‘Screening of Safflower Oil Microemulsion for Enhancing Bioavailability of Lovastatin’

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ABSTRCT

Objective

Microemulsions have been widely studied to enhance the bioavailability of the poorly soluble drugs. They have very low surface tension and small droplet size which results in high absorption and permeation. Interest in these versatile carriers is increasing and their applications have been diversified to various administration routes in addition to the conventional oral route. This can be attributed to their unique solublization properties and thermodynamic stability which has drawn attention for their use as novel vehicles for drug delivery

Method

Lovastatin is systemic lipid lowering drug belonging to statins and is used for lowering blood cholesterol. Like all statins, lovastatin works by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA reductase). Pseudoternary phase diagram was constructed to determine the ME existing zone. Optimised ME was evaluated for its transparency, droplet size, zeta potential, viscosity, conductivity, percentage assay, and phase separation study. Solubilisation capacity of the ME system was also determined. An accelerated stability study of optimised

ME was carried out to check the stability of the formulation. The prepared ME was compared with the pure drug solution and commercially available lovastatin tablet for *in vitro* drug release. Comparative oral absorption of lovastatin from the ME and suspension of the commercially available lovastatin was investigated through an *in vivo* study in a rat model.

Result

Hence microemulsion produced by water titration method showed good particle size (95nm). Refractive index and % transmittance showed good isotropic formulation. In vitro drug release studies revealed overall increase in release from micro-emulsion compared to lovastatin marketed tablet formulation microemulsion showed 76.72% release and marketed formulation showed 52.3% release. There is no physical interaction between drug and excipients, hence collectively it shows microemulsion is promising drug delivery system.

Key Words: Microemulsion, Bioavailability, Lovastatin

INTRODUCTION

Microemulsions are clear transparent, thermodynamically stable dispersion of oil and water, stabilized by interfacial film of surfactant frequently in combination with a co-surfactant. Recently there has been a considerable interest for microemulsion formulation, for the delivery of hydrophilic as well as liphophlic drug as drug carriers because of its improved drug solubilisation capacity, long shelf life, ease of preparation and improvement of bioavailability. In this present review, we have discuss biopharmaceutical aspects, advantages, disadvantage, theories, formulations, marketed lipid based formulations, factors affecting formulation and phase behaviour, preparations, characterization and pharmaceutical application of microemulsions[32].

Microemulsions have been widely studied to enhance the bioavailability of the poorly soluble drugs. They offer a cost effective approach in such cases. Microemulsions have very low surface tension and small droplet size which results in high absorption and permeation. Interest in these versatile carriers is increasing and their applications have been diversified to various administration routes in addition to the conventional oral route. This can be attributed to their unique solublization properties and thermodynamic stability which has drawn attention for their use as novel vehicles for drug delivery. The results obtained have been indeed very promising. In recent past, microemulsion formulation of a poorly soluble immunosuppressant was marketed as a soft capsule which contains a mixture of drug dissolved in oil and surfactant. It converts into an oil-in-water (o/w) microemulsion in situ in an aqueous environment in the stomach and the small intestine. Microemulsion formulation made the bioavailability and plasma concentration profiles of the drug more reproducible which is clinically important in the case of drugs showing serious adverse effects. This is a significant step forward in the

delivery of poorly soluble drugs. Microemulsion systems are also now being increasingly investigated for transdermal, ocular, nasal, pulmonary, vaginal, rectal and intravenous drug delivery. The knowledge on the phase manifestations of the pseudo-ternary (water/amphiphile/oil) or explicitly quaternary (water/surfactant/co surfactant/oil) mixtures has been systematized.

MATERIAL AND METHODS:

Material:

Lovastatin was obtained as gift sample from Macleods pvt.Ltd, Mumbai India. Safflower oil was purchased from Alps emu farm and hatcheries, Vadodara. Tween 80, Labrasol was purchased from Gattefosse, Mumbai, India. Other chemicals used were of analytical grade.

Methods:

DRUG AUTHENTICATION

General Description

The sample of lovastatin was evaluated visually for its physical state, color, odor and solubility.

Melting Point

Melting point is one of the important parameter to identify the purity if the drug. Melting point also helps in understanding crystallinity. Melting point gives an idea about its purity. Many methods are used to determine melting point but widely accepted method is capillary method. Melting point of LOV was determined by open capillary tube method. Briefly, LOV was placed in capillary tube whose one and was sealed and attached to the thermometer. The whole assembly was kept in liquid paraffin oil and heated. The progress in temperature was monitored. The point at which drug started melting was noted. The determination was for repeated three times. The mean melting point was considered as the melting point of LOV.

Fourier Transform Infrared (FTIR) Spectrophotometer Study

FTIR is an important tool to analyze the purity of the drugs. The FTIR spectrum shows the fundamental peaks corresponding to the chemical nature of the drug. FTIR spectra of LOV were recorded using FTIR (Jasco- V-530 model). Briefly, about 2 mg of sample was ground thoroughly with previously dried KBr at 120°C for 30 min; uniformly mixed with drug sample and kept in sample holder. The spectra were recorded over the range of wave number 500 - 4000 cm^{-1}.

Determination of λ_{max}

Standard stock solution containing LOV was prepared by dissolving 10 mg of LOV in 50 mL methanol Flask was sonicated for 10 min and the final volume was made up to 100 mL with the same solvent mixture to obtain a concentration of 100 µg/mL. Solution obtain containing 10 µg/mL of LOV was scanned in the range of 200 – 400 nm in Jasco V-630 spectrophotometer using mixture of methanol as reference. The wavelength of maximum absorbance was considered for further studies. The wave length of maximum absorbance considered for further studies, solution obtained contains lovastatin where methanol is used as reference standard.

DEVELOPMENT OF ANALYTICAL METHODS

Standard Curve in Methanol

Double beam UV-Visible spectrophotometer (Jasco V630), fused silica cuvette of 1 cm in width. Accurately weighed quantity of 10 mg of LOV was taken and transferred to100 mL volumetric flask containing methanol and volume made up to 100 mL. This solution was treated as stock solution of concentration (100 µg/mL) of LOV. From this stock solution 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4 mL aliquots were withdrawn and diluted with methanol to 10 mL to obtain solutions of concentration 5, 10, 15, 20, 25 and 30, 35, 40 µg/mL. Absorbance of solutions was measured using UV-visible double beam spectrophotometer (Jasco V-630) against methanol as blank solution at λ_{max} 238 nm.

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Standard Curve in Phosphate Buffer 6.8

Accurately weighed quantity of 10.0 mg of LOV was taken and transferred to a 100 mL volumetric flask. This was dissolved in phosphate buffer and volume made up to 100 mL. This solution was treated as stock solution of concentration 100 µg/mL of LOV. From this stock solution 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mL were withdrawn and diluted with water to 10 mL to obtain solutions concentration 5, 10, 15, 20, 25.30, 35 and 40 µg/mL. Absorbances of the solution were measured using UV-visible double beam spectrophotometer (Jasco V-630) against Distilled water as blank solution at λ_{max} 238 nm.

SCREENING OF EXCIPIENTS

The solubility of LOV in various oils, surfactants and co-surfactants was determined by dissolving an excess amount of LOV in 2 mL of each of the selected oils, surfactants and co-surfactants in 5 mL stopper vials separately. A combination of oils was also used for the determination of solubility. An excess amount of LOV was added to each 5 mL capacity stopper vial and mixed by vortexing. The mixture vials were then kept at 37°C ± 1°C for stirring for 72 hours to attain equilibrium. The equilibrated samples were removed from the stirring and centrifuged at 3000 rpm for 15 minutes. The supernatant was taken and filtered through a 0.45-µm membrane filter. The concentration of LOV was determined in each oil, surfactant, co-surfactant, and combination of oils by UV spectrophotometer at their respective λmax. The concentration of lovastatin was determined in each oil surfactant and co surfactant by UV visible spectroscopy.

In vivo studies

Approval of protocol

Before starting in vivo studies protocol in prescribed Proforma B for animal studies entitled ‘Screening safflower oil micro-emulsion for enhancing bioavailability of lovastatin’ was submitted on 22 march2014 to IACE of Bharati Vidyapeeth College of Pharmacy Kolhapur. The protocol was approved by IAEC in presence of CPCSEA nominee with Approval no. BVCPK/ CPESEA/ IAEC/ 02/ 16 dated 17 Dec. 2011 at Bharati Vidyapeeth College of Pharmacy, Kolhapur.

Selection of Animals

Albino rats of either sex having weight 200-250 g were used for estimation of pharmacokinetic parameters of LOV micro-emulsion and marketed formulation. The albino rats were kept under standard conditions in animal house of Bharati Vidyapeeth College of Pharmacy, Kolhapur, as per guidelines of CPCSEA.

Experimental Method

Bioavailability study was carried out on 18 rats. All the animals used in the study were fasted over night. Animals were separated into three groups with six animals in every group. The formulations were provided orally using oral feeding needle. Average weight of marketed tablets of LOV providing 10 mg dose was found to be 125.63 mg. So, 250 g rat required 1.2 mg of drug. Hence, fraction of tablet corresponding to the weight of the tablet required to give the desired dose was administered to rat. The rats were anaesthetized using ether anesthesia. Blood samples were withdrawn from retro orbital puncture at an interval of 30 min up to 2 hours and 1 hour interval from 2 hours to 5 hours and last sample at 15^{th} hour from retro orbital puncture. The collected samples were analyzed by HPLC.

Selection of chromatographic conditions

The selection of HPLC method depends upon the nature of the sample, its molecular weight and solubility. The chromatographic variables such as mobile phase, flow rate and solvent ratio were studied. The resulting chromatograms were recorded and the chromatographic parameters such as capacity factor, asymmetric factor, and peak resolution and column efficiency were calculated. The condition that gave the best resolution, symmetry and capacity factor was selected for estimation. The separation conditions used for chromatography are given in Table

Table 1: Separation condition for chromatography

Chromatographic mode	Chromatographic condition
Standard solution	LOV (1000 µg/mL) in mobile phase
Stationary phase	HIQ SII C18 column- (4mm × 250)
Mobile phase	Methanol: Water (70: 30)
Detection	238
Flow rate	1 mL/min
Sample size	20 µl

Preparation of standard drug solution

Standard stock solution containing LOV was prepared by dissolving amount equivalent to 10 mg of LOV in mobile phase and sonicated for 20 min. and volume adjusted to 10 mL with mobile phase to obtain concentration 1000µg/mL.

Selection of detection wavelength

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is one that gives good response for the analyte to be detected. In the present study drug solution of 10µg/ml of ATR were prepared in mobile phase. After observing UV spectra of drug, wavelength of 238nm was selected for further study.

Preparation of blood samples for HPLC analysis

Accurately 0.5 mL of 0.1 M acetic acid was added to 3 mL of blood and centrifuged at 5000 rpm for 35 min. The supernatant was collected as plasma. Accurately 1 mL plasma was added to series of 10 mL of volumetric flasks and 1ml of 0.1µg/mL standard stock solution of ATR was added in 1 mL of plasma and final volume adjusted to 10 mL with mobile phase.

Estimation of pharmacokinetic parameter

Accurately 20 µl of sample solution was injected into the chromatographic system using fixed volume loop injector. The pharmacokinetic parameters the maximum plasma concentration (C_{max}) and corresponding time (t_{max}) the area under the concentration-time curve (AUC) were obtained directly from plasma concentration time data was estimated.

Estimation antihyperlipedemic activity of oil used in formulation

All the animal groups were deprived of food for 8 h prior to evaluate the cholesterol reducing effect. The formulation administered to different animal groups is given in Group I will serve as control group, Group II will serve as test formulation, Group III will serve as test oil, and Group IV will serve as standard group Blood samples were collected into sterile tubes at 0 h and 24 h after triton X-100 (400mg/kg) p.o. saline suspension by retro-orbital venous plexus. The obtained serum samples were centrifuged at 4000g and analyzed for cholesterol by using enzymatic kit.

RESULT AND DISCUSSION

PREFORMULATION STUDY OF SAFFLOWER OIL

Density of Safflower oil

Density of any material is its physical constant and aids in its identification and purity determination. The density of Safflower oil was calculated by following equation (H. N. More et al, 2008).

$$\text{Density of emu oil at T°C} = \frac{W}{V} \quad \text{... (10)}$$

The density of safflower oil was found to be 0.936 g/ mL.

Refractive Index

Refractive index of any material depicts valuable information about characteristics, purity, and composition of the substance. For determination of refractive index of safflower oil was measured by Abbe's refractometer at room temperature and this was found to be 1.321 (Indian Pharmacopoeia, 2007).

Acid Value

The lower acid value of safflower oil indicates possible low free fatty acid compositions which suggest lesser susceptibility to physico-chemical and antioxidant properties In general any oil of low acidity is to be explored for economic, nutritional and health related applications. Acid value of safflower oil was found to be 1.12. Thus it was ideal candidate for formulating it in micro-emulsion (Indian Pharmacopoeia, 2007).

Iodine Value

Iodine value is one of the characteristics properties for determination of identity and purity of safflower oil. Iodine value measures degree of unsaturation in an oil which is a useful parameter in studying oxidative rancidity of oil hence, in determination of iodine value of safflower oil gives explanation that iodine get incorporated into fatty acid chain where double bond exist. Iodine value is one of the characteristics properties for determination of identity and purity of safflower oil. Iodine value of safflower oil was found to be 147.23% that it contains more unsaturated fatty acids

Saponification Value

Saponification value is used to measure of the average molecular weight of fatty acids. Saponification value of Safflower oil was found to be 189.13. The long chain fatty acids found in fats have a low saponification value because they have relatively fewer number of carboxylic functional group per unit mass of the fat as compared to short chain fatty acids (H. N. More et al, 2008).

Gas Chromatography Mass Spectroscopy

Gas chromatography mass spectroscopy is a more powerful analytical technique that provides a method for detecting target compounds in complex at trace levels. The gas chromatography mass spectroscopy helps to separate and determine the individual elements and molecules in a sample. Gas chromatography is a technique for separating closely related compounds (solutes) from a liquid or gaseous mixture. Mass spectroscopy is a method of determining the molecular weights of a chemical compound's component ions. The analysis of safflower oil by GCMS indicates that oil contains 9 different structural components as shown in Fig.7.1 Safflower oil contains 13 different structural components having molecular weight of 185, 172, 154, 278, 290, 256, 254, 308, 440 g/ mol.

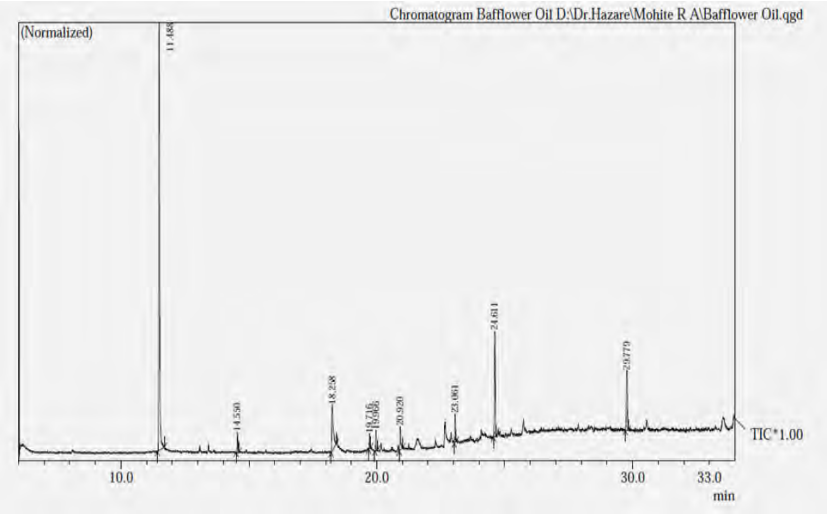


Figure 1: Gas chromatography mass spectroscopy of safflower oil

Table 2: Observed values for GCMS of safflower oil

Peak	Retention time (min.)	Mol. Weight(g/mol)
1	11.684	185
2	16.817	172
3	19.925	154
4	20.058	278
5	20.20	290
6	21.442	256
7	25.608	254
8	26.850	308
9	30.11	440

DRUG AUTHENTICATION

Description

The LOV was white, crystalline powder with characteristic odor.

Melting Point

The melting point of LOV was found to be in the range of 172–175°C.

FTIR

The FTIR study of LOV was performed to identify it's chemical stability and purity. The FT-IR spectrum of LOV is shown in Fig.7.2 The spectrum of pure LOV shows characteristic peaks of aromatic N-H stretching and C=O stretching at 3364.21 cm^{-1}, 1649.81 cm^{-1} respectively. The prominent peak is of 1734 cm^{-1} which is of lactone ring which is responsible for exerting pharmacological activity or therapeutic activity. Where this lactone ring hydrolyses to beta hydroxy acid which exerts the antihyperlipedemic activity. This peak is retained in the formulation IR which confirms there is no interaction so this excipients can be used with drug to formulate microemulsion.

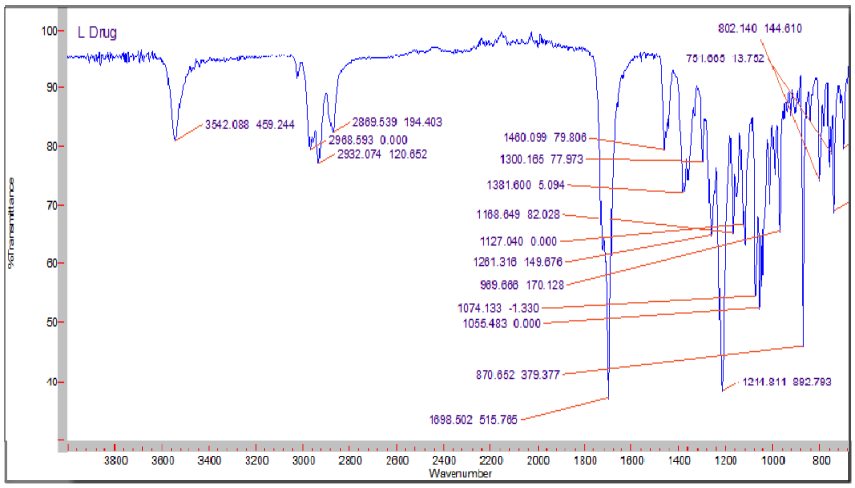


Figure 2: FTIR spectrum of LOV

Comparison between the reported FTIR peaks and the observed FTIR peaks are shown in Table7.2. Principle peaks were found in the range corresponding to functional groups. Appearance of the principle peaks in spectrum confirms the drug sample is LOV and is pure.

Table 3: Reported and observed FTIR peaks of LOV

Chemical entity	Reported Peak (cm^{-1})	Observed Peak (cm^{-1})
Aromatic –OH stretching	3550-3500	3542.08
Aromatic C–H stretching	3100-3000	2968.59
Aliphatic C-H stretching	2870	2869.53
Aromatic ester C=O stretching	1730-1705	1698.50
Aromatic C=C stretching	1600-1430	1454.06
Aromatic ester C-O stretching	1310-1250	1262.18
Aliphatic ester C-O stretching	1300-1100	1214.81

Determination of λ_{max}

For characterization of drug by UV spectroscopy, it is important to know the wavelength of maximum absorption (λ_{max}). The spectrum of LOV in methanol was taken and is given in Fig.7.3 The results of λ_{max} of LOV in methanol are given in Table 7.3.

Table 4: λ_{max} of LOV in methanol

Solvent system	Maximum absorbance wavelength
Methanol	238 nm

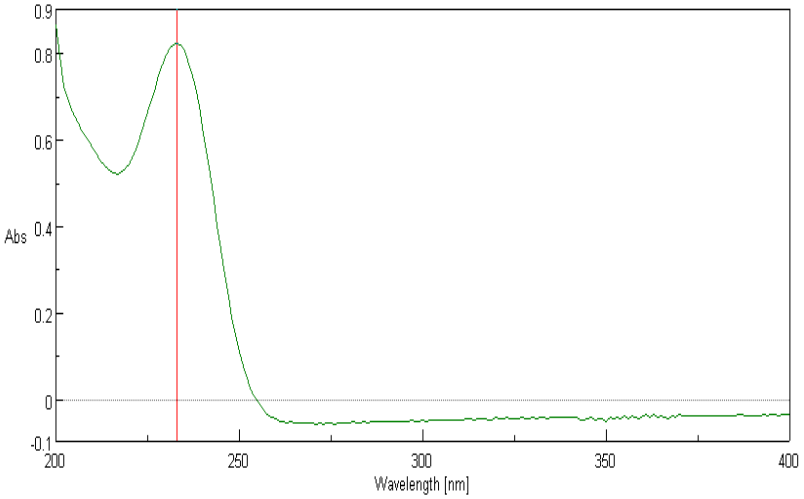


Figure 3: UV spectra LOV in methanol

DEVELOPMENT OF ANALYTICAL METHODS

Calibration Curve of LOV in Methanol

The UV absorption data at 238nm and concentration estimates of pure LOV showed good linearity (r = 0.999) over the concentration range of 5-30 µg/mL. Hence the sample of LOV was found to obey Beer- Lambert's law over this range. Slope and intercept values of calibration curve were 0.0621 and 0.0095 respectively. The absorbance data for calibration curve is given in Table 7.4 and calibration graph is shown in Fig. 7.4

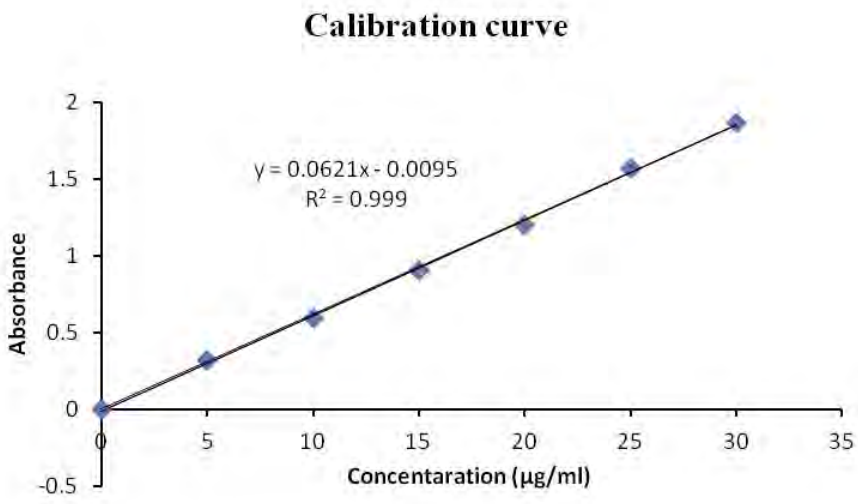


Figure 4: Calibration curve of LOV in methanol

Calibration Curve in Phosphate Buffer 6.8

The UV absorption data at 238 nm and concentration estimates of pure LOV showed good linearity (r = 0.992) over the concentration range of 5-30 µg/mL. The sample of LOV was found to obey Beer- Lambert's law over this range. Slope and intercept values of calibration curve were 0.0037 and -0.004 respectively. The absorbance data for calibration curve is given in Table 7.5. and calibration graph is shown in Fig. 7.5

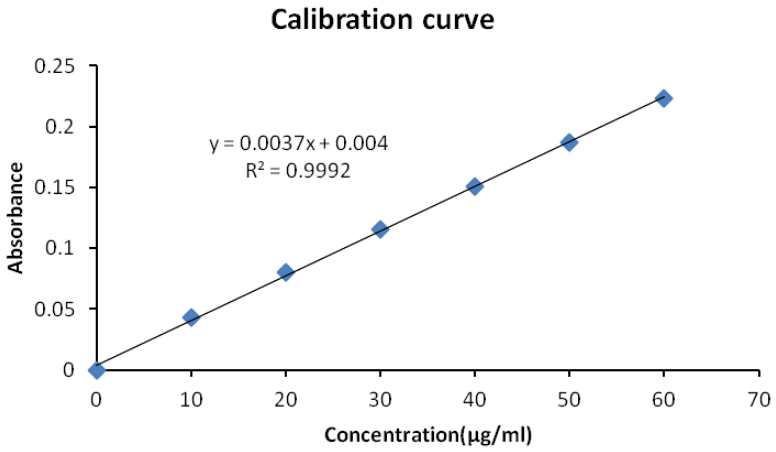


Figure 5: Calibration curve of LOV in phosphate buffer6.8

The summary of different parameters of calibration curve such as slope, intercept and coefficient of correlation is given in Table 7.6

Table 5: Various constants for calibration curves

Parameter	Value for calibration curve in methanol	Value for calibration curve in Phosphate buffer
Slope	0.0062	0.0037
Intercept	0.0095	-0.004
'R'	0.999	0.992

PREFORMULATION STUDIES FOR MICRO-EMULSION

Compatibility Studies

Presences of any interaction between drug and formulation excipients were studied by FTIR. The overlain spectrums of LOV and formulation are shown in Fig.7.6 From FTIR studies it can be seen that the fundamental peaks of LOV are retained. Results show that there is no chemical interaction between LOV and excipients used in the formulation LOV containing the lactone ring. It gives characteristic peak at 1725, 1711, 1700 cm-1. Physical mixing of LOV with surfactant and co-surfactant showed no major changes in position of the characteristic peaks of drug which indicate compatibility of surfactant and co- surfactant with drug. hence, can be used for the formulation of micro-emulsion.

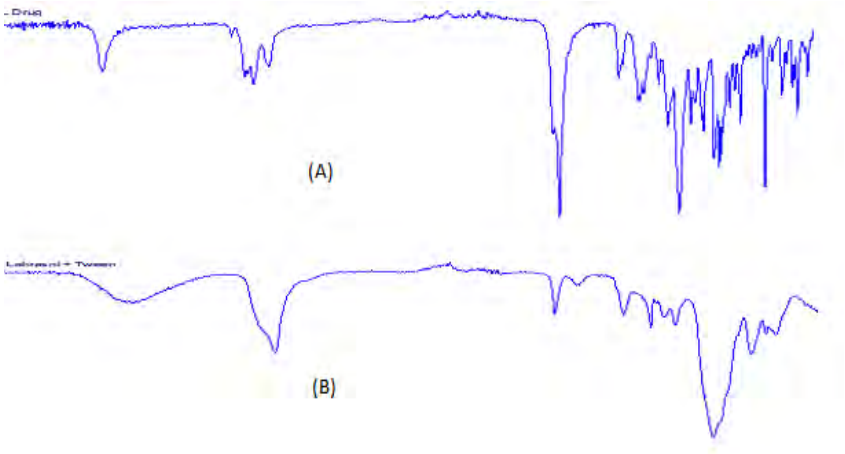


Figure 6: Overlain IR spectra of (A), LOV, (B) Formulation

Determination of Solubility of Drug

The microemulsion formulation consists of one or more surfactant and drug dissolved in oil. The pre-concentrate mixture should be clear, monophasic liquid at room temperature and should have good solvent properties to allow presentation of drug in solution. The objective of saturated solubility study is to identify oil and surfactants with good solubilizing capacity for LOV. The concentration of LOV in various excipients was determined by UV spectrophotometrically

SELECTION OF EXCIPIENTS

The important criterion for selection of materials for the micro-emulsion formulation development is that the excipients selected needed to be pharmaceutically acceptable, non toxic for oral administration and fall under the GRAS (generally regarded as safe) category. Higher solubility of the drug in the oil phase was another important criterion; as it would help the micro-emulsion to maintain the drug in solubilize form. Safety is a major determining factor in choosing a surfactant, as a large amount of surfactants may cause toxicity for oral administration. Non-ionic surfactants are less toxic than ionic surfactants. An important criterion for selection of the surfactants is that the required hydrophilic lipophilic balance (HLB) value to form the micro-emulsions. The right blend of low and high HLB surfactants leads to the formation of a stable micro-emulsion formulation. Transient negative interfacial tension and fluid interfacial film are rarely achieved by the use of single surfactant usually so the addition of a co-surfactant is necessary. The presence of co-surfactant decreases the bending stress of interface and allows the interfacial film sufficient flexibility to take up different curvatures required to form micro-emulsions over a wide range of composition. Thus, oil phase, surfactant and co-surfactant selected for the study were Safflower oil , as oil, labrasol as surfactant and Tween80 as co-surfactant respectively. All these materials are regarded as GRAS excipients by USFDA.

SCREENING OF EXCIPIENTS

The selected excipients need to solubilize the drug. The important criterion for screening of excipients is the solubility of the poorly soluble drug in oil, surfactants, and co-surfactants. Since the aim of this study was to develop an oral formulation, it was important to determine the solubility of the drug. The solubility of LOV in safflower oil was 32.29 mg/mL and in isopropyl myristate it was as 18.42 mg/mL. Both these oil showed higher solubility than other oils in pure form and combinations of oils. Thus, these two oils were selected as the oil phase for the development of formulation. The solubility of the drug in Labrasol was 24.29 mg/mL and in tween80 23.86 mg/mL. Therefore, labrasol and tween80 were selected as surfactant and co-surfactant for the phase study. Comparative histograph showing the solubilities of LOV in various excipients is shown in Fig. 7.7

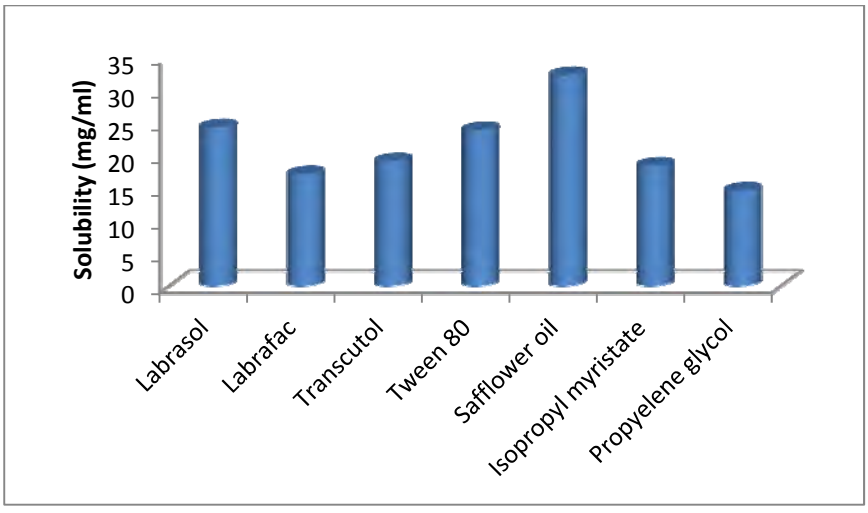


Figure 7: Comparison of solubilities of LOV in tested components

CONSTRUCTION OF PSEUDOTERNARY PHASE DIAGRAM

The micro-emulsion region was determined by plotting data on pseudo ternary phase diagram. Based on results of solubility studies oil, surfactant and co-surfactant were selected for micro-emulsion formulation. Nine different potential combination of surfactant mixture to oil at different K_m values (1, 2, 3, and 4) were selected for phase diagram study of LOV. The boundary layer of o/w micro-emulsion was determined in each phase diagram. Components used for construction of pseudo ternary phase diagram are safflower oil (Oil phase), Labrasol (Surfactant), tween 80(Co-surfactant) and double distilled water (Mandal S et al, 2009

Table 6: Composition of safflower oil /Labrasol/ tween 80/ water at $K_m = 1$

S_{mix}: Oil	Composition (% w/w)		
	S_{mix}	Oil	Water
1:1	50	50	-
1.5:1	52	25	23
2:1	50	24	26
2.5:1	50	19	31
3:1	49	16	35
3.5:1	48	14	38
4:1	46	14	40
4.5:1	42	10	48
5:1	40	10	50

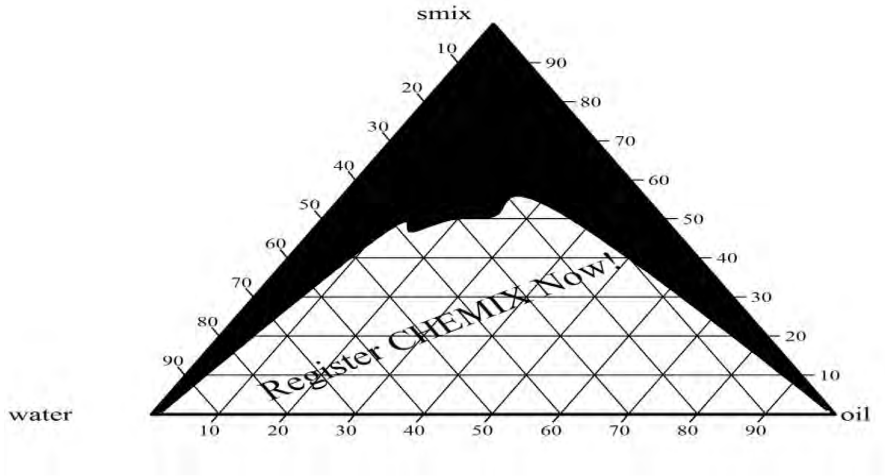


Figure 8: Pseudo ternary phase diagram of safflower oil /Labrasol/tween 80/ water at K_m=1

Table 7: Composition of safflower oil /Labrasol/ tween 80/ water at $K_m = 2$

S_{mix}: Oil	Composition (% w/w)		
	S_{mix}	Oil	Water
1:1	50	50	
1.5:1	40	26	34
2:1	45	27	28
2.5:1	44	20	36
3:1	45	17	38
3.5:1	40	13	47
4:1	38	11	51
4.5:1	36	19	45
5:1	32	6	62

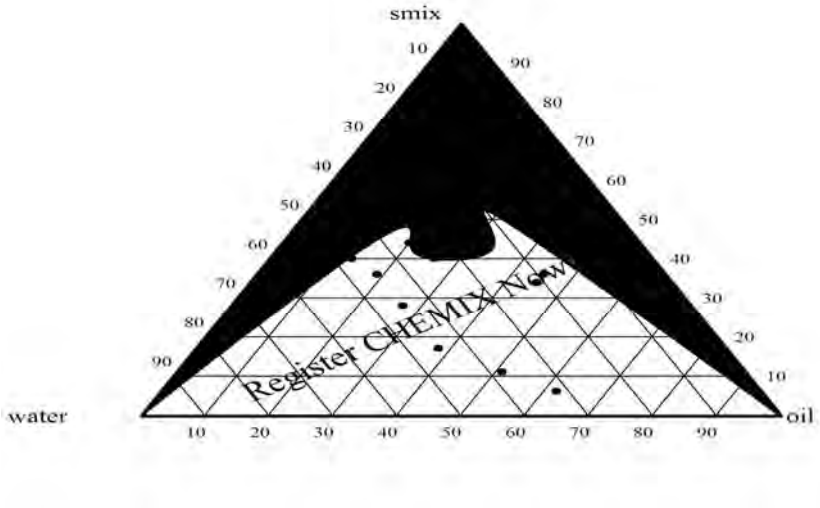


Figure 9: Pseudo ternary phase diagram of safflower oil /Labrasol/tween 80/ water at K_m=2

Table 8: Composition of safflower oil / Labrasol/ tween 80/ water at K_m=3

S_{mix}: Oil	Composition (% w/w)		
	S_{mix}	Oil	Water
1:1	50	50	-
1.5:1	30	36	34
2:1	42	28	30
2.5:1	44	16	40
3:1	42	18	36
3.5:1	40	18	42
4:1	32	10	57
4.5:1	24	5	66
5:1	20	5	75

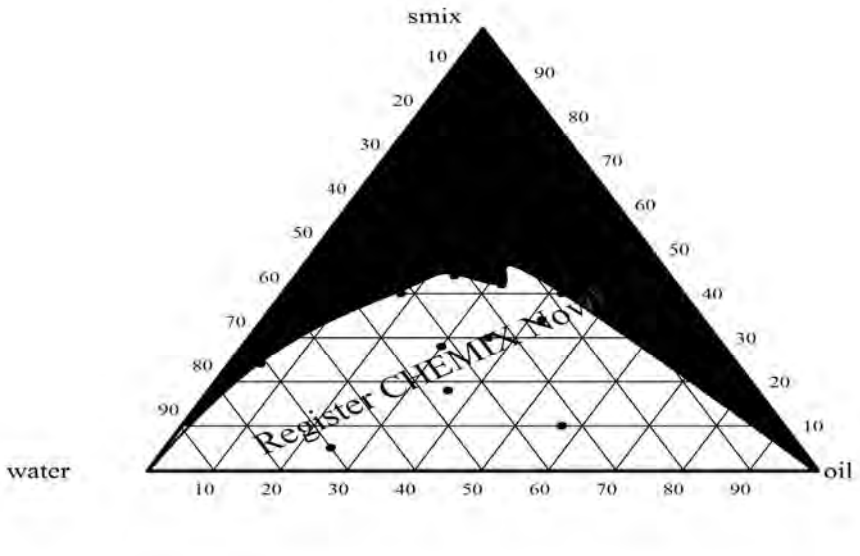


Figure 10: Pseudo Ternary phase diagram of Safflower oil / Labrasol Tween 80/ Water at K_m = 3

Table 9: Composition of safflower oil / Labrasol/ tween 80/ water at K_m = 4

Sr. no.	S_{mix}: Oil	Composition (% w/w)		
		S_{mix}	Oil	Water
1	1:1	50	50	-
2	1.5:1	40	27	33
3	2:1	41	29	30
4	2.5:1	40	17	43
5	3:1	40	10	50
6	3.5:1	36	10	54
7	4:1	28	6	66
8	4.5:1	24	6	70
9	5:1	15	5	80

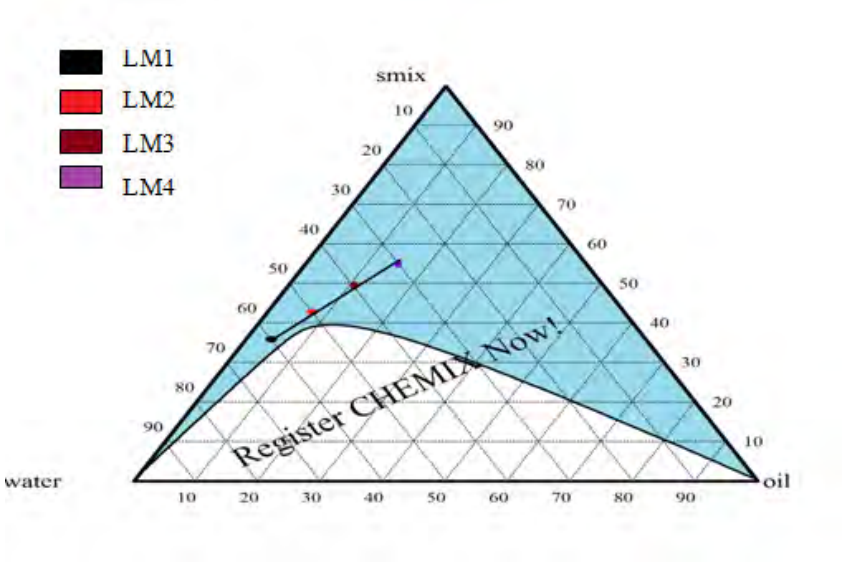


Figure 11: Optimized pseudo Ternary phase diagram of Safflower oil / Labrasol/Tween 80/ Water at K_m=4

Four points were selected from KM ratio 4 showing maximum region which were showing greater water content as microemulsion is o/w

Table 10 Following four batches were made and taken for further evaluation

Formulation	%Composition (w/w)		
	Water	Smix	Oil
ME1	60	35	5
ME2	50	40	10
ME3	40	45	15
ME4	30	50	20

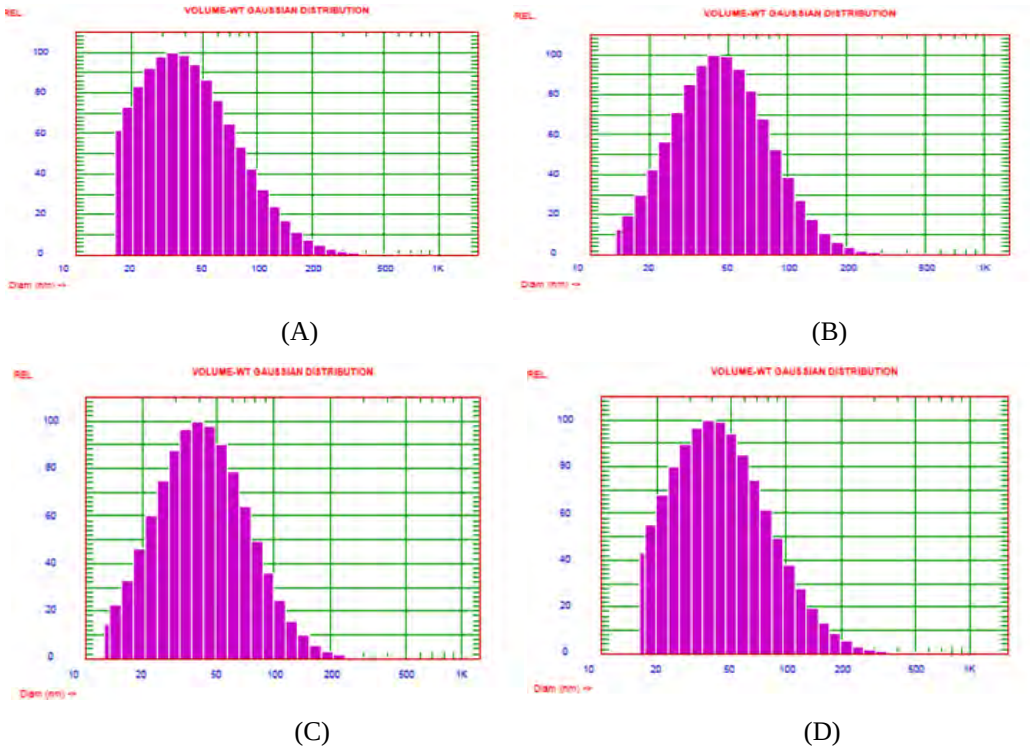
PREPARATION OF MICRO-EMULSION

The aim of the constructing pseudo-ternary phase diagrams was to find out the existence of micro-emulsions region. The pseudo-ternary phase diagram with various weight ratios of the investigated quaternary systems of water-Labrasol (surfactant) - tween 80(co-surfactant) - safflower oil (oil) - is presented in. From constructed pseudo ternary phase diagrams and systems, highest water absorption (highest micro-emulsion region) was selected for formulation. A pseudo ternary phase diagram at Km = 4 shows better micro-emulsion region than 1, 2, 3 so further increase in micro-emulsion existence region was not observed. The micro-emulsion systems (the shaded area) were prepared at room temperature. No distinct conversion from w/o to o/w micro-emulsions was observed. The rest of the region on the phase diagram represents the turbid and conventional emulsions based on visual observation. The area of micro-emulsion isotropic region changed slightly in size with the increasing ratio of surfactant to co-surfactant (Mandal et al, 2009)

Phase behavior investigations of optimized system demonstrated the suitable approach in determining the water phase, oil phase, surfactant concentration and co-surfactant concentration with which the transparent, one phase low-viscous micro-emulsion was formed The phase behavior study revealed that the maximum proportion of oil (31% w/w) and water (82% w/w) was incorporated in micro-emulsion systems when the surfactant to co-surfactant ratio (km) was 4. From a formulation viewpoint, the increased oil content in micro-emulsions may provide a greater opportunity for the solubilization of LOV

Particle Size Determination and Polydispersity index:

The globule size and PDI of the formulations are represented in table. The results show that the droplet size decreases with decreasing ratio of oil: surfactant/co-surfactant. These results are in accordance with the previous report that the addition of surfactant to microemulsion system caused the interfacial film to condense and become stable, while the cosurfactant causes the film to expand. The PDI shows the distribution of particles microemulsion. The PDI values are given in Table 7.13. All values were found to be within limits (<1) which showed that the particles are evenly distributed throughout the microemulsion[18].



(A) (B)

(C) (D)

Figure 12: Particle size distribution of LOV loaded micro-emulsions

Key: A-Batch LM1, B-Batch LM2, C-Batch LM3, D-Batch LM4. (A)= 100.3nm (B)= 86.9 (C)= 95 (D) = 101.6

Table 11 Particle size analysis of various batches

Formulation Code	Particle size(nm)	PDI
LM 1	100.3	0.564
LM 2	86.9	0.350
LM 3	95	0.346
LM 4	101.6	0.482

Labrasol-tween 80 leads for decreasing interfacial free energy in negative terms and reducing surface tension of two immiscible phase's moves for formation of homogenized droplets of safflower oil phase which might be having equal surface area. Due to uniform dispersion of homologous droplets of oil phase in dispersion media of water assisted by HLB theory of surfactant combination system facilitates for formation of LOV loaded micro-emulsion. The combined effect of HLB of surfactant co-surfactant combination system and RHLB (Required HLB) of oil plays an important role in reduction of particle size of drug loaded micro-emulsion up to nanometric range

Zeta Potential Determination

The zeta potential of the particles is used as a measure of particle charge and/or electrostatic repulsion. According to the theory of DLVO system could be regarded as stable if the electrostatic repulsion dominated the attractive Van der Waals forces. The physical stability of disperse system increases with increasing electrostatic repulsion energy. The electrostatic repulsion increases with increasing surface charge and increasing thickness of the diffuse layer. To investigate the surface properties of the LOV micro-emulsion under study zeta potential was measured in distilled water adjusted to conductivity of 0.0850 mS/cm and 0.0357 mS/cm respectively. Usually particle aggregation is less likely to occur for charged particles with high zeta potential > 30 mV due to electric repulsion. Usually lipid nanoparticles are negatively charged on the surface (Schwarz C et al, 1999). However, the zeta potential allows only predicting the electrostatic stabilization. Further stabilizing factors or destabilizing factors cannot be predicted with the zeta potential.

Lov safflower oil micro-emulsion under investigation showed a zeta potential of respectively, measured in distilled water as shown in Table 7.13. and Fig7.13

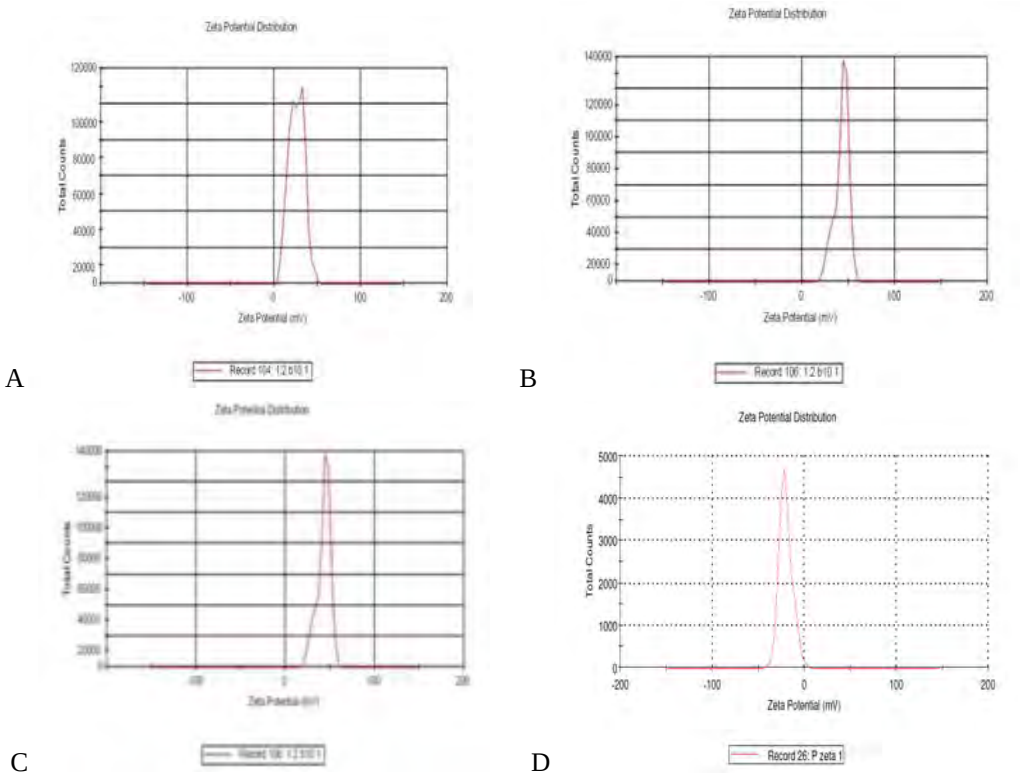


Figure 13: Zeta potential of LOV loaded micro-emulsions

Key: A-Batch LM1, B-Batch LM2, C-Batch LM3, D-Batch LM4. (A)= 52.8mV (B)= 42.8mV (C)= 34.6mV (D) = -20.6mV

Table 12 Zeta potential of various batches

Batch code	Zeta potential(mV)
LM1	25.8
LM2	42.8
LM3	34.6
LM4	-20.3

It has been reported, that in a combined electrostatic and sterical stabilization a zeta potential of about 42mV and 34mVcan be sufficient for physical stability. Hence LM2 and LM3 have potential of physical stability as they have zeta potential of 42.8mV and 34.6mV, respectively which shows that LOV micro-emulsion (LM2) has sufficent charge and mobility to inhibit aggregation of particles.

CHARACTERIZATION OF MICRO-EMULSION

Physical Characterization

The LOV loaded micro-emulsion batches prepared by water titration method were found to be transparent micro-emulsions with characteristic odor, and little viscous in consistency as shown in Fig. 7.14. This is due to presence of surfactant in the dispersions. There was no precipitation in the micro-emulsions stored for 3 days.

Figure 14: Micro-emulsion formulations

Refractive Index

The refractive index of micro-emulsion was measured by using Abbe's refractometer. The refractive index of micro-emulsion was almost same to the refractive index of water. The refractive index values prove the transparency of the system as shown in Table 7.14

Table 13: Refractive index of micro-emulsions

Sample	Refractive Index
Water	1.333
LM 1	1.412
LM 2	1.340
LM 3	1.393
LM 4	1.385

Viscosity

The viscosity of microemulsion was determined by Brookfield viscometer at room temperature. The formulations of LM3 batch exhibit Newtonian flow shown in Fig. 26. Micro-emulsion samples showed relatively low viscosities which were close to the safflower oil. Viscosity of microemulsion increased with increase in water concentration, which might probably due to an interaction between oil and water. The observed viscosities of the microemulsion at different angular velocities are given in Table 7.15 (Mandal et al, 2006).

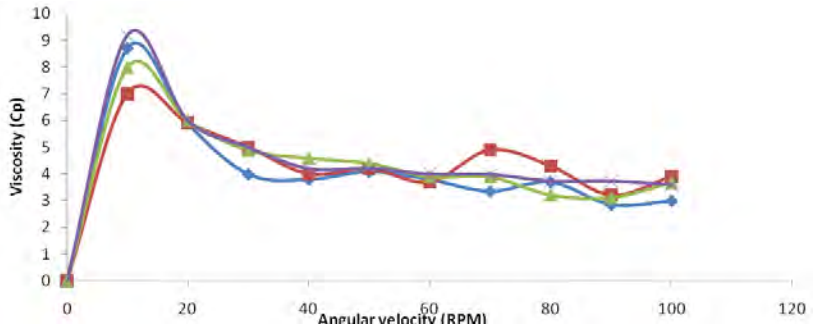


Figure 15: Rheological study of LOV loaded micro-emulsion

Table 14: Viscosity of LOV loaded micro-emulsion

Angular velocity (rpm)	Viscosity (cps)			
	LM1	LM2	LM3	LM4
10	15.62	10.73	14.65	11.65

LOV Content Determination

The LOV content of the micro-emulsions was determined by UV spectroscopy method at 238 nm. The drug content is parameter which is used to optimized the batch drug content means amount of drug present in the formulation. It is desired to have more drug content in the formulation. Drug present in the formulation is analyzed UV spectrophotometrically using the prepared aliquots and the percent drug content is calculated. Drug content was determined in methanol and aliquots were prepared and drug content was measured. Drug content and percentage drug recovery of all batches was found shown in Table 7.14

Table 15: % LOV content of micro - emulsion

Batch code	% LOV content
LM1	88 .23
LM2	93.32
LM3	92.23
LM4	86.35

Based on the above characterization parameters the all four batches were evaluated out of which the LM2 batch were found to have good results which is shown in table below

Table 16 All four batches and their characterization

Sr.no	Batch	Particle size	Zeta potential	PDI	Drug content
1	LM1	100.3	25.8	0.464	88 .23
2	LM2	86.9	42.8	0.350	93.32
3	LM3	95	34.6	0.346	92.23
4	LM4	101.6	-20.3	0.482	86.35

As it is seen from the table that batch LM2 has lesser particle size which is desired in microemulsion formulation, has zeta potential 42.8 which suggest that formulation is stable enough, has Polydispersity index 0.350 that it is less than 1 and close to zero which suggest that formulation is uniformly distributed, and drug content of the LM2 batch is maximum that is 93.32. This concludes that from above results the LM2 batch is optimized.

Thermodynamic Stability of Micro-emulsions

The micro-emulsions has a very large interface between oil and water due to the small droplet size, they can be thermodynamically stable only if the interfacial tension is so low that positive interfacial energy is compensated by negative free energy of mixing. Negative free energy formation is achieved when large reductions in surface tension are accompanied by significant favorable entropy change. In such cases, micro-emulsification is spontaneous and the resulting dispersion is thermodynamically stable

Heating cooling cycle

All the ME formulations didn't showed any type of instability after heating cooling cycle. Phase separation, cracking or creaming of emulsion was not seen. They appeared as a single phase and clear when visually observed.

Centrifugation test

As all the formulations of ME passed the test of heating cooling cycle, they were taken for further centrifugation study. After centrifugation test, all ME samples were seen clear and no phase separation was observed after visual observation.

Freeze thaw cycle

As all the formulation batches of ME passed the centrifugation test, they were studied under freeze thaw cycle for their stability. All ME formulations didn't show phase separation or turbidity after visual observation. Hence all formulation passed this test and showed that they are thermodynamically stable.

Table 17 Thermodynamic stability study

Formulation	Heating cooling cycle	centrifugation	Freeze thaw cycle
LM2	√	√	√

* √ Formulation passed, X Formulation failed

Specific Gravity

Specific gravity is a physical property it is defined as relative density of substance with respect to water. Specific gravity of LM2 micro-emulsion was found to be 0.985 Specific gravity data prove purity of the formulation.

Percent Transmittance

LM2 microemulsion showed greater than 90% percent transmittance. The percent transmittance data prove the transparency of the system and formulation having good stability (Monahan, S.D et al., 2002).

Transmission Electron Microscopy (TEM)

Two best formulations LM2 was optimized based on earlier zeta potential and particle size determination. These formulations were further evaluated in order to provide more insight about the morphology and particle size. The TEM photograph shown in Fig. 28. The photograph of formulation revealed that the particles were spherical with homogeneity. The size of the LM2 formulation was 87nm.

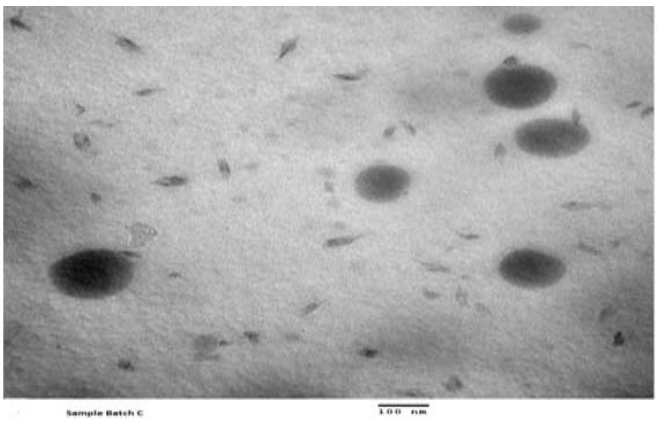


Figure 16: Transmission electron microphotographs of drug-loaded Micro-emulsion

In vitro Drug Release Studies

In vitro drug release studies were carried out in USP dissolution test apparatus II using dialysis bag method. Dialysis membranes retained oil phase of micro-emulsion and allowed LOV molecules to pass through, which were released over the time into the dissolution medium.

The drug release profiles are shown in Fig.7.17. Significant variation in the drug release rate was observed. Results reveal initial burst release in first 2 h. with 20% of drug release. This may be attributed to drug adsorption on to surface of nanoparticle. Drug release in later stage was continuous and indicating slow drug release following diffusion from the oil phase. The drug dissolution results revealed sustained release of LOV from formulation after 12 h it was found to be 78.72%. The drug release from marketed formulation was 55.33%. Data generated shows drug release profile of formulation shows Peppas model. On comparing these results it was observed that drug release from optimized microemulsion was better than reference products. Hence here we may state or conclude that the bioavailability may have increased and the due to increase in release of drug from the optimized microemulsion formulation is greater than marketed formulation. But the increase in bioavailability is further stated by in vivo studies which confirms increase in bioavailability.

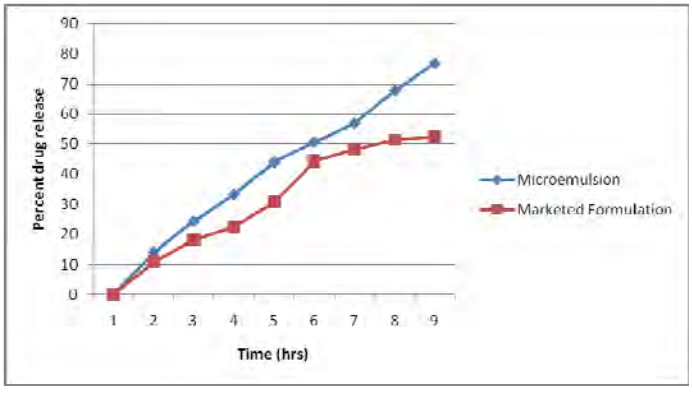


Figure 17: Average % release of LOV from micro-emulsion

Stability Study of Micro-emulsion

Prepared and optimized microemulsion formulation LM2 was subjected to stability studies at 40^0C and 75% relative humidity. Samples were withdrawn after 0 days, 1, 2 and 3 months and were evaluated for parameters such as appearance, particle size studies, drug content,

At given intervals, formulation was observed for clarity. The appearance formulation was clear when observed under black and white background. It showed slight increase in particle size, the observations are shown in Table 7.16. The formulation showed slight decrease in drug content after 3 months (84.35%). From the stability studies it was confirmed that safflower oil LOV microemulsion remained stable

Table 18 Stability studies and parameters evaluated

Formulation	Period of testing	Parameters evaluated	
		Particle size analysis	Drug content (%w/v)
LM2	0 Days	90.22	88.23
	1 Month	102	89.21
	2 Month	108.2	90.53
	3Month	107.6	84.3

In vivo Study

Calibration overlay of lovastatin

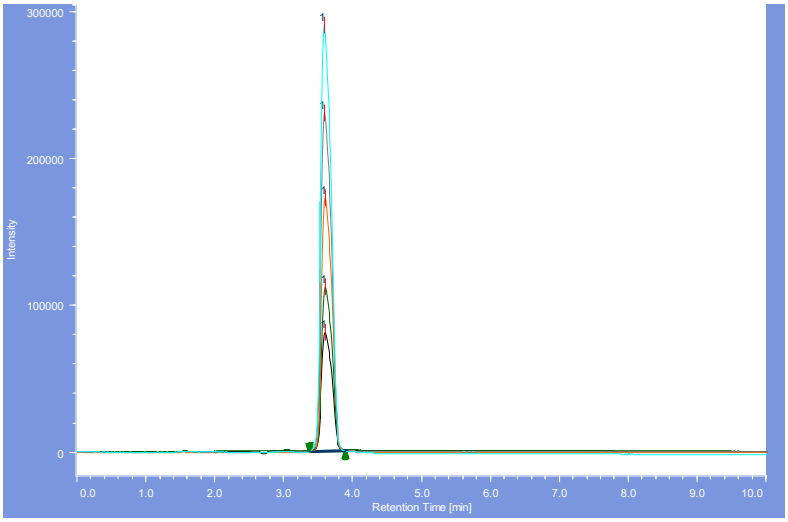


Fig 18 calibration of lovastatin in HPLC

Analysis of LOV in plasma

The in vivo study was performed to quantify LOV, after oral administration of formulations containing drug. The plasma concentration time profiles of LOV in wistar rats following oral administration of the LM2 micro-emulsion and marketed tablet (Atorlip TM 10) suspension formulations were compared

Table 19: Analysis of LOV in plasma

Time interval (mins)	Peak area of LM2	Peak area of STD
30	747178	36452
60	26020	254360
90	36470	14179

The blood samples were withdrawn at 30, 60, 90 mins. The data analyzed by HPLC is given in Table 33. The peak area obtained was used to calculate the concentration at each time interval T_{max} for AM3 and marketed tablet suspension was observed at 1h and 2 h, respectively, the corresponding area and retention time values for both formulations as shown in Fig 32.

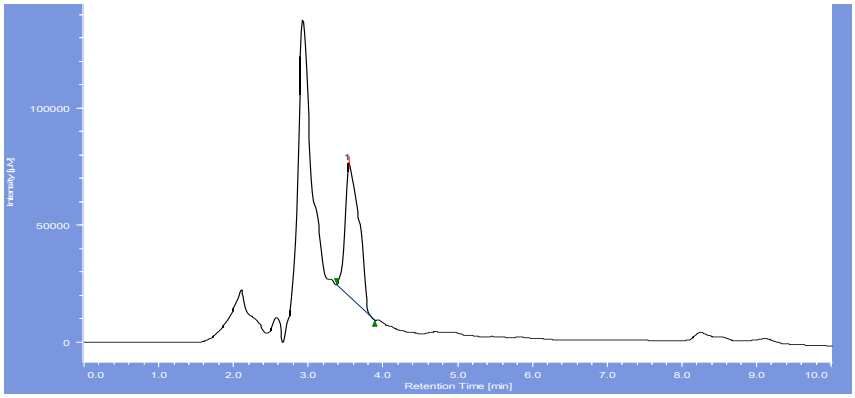


Figure 19: Chromatogram of LOV loaded micro-emulsion in plasma

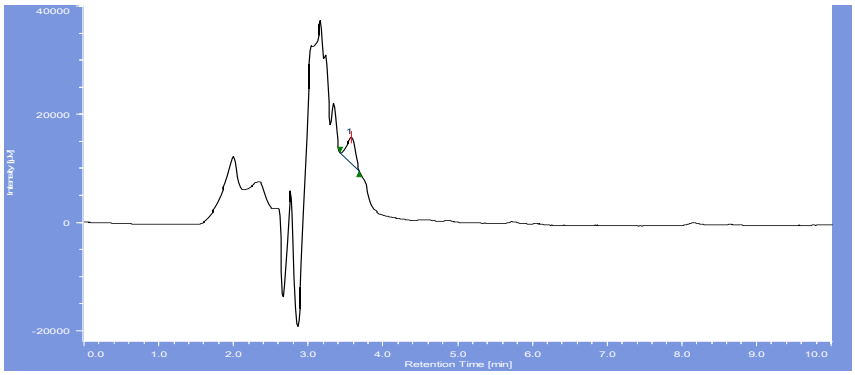


Figure 20: Chromatogram of marketed tablet suspension in plasma

Relative pharmacokinetic parameters of LM2 micro-emulsion and tablet suspension has shown in Table 7.18.

Table 20: Relative pharmacokinetic parameters of different formulations containing LOV

Formulation	C_{max} (µg/mL)	t_{max} (h)	AUC_{0-t} (µg. h/mL)	Rel. BA (%)
LM2	40.94	1	171.07	3.6
STD	19.81	2	47.52	-

STD - Marketed tablet suspension, LM2- micro-emulsion

It was found that the C_{max} for microemulsion formulation (40.94 µg/mL) represents greater improvement than the tablet suspension (19.81 µg/mL).The significance in C_{max} of both formulation clearly observed from Fig. 33

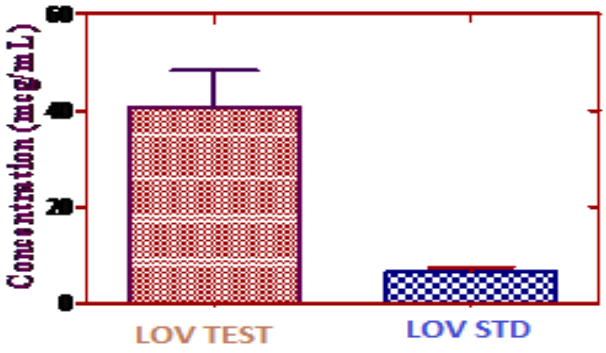


Figure 21: C_{max} of LOV

It was also observed that AUC_{0-t} LM2 formulation was 171.07µg. h/mL and thus the difference was highly significant ($p<0.05$) as compared to AUC_{0-t} (47.52) tablet suspension. Statistically, the difference in t_{max} of LM2 micro-emulsion was found to be significant ($p<0.05$) when compared to tablet suspension. Both the values of

AM3 were very significant ($p<0.05$) as compared to marketed tablet suspension. The % relative bioavailability of AM3 micro-emulsion to that of conventional tablet suspension was 3.6%.

The absorption of LOV micro-emulsion resulted in 3.6 fold increase in bioavailability as compared to conventional tablet suspension. The in vivo studies revealed significantly greater extent of absorption from LOV loaded micro-emulsion than the conventional tablet suspension. Furthermore, this result attributed that the presence of surfactants in micro-emulsion system might have caused changes in membrane permeability of the GI tract and the inhibition of an apically polarized efflux system, which could lead to enhancement of the oral absorption.

Estimation of antihyperlipedemic activity of oil

All the results were compared including group I, II, III, IV of which group III was of formulation which showed decrease in the cholesterol and most prominently decrease in DL which is bad cholesterol. The results of antihyperlipedemic results of safflower oil is shown in table 7.19 safflower oil shows antihyperlipedemic activity hence the formulated dosage form exerts synergistic effect.

Table 21 Effect of safflower oil on serum lipid profile of rats

Treatment	Cholesterol(mg/DL)	HDL(mg/DL)	LDL(mg/DL)
Group I	159.2	51.3	53.93
Group II	89.2	61.25	71.65
Group III	80.5	78.2	60.23
Group IV	97.28	57.25	71.65

CONCLUSION

Successful incorporation of lovastatin, a poorly soluble BCS Class II drug was carried out into micro-emulsion by water titration method. The formulated LOV loaded microemulsion exhibited nanometer size range spherical structure with sustained release profile in-vitro.

This method produced micro-emulsion in nanometer size. Particle size of microemulsion produced by water titration showed least increase in size. Refractive index and % transmittance showed good isotropic formulation. In vivo studies on rat revealed overall increase in bioavailability of the drug upon oral administration of micro-emulsion compared to marketed lovastatin tablet formulation. It also showed that safflower oil which was used as the oil phase showed antihyperlipedemic activity and reduces the LDL i.e. bad cholesterol by 30% all by itself hence formulating lovastatin microemulsion would exert the synergistic effect and reduce the dose and will increase the therapeutic activity.

The experimental findings collectively support micro-emulsions potential to enhance oral absorption and bioavailability of poorly water soluble drugs, and are thus a promising delivery system. The future of microemulsion is in the same way as other fields, the science and technology of the microemulsions is a rapidly growing area, which gained a very high importance during last two decades. The future need for the developing of systems and materials with sustainability and biodegradability requires that biodegradable surfactants and compounds must be developed. Doing this, will be another reason increasing the importance of the microemulsions which can be used in bio systems. As far as the future of this developed microemulsion is concerned the proper long term stability is essential to be performed, long term stability will give idea about the every aspect of stability at different conditions. And proper in vivo studies needed to be carried out which will help to prove that or to state the increase in bioavailability.

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