JOURNAL OF PHARMACEUTICAL SCIENCE AND BIOSCIENTIFIC RESEARCH (JPSBR)

(An International Peer Reviewed Pharmaceutical Journal that Encourages Innovation and Creativities)

# Formulation and Evaluation of Simvastatin SEDDS

**Prajapati Paresh A[1]. Maheshwari Mittal M.[2]*,**

**1. Department of Pharmaceutics, Shankersinh Vaghela Bapu Institute of Pharmacy, Vasana, Gandhinagar, Gujarat, India**

**2. Research Scholar, JJT University, Jhunjhunu, Rajasthan, India**

**Article history:**

Received 31 Jan 2016
Revised 28 Feb 2016
Accepted 20 March 2016
Available online 01 March 2016

Citation: Maheshwari M. M., Prajapati P. A. Formulation and Evaluation of Simvastatin SEDDS J Pharm Sci Bioscientific Res 2016. 6(2):213-219

***For Correspondence:**

Mittal Maheshwari

Research Scholar, JJT University, Jhunjhunu, Rajasthan, India

**ABSTRACT:**

The present study was done to formulate the self emulsifying drug delivery system of Simvastatin, a lipophilic drug for improvement in in-vitro dissolution and thereby oral bioavailability. Final optimized formulation consisted of oil (oleic acid), surfactant (Tween 80) and co-surfactant (Capmul MCM) and solid carrier (Neusilin US2) for adsorption. At first, calibration curve of simvastatin was generated, and then based on saturation solubility studies in different vehicles; oil, surfactant and co-surfactant were screened. Pseudo ternary phase diagrams were used to get micro emulsion region, based on which liquid self emulsifying drug delivery systems of simvastatin were formulated. They were characterized by macroscopic evaluation by visual assessment, self-emulsification time, % Transmittance, globule size, zeta potential, and thermodynamic stability study. In vitro drug dissolution of optimized liquid self emulsifying formulation showed that % release of simvastatin found 99.76±0.21 within 30 min which was comparable to marketed tablet of simvastatin 70.37±0.45. The globule size found was 14.69 nm from the optimized Liquid self emulsifying formulation. Optimized liquid self emulsifying formulation was successfully solidified using carrier Neusilin US2 by adsorption technique. Solid SEDDS was characterized by flow property, particle size, zeta potential measurement, % Transmittance, % drug content and in-vitro dissolution test and found that there was not much difference in dissolution behavior of solid and liquid SEDDS. Even it passed stability studies. So it was concluded that solid self emulsifying drug delivery system was successfully developed to improve its rate and extent of dissolution.

**KEYWORDS**: Simvastatin, BCS class II drug, Self emulsifying drug delivery system, dissolution

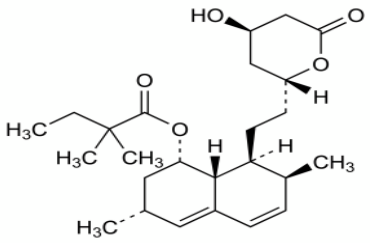
## INTRODUCTION:

Simvastatin is a crystalline and non-hygroscopic powder. Its IUPAC name is (1S,3R,7S,8S,8aR)-8-{2-[(2R,4R)-4-hydroxy-6-oxooxan-2-yl]ethyl}-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate. Its chemical formula is C25H38O5.Its melting point is 135-138 °C. [1][2]

Simvastatin is HMG co-A reductase (3-hydroxy-3-methyl-glutaryl coenzyme A) enzyme competitive inhibitor. Simvastatin is metabolized via hydrolysis by cytochrome-3A system to β-dihydroxy acid (metabolite, structure similarity with HMG co-A), compete with hepatic enzyme HMG co-A reductase. Reduction in enzyme activity leads to inhibition of mevalonate (cholesterol synthesis precursor) in the liver, here main step of cholesterol synthesis is arrested and even LDL cholesterol catabolism is increased via hepatic LDL receptors induction. It is a lipid lowering and hypolipidemic drug and is widely used in treatment hypercholesterolemia orally.[3]

According to Biopharmaceutical Classification system, Simvastatin belongs to Class II drug list having low solubility and high permeability. Its bioavailability is approximately 1%. Its log P value is 4.68, means highly lipophilic drug.[1]it is insoluble in water practically

0.0013-0.0015 mg/ml at 23°C, so it is poorly absorbed from GIT and absorption is dissolution rate limited. It shows high variability in pharmacological effects. Therefore, it's a need to improve its solubility and rate of dissolution of simvastatin for improvement in oral bioavailability. So attempt to increase the solubility by lipid based approach to be made.[4,5]



**Structure of simvastatin**

Lipid based formulation, especially self emulsifying system was chosen to exhibited major role in overcoming the problems related to oral delivery of lipophilic compounds. Self emulsifying systems are isotropic mixtures of drug, oil, surfactant and co-surfactant, which forms O/W micro emulsion in contact with aqueous gastric medium with a very low globule size ranges in nm. They give additional advantages like production ease, physical and thermodynamic stability and improvement in solubility and its bioavailability. Facilitation of para-cellular and trans cellular absorption, protection of drug from hepatic first pass metabolism and reduction of cytochrome p450 metabolism are some different mechanism for this system. Liquid self-emulsifying systems have disadvantages like chemical instability, leakage from hard gelatin capsule, precipitation of drug; these drawbacks can be overcome by converting the liquid system to solid by adsorption onto solid carrier without disturbing its self-emulsification performances.

Here, the main objective of work was to (1) formulate and characterize the liquid self emulsifying formulation of poorly soluble simvastatin to improve its solubility and (2) the solidification of optimized liquid self emulsifying system of simvastatin by adsorption onto carrier without affecting its self emulsification performance and release rate.

## MATERIALS AND METHODS:

Simvastatin was gifted by Ajanta pharmaceuticals Ltd, Mumbai, India. Polyglycolized glycerides vehicles like Labrafil M2125CS, Labrafac CC, Labrasol, Lauroglycol 90, Capryol 340 were gifted from Gatteffose, France. Captex 100, Captex 200, Captex 355**,** Capmul MCM, Peceol, Acconon MC 82 were gifted by ABITEC Co., USA. Cremophore EL was a generous gift sample from Astron research centre, Ahmedabad. Cremophore RH 40 was gifted by BASF Co., Germany. Olive oil, Soyabean oil, Sunflower oil, Peanut oil, Cottonseed oil, Sesame oil were provided by Bright lab, Hyderabad. Oleic acid, Tween 80, Tween 20, span 20, span 80, Propylene glycol, PEG 400 were purchased from S.D. fine chemicals, Mumbai. Acrysol K-140 was gifted by Corel Pharma, Ahmedabad. Neusilin US2 was generous gift from Jay Radhe sales, Ahmedabad. Hard gelatin capsules were provided by Zydus research centre, Ahmedabad. Other chemicals were of analytical grade. All solvents used in the study were also of analytical grade. Fresh distilled water was used in the study.

## METHODOLOGY

- **UV SPECTROMETRY ANALYSIS OF SIMVASTATIN:[6,7]**

Calibration curve of simvastatin in methanol: 100 mg of Simvastatin was accurately weighed taken in 100 ml volumetric flask and dissolved in 100 ml of methanol to get stock solution of 1000 µg/ml (1 mg/ml).From this, pipetted out 10 ml (1mg /ml) was further diluted with same solvent to obtain 100 µg/ml solution. The aliquot was scanned in the wavelength range of 200 to 400 nm on UV spectrophotometer to determine the wavelength of maximum absorbance, which was found at 238 nm. Working standard solutions having concentration of 2-20µg/ml were prepared by diluting 100 µg/ml with methanol. The absorbance of each working standard solution was measured at absorption maxima in UV spectrophotometer using methanol as a blank. Absorbances were plotted against concentration using Microsoft Excel. Beer's law was obeyed in the range of 2-20 µg/ml. same calibration curve tobe plotted in pH 7.0 phosphate buffer also.

- **SATURATED SOLUBILITY STUDIES:[8]**

Saturation solubility study of Simvastatin in various vehicles like oils, surfactants and co-surfactants were done in triplicates. Excess amount of simvastatin (drug) was added into 2 ml of each selected individual vehicle contained in stoppered vials separately and after sealing, the mixture was heated at 40 °C in a water-bath to facilitate the solubilization using a vortex mixer. Then the mixtures in vials were then shaken using shaker at 37°C±1°C for 48 hour then allowed them to reach equilibrium. The equilibrated samples in each vial were centrifuged (5000 rpm) for 5-10 min to separate the undissolved drug and the supernatants of oil were taken out and filtered through a membrane filter (0.45µm,13mm, Whatman, India) and after appropriate

dilution with methanol, the absorbance was measured against respective blank by UV spectroscopy at 238 nm λmax. The concentration of simvastatin was calculated by the UV Spectrophotometer using calibration curve.

Vehicles to be used were Olive oil, Tween 80,Propylene glycol, Soyabean oil, Cremophore ELP,PEG 400, Sunflower oil, Cremophore RH 40, Capmul MCM, Peanut oil, Labrafil M2125CS, Lauroglycol 90, Cottonseed oil, Labrasol, Capryol 340, Sesame oil, Span 20, Peceol, Oleic acid, Tween 20, Acconon MC 82, Captex 100, Span 80, Captex 200, Acrysol K-140, Captex355, Labrafac CC.

- **PSEUDO TERNARY PHASE DIAGRAM:[9,10,11]**

Based on the data observations of solubility studies, excipients of SMEDDS like oil, surfactant and co surfactant having highest solubility of Simvastatin were selected. The phase diagrams of oil, surfactant: co-surfactant and water were constructed using water titration method. Here seven ratios of surfactant: co-surfactant (Smix) ( 1:1, 1:2, 1:3, 1:4, 2:1, 3:1, 4:1 W/W) were mixed together. Each ratio of Smix blended with oil in a proportion of 9:1, 8:2, 7:1, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9 with constant stirring by magnetic stirring. Each blend of mixture was titrated with distilled water in increment of 0.5% (W/W) with proper stirring to form clear transparent micro emulsion. The point which indicates the clear and isotropic mixtures were considered to be within the micro emulsion region. Optimization of the concentration of vehicles (oil, surfactant and co-surfactant) was based on maximum uptake of water by formed micro emulsion. Chemix software was used to optimize the concentration of vehicles. Drug loading capacity was checked on self-micro emulsifying domain of ternary phase diagram.

- **FORMULATIONS OF LIQUID SELF-EMULSIFYING DELIVERY SYSTEM:[11]**

A series of self emulsifying formulations of simvastatin were being prepared using oil (oleic acid), surfactant (Tween 80) and co-surfactant (Capmul MCM). Simvastatin amount in all formulations were kept constant (i.e. 20 mg per unit dose). Here, in a glass vial, accurately weighed simvastatin, oil, surfactant and co-surfactant were mixed be vortex mixing for some time till it dissolves properly and stored till further use.

- **CHARACTERIZATION OF LIQUID SEDS OF SIMVASTATIN:**

**1. Macroscopic evaluation by visual assessment**

Small amount of SMEDDS diluted with distilled water (100 ml) and stirred with glass rod. Macroscopic assessment like self-emulsification efficiency, appearance like color, transparency, phase separation and precipitation of simvastatin was carried out visually immediately after dilution. Any change in color & transparency or phase separation during normal storage condition (37±2°C) was checked in micro emulsion formulation.

All SMEDDS were diluted by distilled water, 0.1N HCl (1.2 pH) and pH 7.0 phosphate buffer and micro emulsions were checked for precipitation or phase separation after 24 hours. Precipitation was checked after 24 hr to categorize the formulation like clear (transparent or transparent with bluish tint), nonclear (turbid), stable (no precipitation after 24 hr) or unstable (if sign of precipitates within 24 h)[8]

**2. Dispersibility test and Determination of self emulsification time**

The emulsification time of SMEDDS was determined According to USP dissolution apparatus. Formulation was added drop wise to 500ml water at 37 °C and agitated by dissolution paddle rotating at 50 rpm. Emulsification time was assessed visually and appearance of dispersion was also being checked and compared from table 1 for grading system. [12]

**Table: 1 Visual assessments of efficiency of self-micro emulsification**

| Grade | Dispersibility & Appearance | Time of self micro-emulsification |
|---|---|---|
| **I.** | Rapid forming micro emulsion which is clear or slightly bluish in appearance | < 1 min |
| **II.** | Rapid forming, slightly less clear emulsion which has a bluish white appearance | < 2 min |
| **III.** | Bright white emulsion (similar to milk) | < 3 min |
| **IV.** | Dull, grayish white emulsion with slightly oily appearance that is slow to emulsify | > 3 min |
| **V.** | Exhibit poor or minimal emulsification with large oil droplets present on the surface | > 3 min |

**3. %Transmittance test**

Stability of optimized micro emulsion of SMEDDS with respect to dilution was checked by measuring % Transmittance. One mL of SMEDDS was diluted to 100 mL with distilled water and observed at 650 nm using UV spectrophotometer and for each sample three replicates were performed[13]

**4. Droplet/Globule size measurement**

Globule Size analysis of micro emulsion of SMEDDS carried out by dynamic light scattering with Malvern

Zetasizer. SMEDDS were dispersed in 100 ml distilled water, at 37 ± 0.5˚C and emulsions were prepared by gentle agitation using a magnetic stirrer. Samples were placed in square glass cuvettes & droplet size analysis was carried out.[14]

**5. Zeta potential measurement**

Zeta potential for micro emulsion was determined using Malvern Zetasizer. SMEDDS were dispersed in distilled water, at 37 ± 0.5˚C and emulsions were prepared by gentle agitation using a magnetic stirrer. Samples were placed in clear disposable zeta cells & results were recorded. Before putting the fresh sample, cuvettes were washed with the methanol & rinsed using the sample to be measured before each experiment.[15]

**6. Refractive index measurement**

Refractive index of Drug loaded SMEDDS and SMEDDS without drugs was measured using Abbes refractometer.

**7. Viscosity determination**

The Rheological properties of the micro emulsion were being evaluated. SMEDDS (0.5 g) was diluted 10 times with distilled water with continuous stirring on magnetic stirrer and the viscosity of SMEDDS and resultant micro emulsion were being determined using Brookfield viscometer (DVIII+ Rheometer) at room temperature.[16]

**8. Thermodynamic stability study**

Thermodynamic stability studies like centrifugation test, heating cooling cycle and freeze thaw cycle were carried out on selected SMEDDS to check physical stability of same.

**a. Heating cooling cycle**

SMEDDS were exposed to six cycles between 4 ºC and 40 ºC with storage at same temperature for at least 48 hrs. Formulation which showed turbidity at the end of test was discarded and stable formulations were subjected to centrifugation test

**b. Centrifugation stress test**

Formulations were exposed to centrifugation at 3500 rpm for 30 mins. Formulation showing phase separation was discarded and remaining were being tested for freeze thaw cycle.

**c. Freeze thaw cycle**

Formulations were passed to 3 cycles at -21ºC and +25 ºC.Formulation which were stable in this test were considered for next characterization.

**9. In-vitro dissolution study**

The quantitative in vitro test of was performed in 900ml pH 7.0 phosphate buffer, at 37 ± 0.5°C using USP method (dissolution apparatus type I, at 100 rpm). Simvastatin SMEDDS filled in hard gelatin capsule was placed in basket during the release period and release profile were compared with conventional marketed tablet. 10ml of sample solution was withdrawn at predetermined 5, 10, 15, 20, 30, 45 min time intervals, samples were filtered through a 0.45 µm membrane filter, dilute suitably & analyzed spectrophotometrically at λmax 238 nm using calibration curve. Equal amount of fresh dissolution medium was replaced immediately after withdrawal of the test sample to maintain the volume. In vitro dissolution was carried out three times & mean value was tabulated. Percentage drug dissolved at different time intervals was calculated using calibration curve.[17,18]

**10. Solidification of liquid SEDDS of optimized liquid simvastatin :**

Solid SMEDDS of Simvastatin was prepared using adsorption technique using Neusilin US2 and aerosil 200 carrier by mixing in a mortar and pestle till uniform distribution of blend and after sieving, it was dried and stored till evaluation tests. [19]

**11. CHARACTERIZATION OF SIMVASTATIN SOLID SEDDS:**

**1. Micromeritics properties like** Angle of repose, Bulk density and tapped density, Compressibility Index (% C.I.) and Hausner ratio (H.R.) were tabulated.

**2. Drug content [20]**

Drug content was estimated by extracting Simvastatin from Solid SMEDDS in methanol and analyzed spectrophotometrically at 238 nm.

**3. Droplet/Globule size measurement**

Solid SMEDDS of Simvastatin were diluted to 100 ml with distilled water at room temperature under magnetic stirrer. The droplet size measurements of the resultant micro emulsions were done like liquid SMEDDS formulations.

**4. Zeta potential measurement**

Diluted solid SMEDDS of Simvastatin were undergone to zeta potential measurements as like liquid SMEDDS formulations.

**5. %Transmittance**

Stability of micro emulsion of solid SMEDDS with respect to dilution was checked by measuring % Transmittance. Solid Simvastatin SMEDDS were accurately weighed and diluted with distilled water to

100 ml. After mixing, resultant emulsion was kept for rest for and emulsion was observed for % transmittance. Each sample were performed triplicates were performed.

**6. In-vitro dissolution study**

The quantitative in vitro test of was performed in 900ml pH 7.0 phosphate buffer, at 37 ± 0.5°C using USP method (dissolution apparatus type I, at 100 rpm). Simvastatin solid SMEDDS filled in hard gelatin capsule was placed in basket during the release period and release profile was compared with conventional marketed tablet. 10ml of sample solution was withdrawn at predetermined 5, 10, 15, 20, 30, 45 min time intervals, samples were filtered through a 0.45 µm membrane filter, dilute suitably & analyzed spectrophotometrically at λmax 238 nm using calibration curve. Equal amount of fresh dissolution medium was replaced immediately after withdrawal of the test sample to maintain the volume. In vitro dissolution was carried out three times & mean value was tabulated. Percentage drug dissolved at different time intervals was calculated using calibration curve and compared with pure drug and marketed product.

Statistical analysis was being used for in-vitro dissolution comparison of liquid-solid SMEDDS and solid SMEDDS-marketed tablet by Model independent mathematical method of similarity (f2) values and ANOVA test.

**7. Solid state characterization**

The simvastatin Solid SMEDDS optimization was done on the basis of globule size and zeta potential results, % drug content, % Transmittance and *in-vitro* dissolution study and optimized formulation was undergone for solid state characterization like Differential scanning calorimetry (DSC), scanning electron microscopy (SEM), powder x-ray diffraction (PXRD) ,FT-IR study.

- **Differential scanning calorimetry (DSC)**

Thermograms of Simvastatin and optimized Solid SMEDDS were obtained using differential scanning calorimeter to check the thermal behaviour of optimized formulation.

- **Scanning electron microscopy (SEM)**

Morphological analysis of optimized simvastatin Solid SMEDDS can be done using Scanning electron microscope and scanning electron micrographs were recorded and analysed.

- **Powder X- ray diffraction (PXRD)**

The physical state and changes in crystallinity of drug Simvastatin and its optimized solid SMEDDS were characterized by powder X-ray powder scattering (XRD) measurements using X ray diffractometer.

- **FT-IR studies**

FTIR studies were performed to check interaction of drug and excipients in the optimized solid SMEDDS formulation.

**12. STABILITY STUDY**

Stability study was conducted as per ICH guidelines for final selected solid SMEDDS formulation. Hard Gelatin Capsule filled with final selected solid SMEDDS of Simvastatin were stored in air-tight screw capped containers protected from light and maintained under real time (25 ± 2 $^0$C / 60 ± 5% RH) for 6 months and accelerated conditions (40 ± 2 $^0$C, 75 ± 5% RH) for 3 months. Samples were taken at 0, 1, 2 , 3 and 6 month for long term and at 0 ,1 ,2 and 3 month for accelerated conditions and evaluated for appearance, self-emulsifying properties, drug content and in-vitro drug release.

## RESULT AND DISCUSSION:

- **UV SPECTROMETRY ANALYSIS OF SIMVASTATIN**

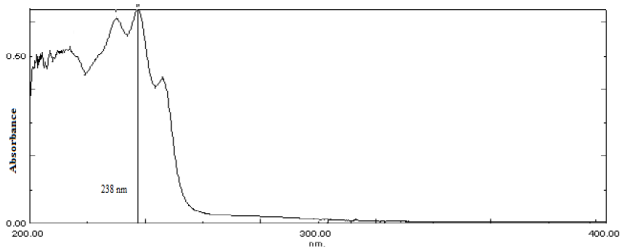



**Figure 1: Simvastatin UV absorption maxima**

From the scanning of Simvastatin from 200-400 nm in UV spectra, Maximum absorption (λmax) of Simvastatin found tobe 238 nm in Figure 1.

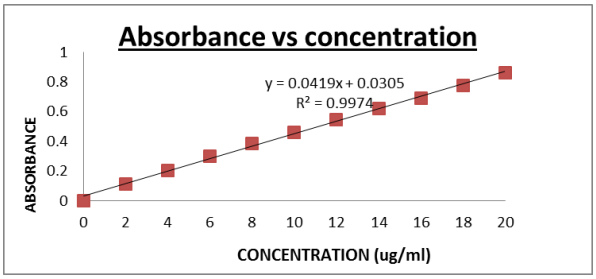



**Figure 2 Standard calibration curve of Simvastatin in methanol**

From the figure 2 and figure 3, Graphs of absorption Vs concentration for pure Simvastatin were found to be linear in concentration range 2-20 µg/ml at 238 nm. So pure Simvastatin obeys Beer-Lambert's law in the range 2-20 µg/ml in both medium.

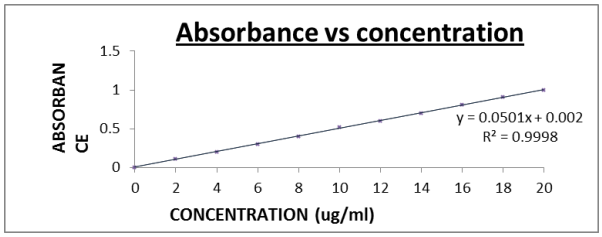



**Figure 3 Standard calibration curve of Simvastatin in phosphate buffer**

- **SATURATED SOLUBILITY STUDIES:**

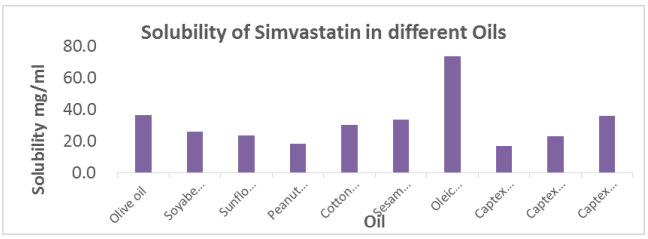



**Figure 4 Saturation solubility of Simvastatin in oils**

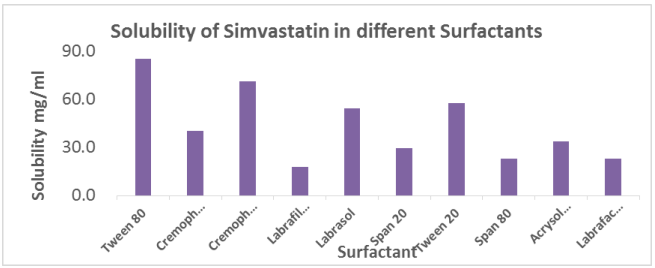



**Figure 5 Saturation solubility of Simvastatin in different surfactants**

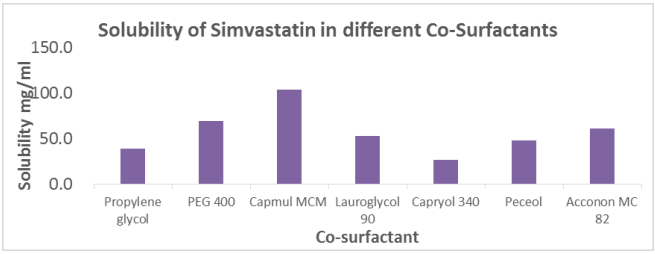



**Figure 6 Saturation solubility of Simvastatin in different co-surfactants**

Figure 4, 5, 6 shows solubility of simvastatin in oils, surfactants and co-surfactants respectively. As per maximum solubility data of Simvastatin in different oils, surfactants and co-surfactants; oleic acid as oil, Tween 80 as surfactant and Capmul MCM as co-surfactant were selected for ternary phase diagram.

- **PSEUDO TERNARY PHASE DIAGRAM:**

From the Simvastatin ternary phase diagrams shown in figure 7, the highest microemulsion zone was found in S mix =s/c = 1:3 with 40 mg simvastatin loading in comparison to other phase diagrams. So that was selected for preparation of Simvastatin liquid SMEDDS.

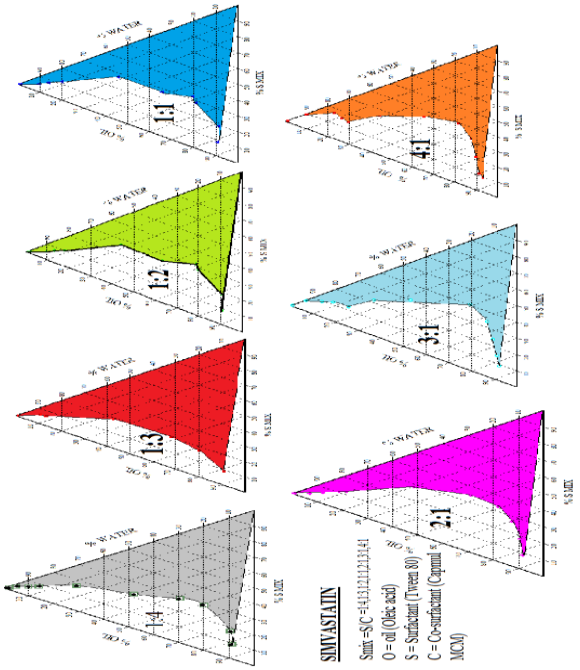



**Figure 7 Pseudo ternary phase diagrams of Simvastatin with O= Oleic acid , S= Tween 80 , C= Capmul MCM at different S mix=S/C = 1:4,1:3,1:2,1:1,2:1,3:1,4:1**

- **FORMULATIONS OF LIQUID SELF-EMULSIFYING DELIVERY SYSTEM:**

From the ternary phase diagram study, boundary formulations were being selected for the formulation (table 2) and characterization study.

**Table: 2 Formulations of liquid SMEDDS of simvastatin**

| Sr no. | Formulation code | Oil % | % Smix | Ratio of sur/co-sur = S mix |
|---|---|---|---|---|
| 1 | S1 | 25 | 75 | 1:3 |
| 2 | S2 | 30 | 70 | 1:3 |
| 3 | S3 | 35 | 65 | 1:3 |

**Oil (oleic acid), surfactant (Tween 80) and co-surfactant (Capmul MCM)**

**Simvastatin amount in unit dose = 20 mg**

- **CHARACTERIZATION OF LIQUID SEDDS OF SIMVASTATIN:**

Table 3 shows characterization of liquid SMEDDS like visual assessment, dispersion, self-emulsification time, % Transmittance, particle size, zetapotential, refractive index, viscosity, thermodynamic study etc.

**Table 3: Characterization of liquid SMEDDS of simvastatin**

| Parameter/ Formulation | S1 | S2 | S3 |
|---|---|---|---|
| **Visual assessment** | Clear and uniform emulsion without precipitation; Robust to dilution without pH effect | Clear and uniform emulsion without precipitation; Robust to dilution without pH effect | Less clear emulsion; Robust to dilution without pH effect |
| **Dispersion** | Grade II slightly bluish emulsion | Grade I microemulsion | Grade III bluish white emulsion |
| **Self-emulsification time** | within 1.1 min | Spontaneous within 30 seconds | 2.1 min |
| **% Transmittance** | 88.82±0.08 | 99.42±0.008 | 77.76±0.098 |
| **Particle size (nm)** | 19.07 | 14.69 | 41.7 |
| **Polydispersity index** | 0.273 | 0.167 | 0.418 |
| **Zeta potential** | -7.8 | -19.81 | NOT found |
| **Refractive index** | 1.495 ± 0.08 | 1.392 ± 0.04 | 1.48 ±0.11 |
| **Viscosity (cps)** | 218 | 212 | 225 |
| **Thermodynamic stability study** | Passed | Passed | Passed |

**REFERENCE:**


1. WHO fact sheet No. 164 and 204, October 2000

2. NIDDK/NIH. An action plan for liver disease research http://liverplan.niddk.nih.gov.

3. Humbe r JM. The role of complementary and alternative medicine: Accommodating pluralism. J Am Med Association; 2002; 288: 1655-56.

4. Dorai A. A. Wound care with traditional, complementary and alternative medicine. Indian Journal of Plastic Surgery; 2012; 45(2): 418-24.

5. Nordeng H., Diallo D., Al-Zayadi W., Ballo N., Berit Smestad Paulsen. Traditional medicine practitioners' knowledge & views on treatment of pregnant women in three regions of Mali. Journal of Ethno-biology, Ethno-medicine; 2013; 9: 67.

6. Sari L. M., Suyatna Fd, Utami S, Chairul C, Subita GP, Whulandhary YS, Auerkauri EI. Acute oral toxicity study of areca catechu linn. Aqueous extract in sprague-dawley rats. Asian Journal of Pharmaceutical Clinical Research; 2014; 7(5): 20-2.

7. Ghosh M. N. Fundamentals of Experimental Pharmacology, 3rd Edition. S.K. Ghosh & others publications; 2005; 190-7.

8. Gatne M. M., Adarsh and Ravikanth. Acute oral toxicity study of poly-herbal formulation AV/KPC/10, IJBAR; 2015; 6 (03): 281-283

9. Vinod D. Rangari. Pharmacognosy and phytochemistry: Part 1. 1st ed. Pune: Published by Career Publication; 2002; 129-139.

10. Pulok K Mokherjee. Quality control of crude drugs. 1st Ed. New Delhi: Published by Business Horizones Pharmaceutical Publishers; 2002; 403-405.

11. Lipnick R. L., Cotruvo J. A., Hill R. N., Bruce R. D., Stitzel K. A., Walker A. P., Chu I., Goddard M., Segal L., Springer J. A., Myers R. C. Comparison of the Up-and-Down, Conventional LD50 and Fixed dose Acute Toxicity Procedures. Fd Chem Toxicology; 1995; 33: 223-231.