$ was considered as a statistically significant difference.

3. Results

3.1 Saturation solubility and compatibility studies

Figure 1 depicts the solubility of CyA and Vitamin E TPGS in various oils. Capmul MCM showed highest solubilization potential for both CyA and Vitamin E TPGS. Although no statistical significant difference in solubility was noted for Capmul MCM and Capmul MCM C8, the former was preferably chosen owing to its greater ease of emulsification being a mixture of mono and diglycerides [31]. Further the compatibility studies were also carried out which revealed no signs of interaction. HPLC chromatogram of blank Capmul showed a peak at 0.67 min which could be due to the caprylic acid content of the Capmul MCM. HPLC chromatogram of CyA extracted from Capmul MCM showed an intense peak at 2.833 min whereas the vitamin E TPGS extracted from Capmul MCM showed a peak at 7.87 min. In the similar manner, oily sample containing CyA and vitamin E TPGS showed the peaks at same retention time as revealed in drug sample solubilized in Capmul MCM separately (Supplementary information).

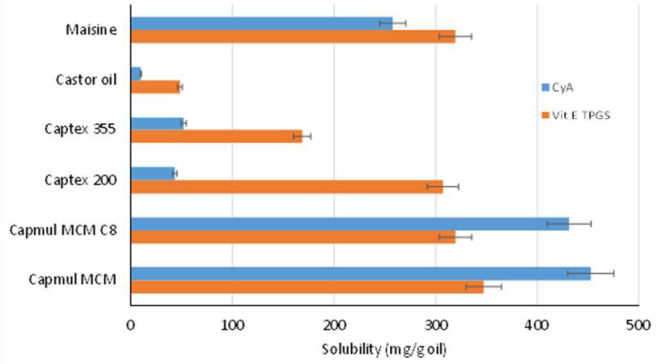


Figure 1: Solubility of CyA and Vitamin E TPGS in various oils. Each data point represents mean±SD (n=6)

3.2 Screening of surfactants and co-surfactants

Table I depicts various quality attributes of the formulations subjected to surfactant screening. Remarkable difference in droplet size, PDI and % transmittance values were noted with different surfactants. Based on the observed quality attributes, Cremophor RH 40 was chosen as preferred surfactant. Tween 20, Tween 80 and Cremophor EL also showed marginally acceptable results hence a secondary screening step was employed which comprised of estimation of the CyA solubility in presence of 10% surfactant solution. The results revealed remarkably higher solubilisation potential of Cremophor RH 40 and hence was selected for further studies (Supplementary information).

On similar line of action, co-surfactant screening was also carried out which revealed Labrafil 1944 CS to yield the best quality attributes among the tested combinations (Table II). Although % transmittance of ethanol, ethylene glycol and transcutol was relatively better but no statistical difference was noted hence on the basis of droplet size and PDI, Labrafil 1944 CS was selected as a co-surfactant for further studies.

Table I: Emulsification ability of various Surfactants (Oily Phase-Capmul MCM)

Surfactants	Particle size	PDI	%Transmittance
Tween 20	162.3±13.02	0.56±0.052	72.00±0.156
Tween 40	188.8±20.48	0.987±0.240	69.39±0.52
Tween 60	256.9±14.05	0.581±0.134	68.28±0.243
Tween 80	194.7±12.07	0.461±0.042	73.17±0.286
Gelucire	214.7±16.26	0.457±0.321	36.92±0.168
Cremophor EL	147.3±8.24	0.49±0.025	75.10±0.137
Cremophor RH 40	**89.12±12.37**	**0.37±0.049**	**88.24±0.581**

Values are expressed as mean ± S.D. (n=6)

Table II: Emulsification studies on Capmul MCM: Cremophor RH 40: Co-surfactant

Co-surfactant	Droplet size	PDI	%Transmittance
Labrafil 1944 CS	38.91±4.57	0.289±0.146	85.90±2.84
Ethylene glycol	101.8±12.05	1±0.025	87.16±6.22
PEG	63.67±9.57	0.92±0.752	85.83±0.57
Transcutol	117.7±11.25	0.675±0.54	87.23±4.73
Ethanol	96.86±3.45	0.637±0.613	88.30±6.64
Plurol oleque	252.8±3.47	0.474±0.428	57.7±7.24

Values are expressed as mean ± S.D. (n=6)

3.3 Construction of pseudo ternary phase diagram and optimization of drug loading

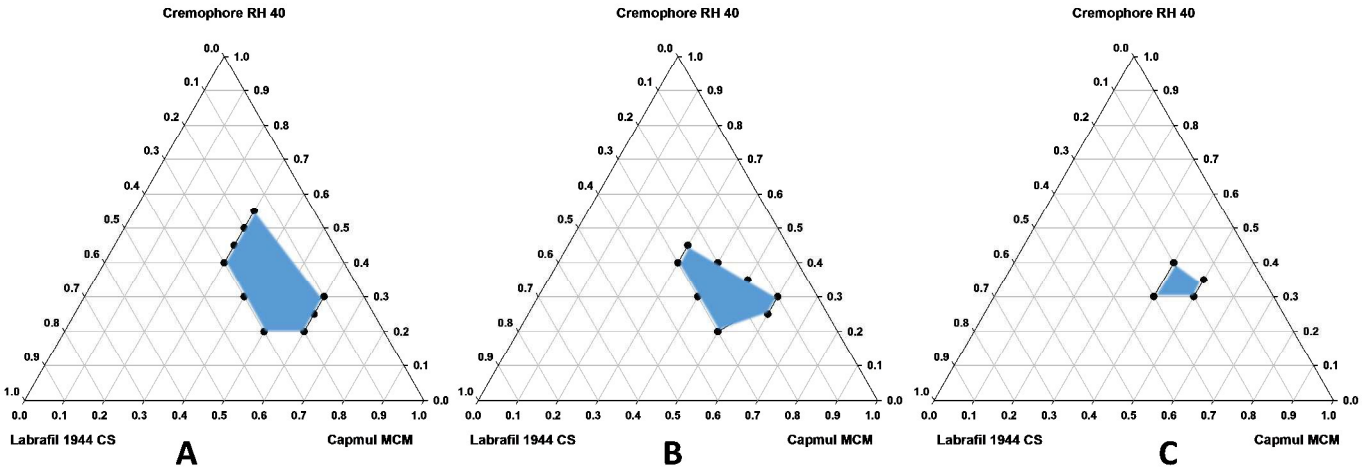
Pseudo ternary phase diagrams were constructed using Capmul MCM as oil, Cremophor RH 40 as surfactant and Labrafil 1944 CS as co-surfactant, representing three apexes of the triangle. Figure 2A represents the ternary phase diagram constructed with blank formulation and filled area denotes the nanoemulsion region. Figure 2B denotes the effect of CyA loading on the nanoemulsification region as noted for the blank formulation whereas Figure 2C depicts the effect of co-loading of CyA and Vitamin E TPGS. Based on preliminary studies, the quantum of CyA and Vitamin E TPGS were set as 25 mg and 80 mg per gram of formulation, respectively (Supplementary information). A representative formulation from the nanoemulsion region of Figure 2C i.e. 40:40:20 (oil: surfactant: co-surfactant), was considered as optimized and selected for further studies. The quality attributes of said formulation (CyA and Vitamin E TPGS loaded) were as follows: droplet size 72.19±7.48 nm, PDI 0.24±0.186, and % transmittance 89.53±4.26.

Figure 2: Ternary phase diagram depicting the nanoemulsion region of (A) blank formulation, (B) CyA-SNEDDS and (C) CyA-TPGS-SNEDDS

Table III: Stability of

Medium	Initial			After 2h			After 6h		
	Size (nm)	PDI	ZP (mV)	Size (nm)	PDI	ZP (mV)	Size (nm)	PDI	ZP (mV)
Water	72.19±7.48	0.243 ±0.01	-7.41±1.88	79.22 ±4.22	0.216 ±0.01	-8.11±2.17	81.39 ±7.34	0.251 ±0.01	-10.79±1.52
SGF, pH 1.2	80.62±5.11	0.292 ±0.081	-4.19±2.33	89..34 ±5.62	0.312 ±0.042	-5.68±3.98	86.85 ±9.06	0.303 ±0.038	-6.95±3.02
SIF, pH 6.8	89.28±7.12	0.266 ±0.037	-11.24±3.96	96.53 ±12.94	0.293 ±0.015	-12.25±3.14	98.54±4.79	0.344 ±0.043	-12.52.91

ZP: Zeta potential; Values are expressed as mean ± S.D. (n=6);



3.4 Stability studies

STABILITY IN SIMULATED GASTROINTESTINAL FLUIDS AND ROBUSTNESS TO DILUTION: Table III reflects the critical quality attributes of developed CyA-TPGS-SNEDDS formulation when reconstituted using various media. As evident, no statistical significant difference in the quality attributes was noted either as a function of nature of diluting media or time period. The attributes were found to be consistent over a period of time irrespective of diluting agent and no evidence of drug precipitation or phase separation was noted. On similar line of action, the developed formulation was also challenged for robustness to dilution and quality attributes of the formulations were found to consistent (data not shown).

LONG TERM STABILITY STUDIES AND AT STRESS CONDITIONS OF FREEZE-THAW: The stability of the developed formulation was further assessed as a function of freeze thaw stresses and long term storage. Table IV and Table V depicts the quality attributes of the reconstituted nanoemulsion from CyA-TPGS-SNEDDS subjected three freeze-thaw cycles and long terms stability studies, respectively. As evident no statistical significance in the quality attributes were noted revealing the robustness of the developed formulation against temperature fluctuations and long term standing.

Table IV: Freeze thaw stability studies of CyA-TPGS-SNEDDS

Stress condition	Initial		After	
	Size	PDI	Size	PDI
Room Temperature	72.69±8.44	0.254±0.029	81.68±7.50	0.204±0.045
Freeze Thaw Cycle	78.15±4.25	0.242±0.027	82.56±5.99	0.257±0.034

Values are expressed as mean ± S.D. (n=6)

Table V: Long term stability of CyA-TPGS-SNEDDS

Time period	Size	PDI	Zeta Potential (mV)	CyA content
Initial	57.95±9.46	0.221±0.01	-8.55±1.44	97.24±1.73
1 month	52.99±5.24	0.229±0.01	-9.12±2.56	96.51±1.99
2 months	60.69±8.56	0.230±0.02	-7.94±2.41	96.18±2.62
3 months	50.25±4.75	0.232±0.02	-9.36±2.27	95.90±3.98

Values are expressed as mean ± S.D. (n=6)

3.5 Physicochemical evaluation and characterization of CyA-TPGS-SNEDDS

TEM ANALYSIS: Figure 3 depicts the representative TEM image of reconstituted CyA-TPGS-SNEDDS nanoemulsion. As evident, all droplets upon dilution possess spherical shape, thus confirmed the formation of nanoemulsion and droplet size well correlating with that of dynamic light scattering results.

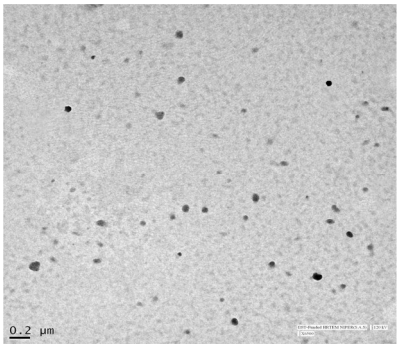


Figure 3: Representative TEM image of reconstituted CyA-TPGS-SNEDDS nanoemulsion

3.6 In vitro drug release studies

Figure 4 shows the cumulative in vitro drug release profile of free CyA, CyA-SNEDDS and CyA-TPGS-SNEDDS formulations. It was observed that about ~27%, ~59% and ~90% of CyA was released from the developed formulation when incubated with SGF (pH 1.2) for 2 h, SIF (pH 6.8) for 8 h and PBS (pH 7.4) for 24 h, respectively. Of note, ~33%, ~46% and ~68% of CyA could be released from the CyA SNEDDS. In contrast, ~20% of the CyA was released from the free CyA over a period of 24 h. The results are suggestive of positive contribution of the Vitamin E TPGS in improving the drug release from the developed formulation.

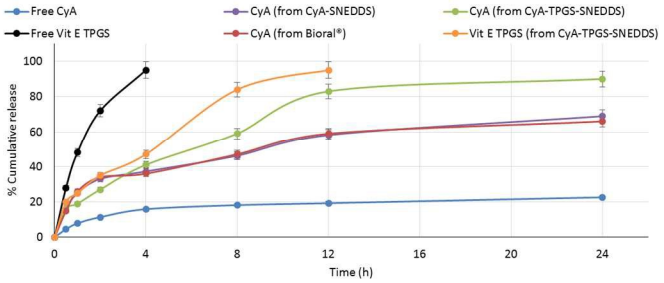


Figure 4: *In vitro* drug release profile of various formulations. Each data point represents Mean±SD (n=6)

3.7 In vivo Pharmacokinetics

Figure 5 represents the plasma concentration time profile of various formulations. The co-administration of Vitamin E TPGS solution along with the free CyA marginally increased the drug plasma concentration of CyA (Table VI). Loading of CyA in the SNEDDS formulation significantly increased the drug plasma concentration as compared to free CyA and free CyA co- administered with Vitamin E TPGS. CyA-SNEDDS significantly increased the total plasma concentration of CyA at all the time points as compared to that of free drug and that co-administered with Vitamin E TPGS. However the plasma concentration time profile of CyA-SNEDDS and Bioral® were found to be comparable. Of note, CyA-TPGS-SNEDDS could significantly increase the oral bioavailability of CyA as compared to that of both CyA-SNEDDS and Bioral®. Overall, 4.48 fold, 1.96-fold and 1.46-fold increase in the AUC was noted in case of developed formulation as compared to that of free CyA, free CyA + Vitamin E TPGS and Bioral®, respectively. Similar appreciation in Cmax was also noted in case of CyA-TPGS-SNEDDS exhibiting 7.04-fold, 3.95-fold and 1.49-fold increase as compared to that of free CyA, free CyA + Vitamin E TPGS and Bioral®, respectively (Table VI).

3.8 CyA induced intracellular reactive oxygen species (ROS) in HEK cell lines

The intracellular ROS generation potential of CyA and capabilities of the vitamin E TPGS to combat the generated ORS were assessed in HEK cell lines to establish the rationale for selected combination. H2DCFDA assay revealed remarkably higher ROS generation within HEK cells treated with CyA alone in contrast to that of CyA + vitamin E TPGS (Figure 6). Interestingly, the ROS generation was also remarkably higher in treatment group of CyA SNEDDS as compared to that of plain CyA, CyA-TPGS SNEDDS and CyA + vitamin E TPGS.

Table VI: Mean pharmacokinetic parameters in various formulations

Formulation	C_{max} (ng/ml)	T_{max} (h)	$AUC_{0-\infty}$(ng/ml-h)	$t_{1/2}$ (h)
Free CyA	1171.85	6	17195.12	7.35
Free CyA + Vit E TPGS	2086.065	6	39325.39	18.05
CyA-SNEDDS	5438.56	6	63335.52	28.55
CyA-TPGS-SNEDDS	8248.56	6	76970.65	32.62
Bioral®	5500.58	6	52677.2	22.4

Values are expressed as mean ± S.E.M (n=5)

3.9 In vivo nephrotoxicity

Remarkable increase in the levels of creatinine and BUN were noted in free CyA treated group suggestive of the potential nephrotoxicity potential of CyA (Figure 7). Statistically significant reduction ($p<0.05$) in the levels of biochemical markers were noted when Vitamin E TPGS was co-administered with free CyA, however the levels still remained quite higher than that of vehicle treated animal group. Noteworthy, no statistical significant difference ($p>0.05$) in the creatinine and BUN levels of CyA-SNEDDS and Bioral® treated animal groups were noted. However, the levels were remarkably higher ($p<0.001$) than control group in both the cases. Interestingly, CyA-TPGS-SNEDDS significantly reduced the BUN levels ($p<0.05$) and Creatinine levels ($p<0.001$) as compared to Bioral® as well as with the free CyA and levels were normalized as compared to that of control group indicative of potential antioxidant potential of Vitamin E TPGS present as functional excipient in the developed system.

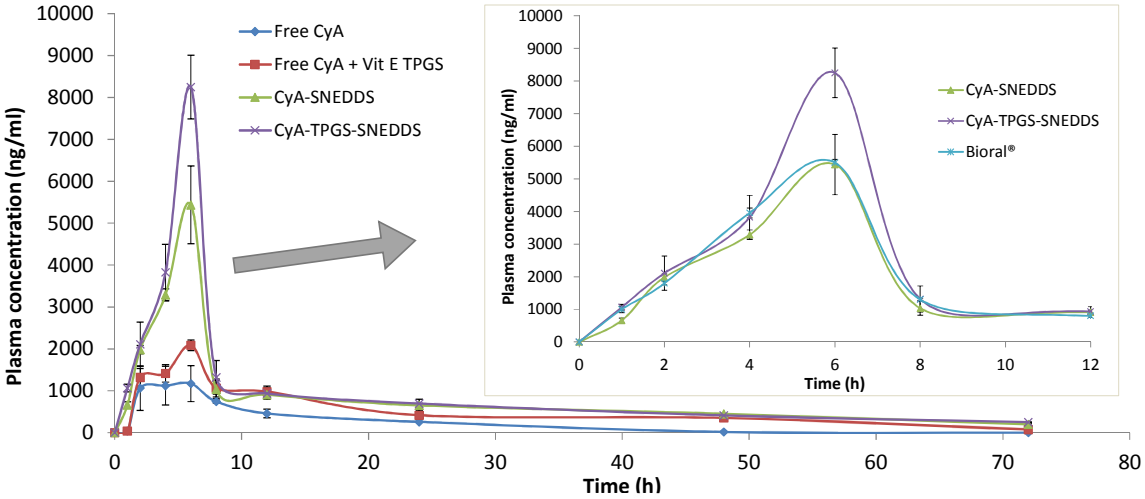


Figure 5: Plasma concentration time profile of various formulations; Each data point represents mean ± S.E.M (n=5)

4. Discussion

Self-nanoemulsifying drug delivery systems (SNEDDS) pose great potential in co-encapsulation of multiple bioactives owing to its high solubilization potential of oil. Solubility studies were extensively carried out to identify the suitable oily phase for rationalized development of SNEDDS. Capmul MCM, being a mixed composition of lower order synthetic glycerides such as caprylic acids preferably solubilized both the drug and functional excipient (antioxidant) in highest amounts (Figure 1). The implementation of synthetic analogues as compared to the natural origin pose

advantages in terms of chemical uniformity and relatively lesser amounts of known impurities. Further, Cremophor RH 40 exhibited excellent solubilizing effects vis-à-vis good emulsification capabilities and hence was chosen as preferred surfactant for further development of SNEDDS (Table I). Subsequently, co-surfactant usually improves upon the PDI of the SNEDDS formulation [25]. The co-surfactant screening studies revealed potential droplet stabilization effects of Labrafil 1944 CS (PEG-8-oleate/linoleate) for the developed formulation in contrast to ethylene glycol, ethanol, transcutol, plurol oleque, etc. and hence was further selected (Table II). Drug loading of the bioactives within the developed formulation was further optimized using pseudo ternary phase diagram. The shaded region within the ternary diagrams represents the nanoemulsification region which yields desired droplet size and PDI. A gradual decrease in the nanoemulsification region was noted from blank formulation to simultaneous co-loading of bioactives reflecting the limited loading capacity of the system (Figure 2). The results were in line with our previous experiences on the development and optimization of SNEDDS [24].

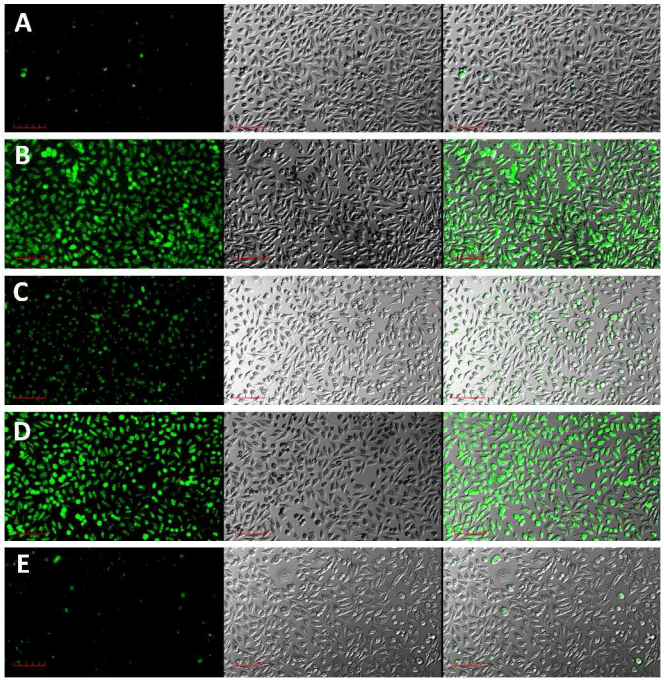


Figure 6: Intracellular ROS generation S. cerevisiae exposed to (A) vehicle treated (B) CyA, (C) CyA + Vitamin E TPGS, (D) CyA SNEDDS and (E) CyA-TPGS SNEDDS. Channel 1 represents the fluorescence emerging from intracellular DCF converted from H_2DCFDA in response to oxidative stress. Channel 2 represents the phase contrast images of cells and Channel 3 represents the overlay of Channel 1 and Channel 2. Scale bar represents 100 µm.

Further, the developed formulation exhibited superior stability in simulated gastrointestinal fluids (Table III) which could be in part attributed to exhaustive optimization and in part inclusion of multifunctional excipient, Vitamin E TPGS, which can act as solubilizer, stabilizer along with therapeutic effects of antioxidants [32]. In addition, the formulation was also found to be stable at long term standing and against temperature fluctuations in freeze thaw cycles (Table IV-V).

Morphological evaluation of the reconstituted nanoemulsion using TEM analysis further revealed the spherical shape of droplets and droplet size being in well accordance with dynamic light scattering results (Figure 3). In vitro drug release studies of the developed formulation revealed some interesting aspects with respect to influence of the release characteristics as a function of physicochemical properties. The drug release of highly lipophilic drug, CyA was enhanced upon incorporation in to SNEDDS formulation. However, no statistical significant difference ($p>0.05$) was noted among drug release of CyA from in-house CyA-SNEDDS and Bioral®. Interestingly, among Bioral® and CyA-TPGS-SNEDDS, the drug release was relatively rapid in later case suggesting strong influence of the formulation components on the physicochemical performance of the system (Figure 4). Further it was also noted that drug release was affected as a function of variety of factors such as droplet size, dilution media, physiochemical properties, localization of drug within the droplet (either within the droplet or at oil-surfactant/water interface), etc. Interestingly, such balancing behavior of the SNEDDS could be correlated to their spontaneity in formation upon exposure to physiological media, which in actual depends upon careful selection of surfactants and co-surfactants in appropriate concentrations, thereby highlighting the significance of exhaustive optimization for development of SNEDDS compositions.

In vivo pharmacokinetic studies were further carried out to assess the delivery potential of the developed formulation. The results were quite evident that CyA (free base) is poorly absorbed owing to its higher lipophilicity (Table VI). Interestingly, co-administration of vitamin E TPGS along with CyA did result in to marginal bioavailability enhancement (Figure 5). However, remarkable appreciation in bioavailability was noted only for developed formulation which could be attributed to the promising role of co-encapsulation strategy in simultaneous delivery of therapeutics with opposite physicochemical properties. The results were in line with our previous results [33, 34]. The key reasons attributed to bioavailability enhancement includes high solubilization potential of SNEDDS, P-gp inhibition effects of vitamin E TPGS, alternate absorption pathway and superior encapsulation of CyA within Nano emulsion droplets [35].

The safety profile of the developed formulation was further assessed in vitro as a function of CyA induced ROS generation within HEK cell lines. CyA induced distress in kidney cell lines is well reported in literature and hence was adopted as a marker to predict the nephrotoxicity potential of the developed formulations [36]. Clinically, acute doses CyA leads to transient reduction in the glomerular filtration rate while chronic administration is reported to induce renal impairment and fibrosis [7]. These detrimental effects have been primarily mediated by series of mediators including ROS [37]. Cell culture studies revealed remarkable potential of vitamin E TPGS in combating the CyA induced intracellular ROS in the developed formulation (CyA TPGS SNEDDS) in contrast to the plain drug combinations and CyA SNEDDS (Figure 6). The results reveal the complexities associated with potent molecule CyA wherein a concomitant approach of bioavailability enhancement and toxicity reduction is desirable. The developed formulation super passes the clinical relevance as compared to that of clinical formulation, BioralTM, wherein only bioavailability enhancement has been addressed.

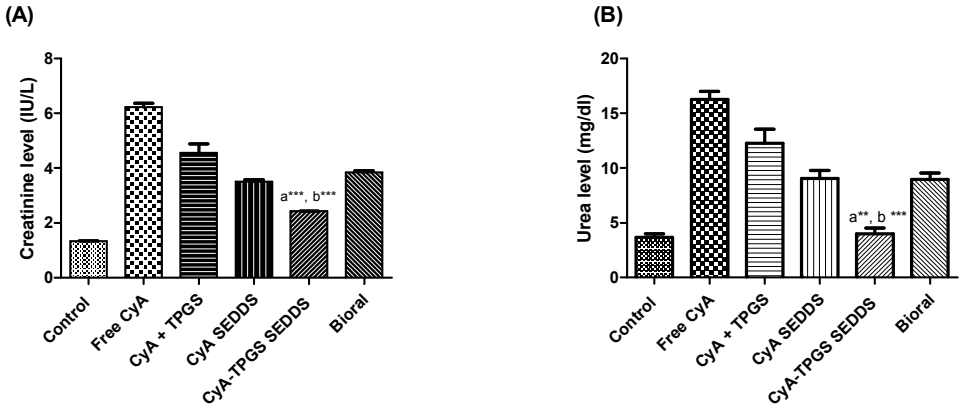


Figure 7: Effect of various formulations on the nephrotoxicity markers (A) Creatinine level (B) BUN level. Each data point represents mean ± S.E.M (n=5)

The potential of the developed formulation was further established at pre-clinical level by assessing the levels of nephrotoxicity biochemical markers in animals treated with various CyA formulations. The results revealed significant reduction in these parameters in case of CyA-TPGS-SNEDDS as compared to marketed formulation (Bioral®) as well as with the free CyA. (Figure 7). The observed advantages with the developed formulation could be attributed to the potent antioxidant of Vitamin E TPGS, playing critical role in arachidonic acid metabolism mediated by processes such as prostaglandin formation, affecting the release of arachidonic acid and alterations in the lipoxygenase activities.

5. Conclusions

The developed formulation pose great potential in increasing the oral bioavailability and reducing the toxicity of CyA induced nephrotoxicity. Further, the said formulation also offers unique advantages high industrial adaptability and relatively lower scalability challenges. The conversion of solid dosage form of the said formulation could be further line of action for remarkable improvement in the pharmaceutical stability of the dosage form. Further, multiple dose kinetics can sought for deeper insights on the delivery potential of the formulation.

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7. Notes and references

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