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A Novel Lipid-based Oral Drug Delivery System of Nevirapine

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Abstract: Nevirapine has been formulated in lipid-based system, a Self Emulsifying Drug Delivery System (SEDDS) to target the drug to lymphoid organs where HIV-1 virus resides in large population. The formulation of nevirapine SEDDS was optimized by solubility assay and pseudo-ternary phase diagram analysis. The optimum formulation consisted of 8.56% of Caprylic acid oil, 74.50% of surfactant Soluphor P and 16.93% of co-surfactant Transcutol P. The SEDDS were characterized by Transmission Electron Microscopy (TEM) observation, droplet size determination, cloud point measurement, *in vitro* diffusion and *ex vivo* intestinal permeability studies. Based on the distribution data obtained by Photon Correlation Spectroscopy (PCS) the morphology of droplet appears to be spherical in the size range of less than 200 nm. Cloud point determination demonstrated the efficiency of the formulation to self emulsify at physiological pH and temperature. *In vitro* diffusion study showed 99.18 ± 2.85% release of nevirapine from SEDDS as compared to 65.14 ± 2.98% from the marketed suspension, approximately in 5 hours. The *ex vivo* intestinal permeability of nevirapine was 69 ± 4.58% and 57 ± 6.77%, respectively from SEDDS and marketed suspension. Nevirapine in SEDDS had higher *ex vivo* intestinal permeability than the marketed conventional suspension, suggesting that the SEDDS may be a useful delivery system for targeting nevirapine to lymphoid organs.
Key words: Nevirapine; self emulsifying drug delivery system; anti-HIV; pseudoternary phase diagram; droplet size; intestinal permeation.

Introduction
AIDS, caused by the HIV virus, is one of the world's leading causes of death with a major medical and economic impact on a society. Lymphoid tissue constitutes the major reservoir of virus and infected cells in HIV-infected patients.[1,2] HIV, generally, enters the human host via mucosal surfaces and is subsequently disseminated throughout the lymphatic tissues, a major reservoir of virus throughout the course of infection.[3-5] Although lymphoid tissues are considered the primary site of CD4+ T cell infection over the course of the disease, follicular dendritic cells (FDCs) are the major source of viral RNA in lymphoid tissue. Measurement of viral pools in lymphoid tissue during the asymptomatic phase of infection has revealed an extremely large FDC pool.[6]

Nevirapine is a non-nucleoside reverse transcriptase inhibitor of human immunodeficiency virus, type 1 (HIV-1). The drug is practically insoluble in water (0.1 mg/ml) with a log octanol–water partition

coefficient (log P) = 2.5 and pKa = 2.8.[7,8] A self emulsifying drug delivery system (SEDDS) is defined as an isotropic mixtures of oil, surfactant, co-surfactant and the drug substance. Upon mixing with water the system has an ability to form fine colloidal droplets with very high surface area. In many cases, this accelerates the digestion of the lipid formulation, improves absorption, and reduces food effect and inter-subject variability.[9-12] The self-emulsification process occurs spontaneously because the free energy required to form the microemulsion is either low and positive or negative.[9] The self-emulsification process was shown to be dependent on the lipid/surfactant pair, the surfactant concentration, the ratio between lipid and surfactant.[13,14] However, only specific combinations can lead to efficient self-emulsifying system.[15,16] Drug substances with adequate solubility in lipid/surfactants blends are suitable candidates for this formulation concept.[17] The SEDDS are believed to be superior compared with lipid solutions due to the presence of surfactants in the formulations leading to a more uniform and reproducible bioavailability as seen for cyclosporine.[18]

This delivery system can specifically target nevirapine to the lymphatic system where the viral load remains very high even in presence of high drug concentration in the circulating blood system.[19,20] Targeting lymphatic system is based on the concept that the drug in the lipid-based system reaches specifically the lymphatic system, bypassing first pass hepatic metabolism, thus resulting in high drug concentration in it.[21]

Keeping this objective in mind, an attempt has been made to develop lipid-based formulation of nevirapine in a system called self emulsifying drug delivery system which would improve the solubility and absorption of drug via intestinal lymphatics.

Lipid-based drug delivery systems have gained considerable interest after the commercial success of Sandimmune Neoral[TM] (Cyclosporine A), Fortovase (Saquinavir) and Norvir (Ritonavir). Much attention has been on SMEDDS/SEDDS; an increase in bioavailability was found for L-365,260[22], WIN 54954[23], Ro 15-0778[16], ontazolast[24], halofantrine[25] and danazol[26] when administered in self-emulsifying systems, compared to solid dosage forms.

Materials and methods
Materials
Nevirapine was a generous gift from Matrix Laboratories Ltd. (Hyderabad, India), Caprylic and Oleic acids were obtained from Soofi Traders (Mumbai, India); Polyoxyl 35 castor oil (Cremophor EL) and 2-Pyrrolidone (Soluphor P®) were obtained from BASF Corp. (Mumbai, India). Diethylene glycol monoethyl ether (Transcutol P®), Propylene glycol

monocaprylate (Capryol 90®) were provided by Gattefosse, France and Glycerol Monocaprylocaprate (Capmul MCM®) was provided by Abitec Corp., Janesville WI. PEG-400, Tween 20, Tween 80 and Propylene glycol were purchased from S D Fine chemicals (Mumbai, India). All other chemicals and solvents used were of analytical grade.

Solubility studies
The solubility of nevirapine in various oils, surfactants and co-surfactants was determined. An excess of nevirapine (approximately 500 mg) was placed in 2 ml of a vehicle in a screw-capped glass vial and the mixture was heated at 60°C in a water-bath (Equitron, Chennai, India) to facilitate the solubilisation using a cyclomixer (Remi Instrument Ltd., Mumbai, India). Mixtures were equilibrated at 30°C for 48 h in a water bath and then centrifuged in a laboratory centrifuge (Sorvall Biofuge Primo R Centrifuge, Thermo Electron Corp. USA) at 2000 rpm for 10 min to separate the undissolved drug. Aliquots of supernatant were diluted with methanol and analysed for the dissolved drug by HPLC (Jasco, Japan), using RP column (LCGC Qualisil BDS C18; 5 µm 250 mm x 4.6 mm i.d) and methanol: water (50:50) as a mobile phase.[27,28]

Construction of pseudo-ternary phase diagrams
In order to find out the concentration range of components for the existing range of microemulsions, pseudo-ternary phase diagram was constructed using the water titration method.[29] Ternary plots were constructed using Caprylic acid, Soluphor P and Transcutol P as oil, surfactant and co-surfactant respectively, in different ratios of Soluphor P : Transcutol P (1:4, 1:3, 1:2 1:1 and 4:1 w/w) with Caprylic acid in ratios of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1 w/w.

The mixtures of oil and surfactant at certain weight ratios were diluted with water, under moderate stirring. After being equilibrated, the mixtures were assessed visually and determined as being microemulsions or coarse emulsions. The data obtained was subjected to Tri plot v1.4 software for fabrication of ternary plot.

Preparation of nevirapine self emulsifying drug delivery system
SEEDS formulation was developed based on the microemulsion regions and maximum amount of drug that can be solubilised in a particular ratio of surfactant and co-surfactant with Caprylic acid meeting the desired criteria for formation of microemulsion after dispersing in aqueous media. The developed formulation consisted of nevirapine: caprylic acid: Soluphor P: Transcutol P (4.5: 8.17: 71.16: 16.17) (%, w/w). The formulation was prepared by dissolving the

nevirapine in the mixture of Transcutol P and Soluphor P at 50°C in a water bath. Caprylic acid was then added. This mixture was mixed by vortexing until a transparent preparation was obtained. The prepared nevirapine SEDDS can be delivered in hard gelatin capsule.

Characterization of self emulsifying drug delivery system

Drug content
SEDDS formulation equivalent to 50 mg of nevirapine was taken and diluted in acidified methanol (1% v/v 35% HCl). Volume was made up to 50 ml with acidified methanol (1mg/ml). From the above stock solution, 0.2 ml (200 mcg/ml) was withdrawn and diluted up to 10 ml with acidified methanol (20 mcg/ml). Samples were prepared in triplicate and absorbance measured at 313 nm using UV-Visible Spectrophotometer (Shimadzu UV-2450, Japan).[28,30] Placebo sample was also treated in the same way to check the interference, if any. Acidified methanol was used as a reference solution.

Spectroscopic characterization of optical clarity
The optical clarity of aqueous dispersions of SEDDS formulation was measured spectrophotometrically. Composition was prepared according to the design and diluted to 100 times with distilled water and Simulated Gastric Fluid (USP 30).[31] The % transmittance of solution was measured at 650 nm, using distilled water as a reference.[32-34]

Morphological characterization
The morphology of self emulsifying formulation was observed by using a transmission electron microscope (TEM) (Phillips Tecnai 20, Holland) at an acceleration voltage of 200 kV and typically viewed at a magnification of 43,000×. The size of the colloidal structures was determined using AnalySIS® software (Soft Imaging Systems, Reutlingen, Germany).

Formulation was diluted with distilled water 1:25 and shaken. Carbon-coated copper grids were glow-discharged (Edwards E306A Vacuum Coater, England) and 10 µl of sample adsorbed on to these holey film grid and observed after drying.

Determination of droplet size
Droplet size of the formed emulsion was determined by photon correlation spectroscopy that analyzes the fluctuations in light scattering due to Brownian motion of the particles, using a Zetasizer ZS 90 (Malvern Instruments, UK). Light scattering was monitored at 25°C at a 90° angle. All the measurements were performed at room temperature (20°C) in triplicate.

In vitro diffusion study
In vitro diffusion study of the nevirapine SEDDS was compared with a conventional marketed suspension (Nevimune®, Nevirapine Oral Suspension, CIPLA) using a dialysis technique. The dialyzing medium was 0.1N HCl. One end of dialysis tubing (Dialysis membrane 70, HIMEDIA; MWCO 12,000-14,000 daltons; pore size: 2.4 nm) was clamped and then the experimental formulation sample, equivalent to 100 mg drug, was placed in it. The other end of the tubing was also secured with clamp and was placed in 900 ml of dialyzing medium and stirred at 100 rpm over a magnetic stirrer (Remi Instrument Ltd., Mumbai, India) at 37°C. Aliquots of 1 ml were removed at 15, 30, 45, 60, 120, 180, 240 and 300 min time intervals and suitably diluted further. Each time the volume of aliquots was replaced with the fresh dialyzing medium. These samples were analyzed for nevirapine present in the dialyzing medium at corresponding time by UV-visible spectrophotometer at 313 nm.

Ex-vivo intestinal permeability studies
Male Sprague- Dawley rats (250-300 g) were euthanized in carbon-dioxide vacuum chamber. All experiments and protocols described in this animal study were approved by the Institutional Animal Ethics Committee of B V Patel PERD Centre, Ahmedabad, India and were in accordance with the guidelines of the Committee for Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India. To check the intestinal permeability, small portion of small intestine was isolated and used for the *ex vivo* permeability study. The tissue was thoroughly washed with pH 7.4 phosphate-buffered saline (USP 30)[35] to remove any mucous and lumen contents. The nevirapine SEDDS, 0.5g (equivalent to 25mg nevirapine) and conventional marketed suspension, 1ml (equivalent to 10 mg nevirapine) were diluted upto 10 ml and 4 ml with distilled water, respectively and mixed over cyclomixer for 1 minute. The resultant samples (2.5 mg/ml) of both nevirapine SEDDS and conventional marketed suspension were injected separately into the lumen of the small intestine tissue using a syringe, and the two sides of the intestine were tightly closed with the help of a thread. The tissue was placed in a beaker filled with 30 ml of pH 7.4 phosphate-buffered saline containing 20% PEG-400 with constant stirring at 37°C. The two ends of tissues were fixed horizontally on to a beaker with the help of a thread. Aliquots of 3 ml were withdrawn at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4 and 5 hours time intervals and suitably diluted further. The absorbance was measured using a UV-Visible spectrophotometer at a wavelength of 283 nm.[36,37] The amount of drug diffused (%) was calculated against time and plotted on a graph.

Statistical analysis

Both *in vitro* diffusion and the *ex vivo* intestinal permeability experiments were repeated six times and data expressed as the mean value ± SD. Statistical analysis for the determination of differences in diffusion and permeability profiles of nevirapine SEDDS and the marketed preparation was assessed by the use of Student's *t*-test. Statistical probability (*P*) values less than 0.05 were considered significantly different.

Results and discussion

Screening of oils and surfactants

Acceptable SEDDS formulation should be simple, safe, compatible and possess efficient droplet size after forming a microemulsion.[9,32,38,39] The appropriate vehicles should have good solubilizing capacity for the drug substance, which is essential for composing a SEDDS. The solubility of nevirapine in various vehicles is shown in Table 1. The components and their concentration ranges can be obtained by the construction of a pseudo-ternary phase diagram with constant drug level fixed at 4.5% (w/w). The drug loading capability is the main factor when screening the oil phase.

Nevirapine has the highest solubility in Caprylic acid as shown in Table 1 and was, therefore selected as an oil phase in the present study. On the

basis of the solubility profile Soluphor P was considered as a surfactant and Transcutol P as a co-surfactant.

Table 1 Solubility of nevirapine in various vehicles at 25°C (*n*=3)

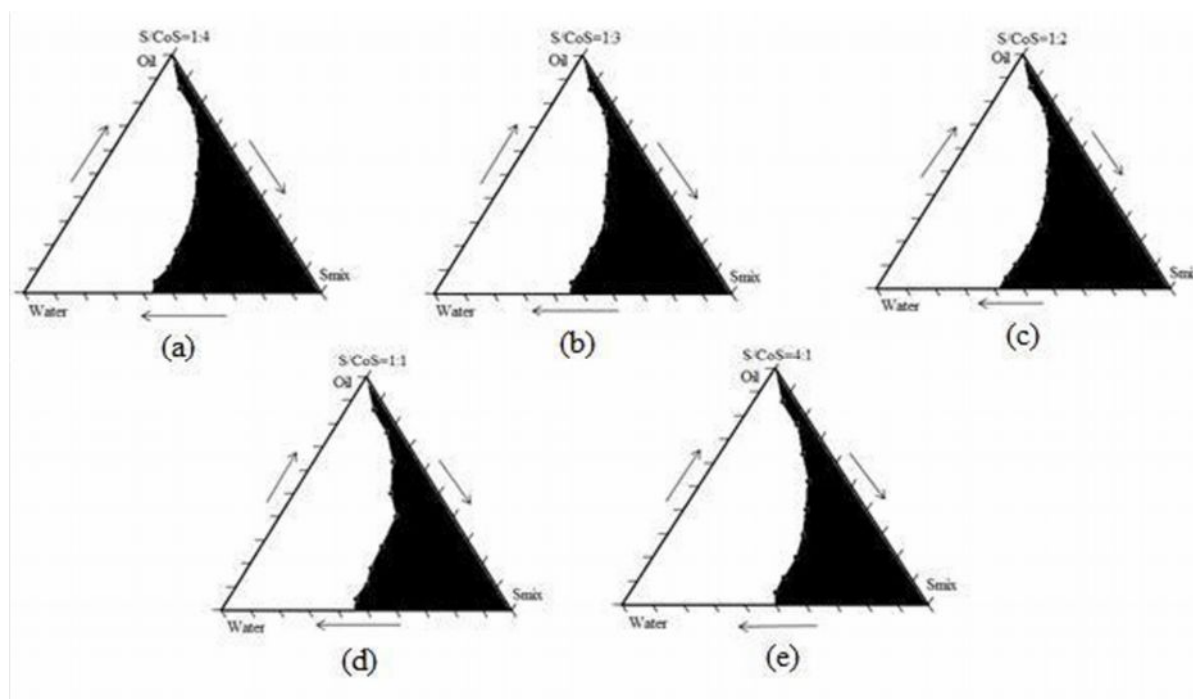| Vehicles	Solubility (mg/ml ± SD)
Oils	
Oleic acid	12.22 ± 2.98
Caprylic acid	20.88 ± 2.84
Capryol 90	3.337 ± 1.09
Capmul MCM	6.095 ± 1.98
Surfactants	
Cremophor EL	1.077 ± 0.47
Tween 20	2.76 ± 0.86
Tween 80	2.76 ± 0.86
Transcutol P	5.086 ± 2.04
Soluphor P	22.178 ± 3.46

Figure 1 Pseudo-ternary phase diagrams of the formulations composed of oil, surfactants and co-surfactant dispersed with distilled water at 37 ∘C. Surfactants = Soluphor P; Co-surfactant = Transcutol P . Surfactant to co-surfactant ratios (S/CoS) were as follows: (a) 1:4, (b) 1:3, (c) 1:2, (d) 1:1, (e) 4:1. The shaded area represents microemulsion region.

Pseudo ternary phase diagrams

Phase diagrams of the systems containing Caprylic acid as an oil phase, Soluphor P as a surfactant and Transcutol P as a co-surfactant were constructed at the surfactant /co-surfactant ratio of 1:4, 1:3, 1:2, 1:1 and 4:1 (w/w) to determine the existence of microemulsion region as shown in Figure 1. The phase study revealed that the obtained microemulsion regions at surfactant/ co-surfactant ratios of 1:4 and 1:2 (Figure 1(a) and (c)) were similar with maximum of 4.65% oil incorporation. At surfactant/ co-surfactant ratios of 1:3 and 1:1, about 4.88% of oil can be incorporated and gave the same microemulsion regions (Figure 1(b) and (d)). The ratio 4:1 of S/Cos showed maximum drug solubilisation capacity with 6% of oil incorporation (Figure 1(e)). It was observed that there was an improvement in the solubilisation of nevirapine with an increase in the proportion of the Soluphor P. Based on our results, SEDDS formulation comprised of 8.56 % oil Caprylic acid, 74.50% of Soluphor P as surfactant and 16.93% of Transcutol P as co-surfactant. This selected composition of oil and surfactants should readily form microemulsion in the body on dilution with physiological fluids at 37°C.

Characterization of self emulsifying drug delivery system

Drug content

Assay of prepared nevirapine SEDDS was carried out by UV-visible spectrophotometer and found to be in the range of 100.9-105.1% with a standard deviation of ± 2.124 %, indicating uniform dispersion of droplets.

Spectroscopic characterization of optical clarity

SEDDS was diluted with water to confirm the formation of microemulsion with the external phase of the system without phase separation. In order to asses the optical clarity quantitatively; UV-visible spectrophotometer was used to measure the transmitted light at 650 nm wavelength transmitted by the solution. Higher transmittance should be obtained with optically clear solutions, since cloudier solutions will scatter more of the incident radiation, resulting in lower transmittance. Aqueous dispersions with small absorbance are optically clear and oil droplets are thought to be in a state of finer dispersion. A clear o/w micro-emulsion was formed in both the dilution media. On 100 fold dilution percent transmittance of the studied aqueous dispersion of nevirapine SEDDS was found to be 99.187 with distilled water and 98.158 with Simulated Gastric Fluid.

Morphological characterization

The nevirapine SEDDS turned into microemulsion when diluted with distilled water. The TEM picture is shown in Figure 2. The microemulsion droplets were observed to be spherical and homogenous with large population of the smaller droplet in the size range of less than 200 nm. The droplet size of formed emulsion determined by TEM is in correlation with that of measured by photon correlation spectroscopy (PCS).
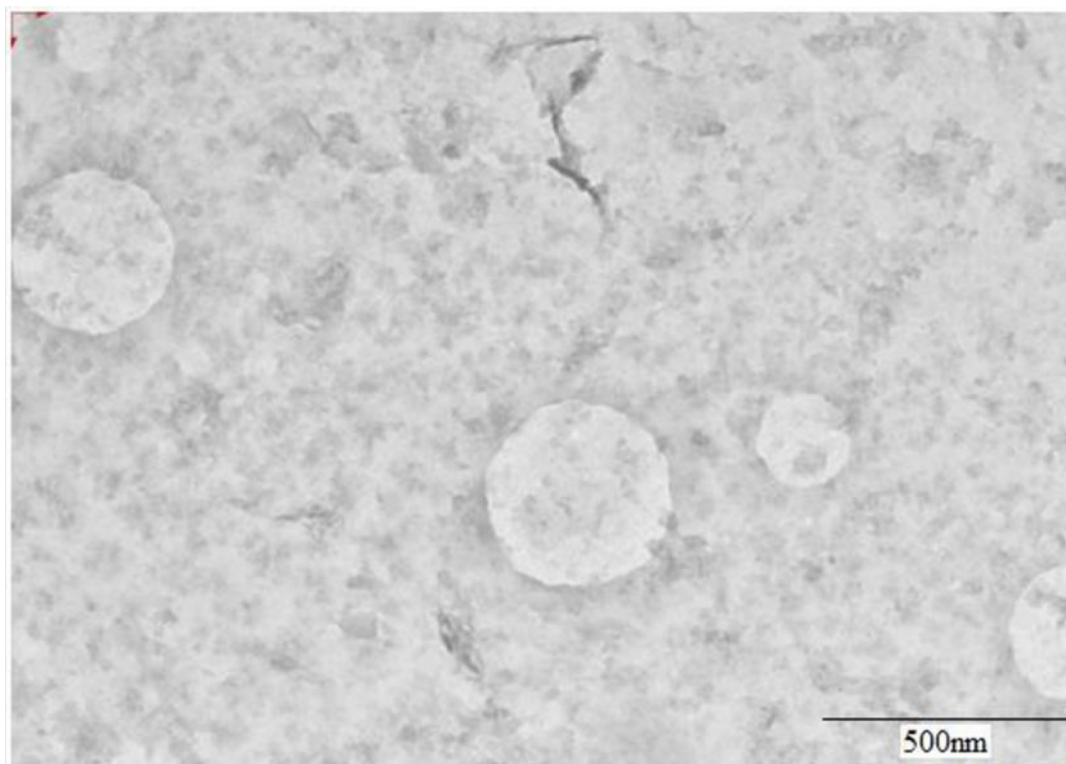

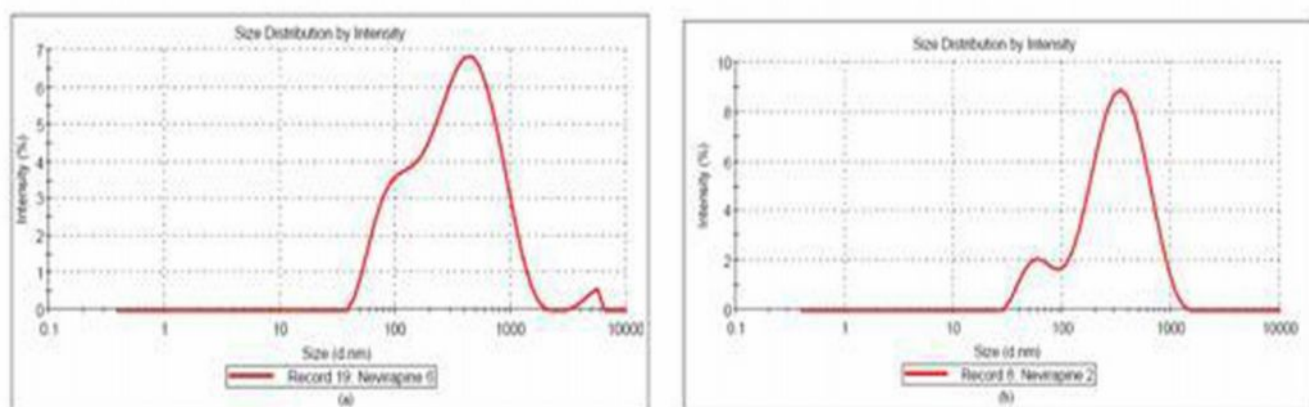**Figure 2** TEM photo of nevirapine microemulsion (x 43,000) (Scale: 500 nm)

Figure 3 Droplet size distribution after dilution in a) Distilled Water and b) Simulated Gastric Fluid

Droplet size analysis

Droplet size distribution following self-emulsification is a critical factor to evaluate a self-emulsifying system. The smaller the droplet size, the larger the interfacial surface area will be provided for drug absorption.[12,17,40] In our study, we investigated the effect of distilled water and Simulated Gastric Fluid as two different dilution media on droplet size. The average droplet size of microemulsion dispersed from the nevirapine SEDDS after 100 times dilution was found to be 203.5 ± 2 nm in distilled water and 198.6 ± 1.76 nm in Simulated Gastric Fluid with Gaussian distribution (Figure 3). The polydispersity of microemulsion dispersed from the nevirapine SEDDS was found to be 0.514 and 0.534 in distilled water and Simulated Gastric Fluid, respectively. There is only a marginal difference in the mean droplet size and polydispersity when nevirapine SEDDS dispersed in distilled water and Simulated Gastric Fluid suggesting uniformity of droplet size of the formed microemulsion. This indicated that the investigated dilution media have no impact on the droplet size and its distribution. This suggests that nevirapine SEDDS when taken orally it will form a microemulsion with uniform distribution of globules under normal GIT physiological environment.

Cloud point measurement

The cloud point is the temperature above which an aqueous solution of water soluble surfactant, especially non-ionic, becomes turbid. It is an indicator of the successful formation of a stable microemulsion.[41] When the temperature is higher than the cloud point, an irreversible phase separation will occur and the cloudiness of the preparation would have a bad effect on drug absorption, because of the dehydration of the polyethylene oxide moiety. Hence, the cloud point for SEDDS should be above 37°C, which will avoid phase separation occurring in the gastrointestinal tract. Nevirapine SEDDS was diluted with water in the ratio of 1:250, and the sample was placed in a water bath with the temperature increasing gradually, at 5°C intervals (or at 2 °C intervals when approaching the cloud point), spectrophotometric analysis was carried out to measure the sample transmittance using an empty glass cuvette as a blank. The cloud point of the SEDDS was found to be 62°C, suggesting formulation of a stable microemulsion of nevirapine at the body temperature.[34]

In vitro diffusion study

In vitro diffusion study was performed to compare the drug release form the developed nevirapine SEDDS and conventional marketed suspension. Nearly 41.45 ± 2.03 % of drug was released from nevirapine SEDDS within 1 hour compared to the marketed suspension which released 21.04 ± 1.81% of the drug. At the end of five hour period, almost all the drug (98.18 ± 2.81%) diffused from the SEDDS formulation compared to 65.13 ± 2.98% drug released from the conventional marketed suspension (Figure 4). Thus, the drug release from the nevirapine SEDDS was found to be significantly higher (P < 0.05) as compared to that of the marketed suspension. It could be suggested that the SEDDS formulation resulted in spontaneous formation of a microemulsion with a small droplet size, which permitted a faster rate of drug release into the aqueous phase. Thus, this greater availability of dissolved nevirapine from the SEDDS formulation could lead to higher absorption and higher oral bioavailability.
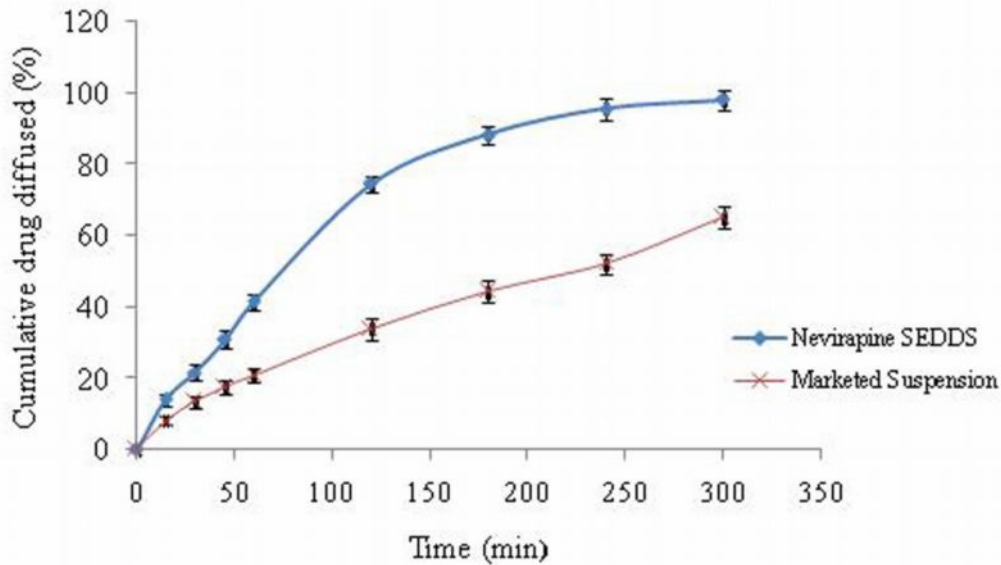
Figure 4 *In vitro* diffusion profile of Nevirapine SEDDS and Marketed Suspension in 0.1N HCl
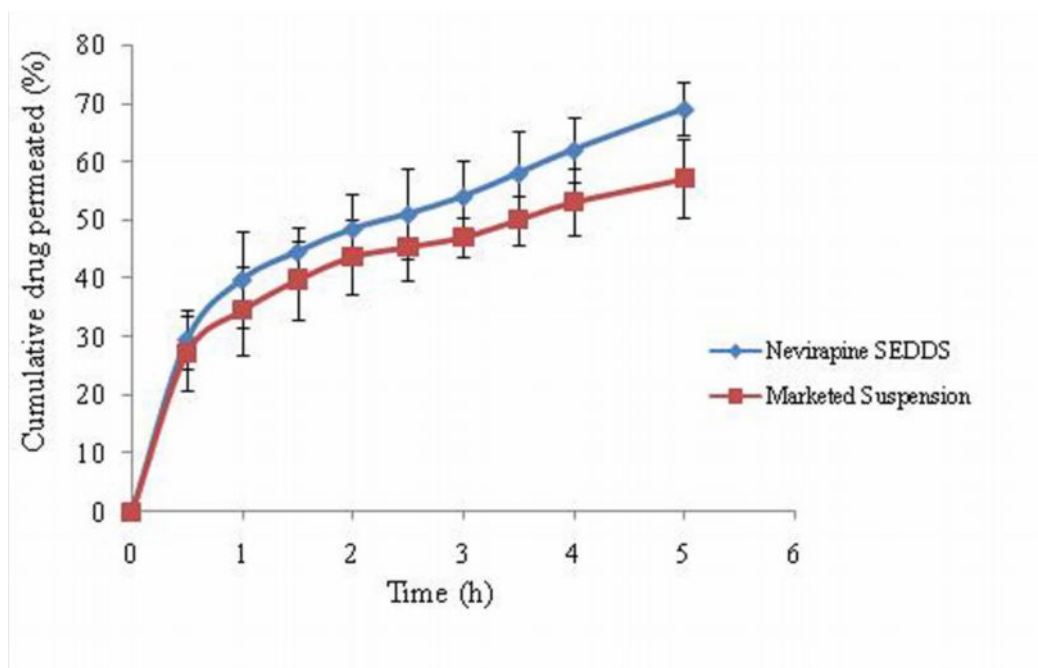

Figure 5 *Ex vivo* intestinal permeation profile of Nevirapine SEDDS and Marketed Suspension in pH 7.4 PBS

Ex-vivo intestinal permeability study

To understand the characteristics of drug permeation, *ex vivo* intestinal tissue permeation study was carried out across the small intestine of male Sprague-Dawley rats.[42-45] The profile of drug permeation is shown in Figure 5. In the first half hour, the permeation of drug through intestinal membrane from the nevirapine SEDDS and marketed suspension was 29.55 ± 5.16% and 27.22 ± 6.32%, respectively. More than 50 % of the drug permeated across the intestine tissue within 2 hours from nevirapine SEDDS. On the other hand it took 3 hours for the marketed suspension. After 3 hours of permeation, more than 69% of the drug was accumulated from the SEDDS, as compared to 57% from the marketed suspension. The total percentage permeation from rat intestine was significantly higher (P < 0.05) for the nevirapine SEDDS than for the marketed suspension.

The main mechanism reported to improve the oral absorption of lipophilic drugs when incorporated into SEDDS include, increasing membrane fluidity, opening of tight cellular junctions, inhibiting P-gp and/or CYP450 by surfactants and stimulating lipoprotein/chylomicron production by lipid.[10,46] The better permeation observed with the developed nevirapine SEDDS formulation might be due to the higher surfactant content that could have made the intestinal wall more permeable by partial disruption of membrane. Oral absorption of the SEDDS and micelles showed that the surfactant-induced membrane fluidity and inhibition of P-gp might play an important role in the permeability change and the increase of drug absorption in the gastrointestinal tract.[39,47]

Conclusions

Our studies highlighted the potential of using SEDDS as an efficient strategy for the oral delivery of hydrophobic nevirapine. Since nearly 40% of drugs are hydrophobic, SEDDS can be a promising formulation approach for improving oral bioavailability of drugs with poor aqueous solubility.[48] A SEDDS containing poorly water-soluble drug, nevirapine, was formulated for oral administration. The components and their ratio ranges for the formulation of SEDDS were obtained by solubility study, pseudo-ternary phase diagram construction, and droplet size analysis. The optimum formulation of the SEDDS consisted of 8.56 % of Caprylic acid as oil, 74.50% of Soluphor P as surfactant and 16.93% of Transcutol P as co-surfactant., which had sufficient drug loading, rapid self-emulsification in aqueous media, and forming droplet size in the range of microemulsion. Our study indicated that the developed nevirapine SEDDS formulation showed greater diffusion and intestinal permeability than the marketed suspension. The results obtained suggested that the developed nevirapine SEDDS could form a fine microemulsion which might enhance the accumulation in Peyer's patch for lymphatic transport of the drug.[25] The developed formulation is expected to be a welcome addition to the clinical arsenal for achieving better therapeutic concentration of nevirapine in lymphoid organs and effective control of the viral load in HIV-infected patients.

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