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Oil based nanocarrier for improved oral delivery of silymarin: *In vitro* and *in vivo* studies

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ABSTRACT

Silymarin, obtained from *Silybum marianum* is used for hepatoprotection and having poor aqueous solubility and low bioavailability. Therefore, it was thought to incorporate the drug into oil-in-water (o/w) based nanocarrier to increase its oral bioavailability. In the present study, o/w nanocarrier was prepared by titration method and was characterized for droplet size, viscosity, etc. *In vitro* drug release was carried out by dialysis membrane method. A pharmacokinetic study was performed to determine maximum plasma concentration (C_{max}), area under the curve (AUC), etc. and hepatoprotective activity was evaluated in terms of serum enzyme estimation. The optimized nanoemulsion formulation consisted of sefsol-218 as oil, tween 80 as a surfactant and ethanol as a co-surfactant having nano-droplet size and low viscosity. *In vitro* dissolution studies showed higher drug release from nanoemulsion as compared to bulk drug suspension. The AUC and C_{max} of nanoemulsion after oral administration were 4-fold and 6-fold higher than those of drug suspension of silymarin. The results of pharmacokinetic studies showed better effects of developed nanoemulsion than drug suspension and marketed formulation. The present study showed that the nanoemulsion being a versatile technology has the potential to improve the biopharmaceutics properties of silymarin.

1. Introduction

Silymarin is a complex mixture of four flavonolignan isomers: silybin (70–80%), silychristin (20%), silydianin (10%), and isosilybin (0.5%), obtained from *Silybum marianum* (Luper, 1998). Among the isomers, silybin is the major and most active component and responsible for its pharmacological activity. It has been used for centuries to self-treat liver disorders (Fraschini et al., 2002; Pradhan and Girish, 2006). Its mechanism of action includes inhibition of hepatotoxin binding to receptor sites on the hepatocyte membrane; reduction of glutathione oxidation to enhance its level in the liver and intestine; antioxidant activity; and stimulation of ribosomal RNA polymerase and subsequent protein synthesis, leading to enhanced hepatocyte regeneration (Dixit et al., 2007). It is a natural lipotropic agent with low bioavailability i.e. 23–47% and lipophilic in nature having a log *p* value of 1.41. Poor bioavailability is due to poor entral absorption, poor solubility or degradation by gastric fluid (Giacomelli et al., 2002). Hence, silymarin is required in large dose to achieve therapeutic plasma levels. This led to the development of novel drug delivery system to increase its solubility and oral absorption. A number of approaches have been used to increase its solubility and thereby bioavailability. These include complexation with cyclodextrin (Arcari et al., 1992) and phospholipids (Yanyu et al., 2006), incorporation in solid dispersion (Chen et al., 2005), solid lipid nanoparticles (He et al., 2007) and formulation of self emulsifying drug delivery system (Woo et al., 2007; Wei et al., 2006).

Numerous potent lipophilic drugs exhibit low oral bioavailability due to their poor aqueous solubility and cannot be delivered by the oral route of administration in their original form due to instability, low membrane permeability, poor solubility and efflux transport mechanisms, etc. (Leonard et al., 2006). In recent years, lipid-based formulations (incorporation of the active lipophilic component into inert lipid vehicles) are used to improve the oral bioavailability of poorly water-soluble drug compounds, which include micro or nanoemulsions, oils, self-emulsifying formulations, surfactant dispersions, liposomes, solid lipid nanoparticles and lipid nano carriers etc. Nanoemulsion offers several advantages over these drug delivery systems like higher solubilization capacity, rapid onset of action (no extra time for dispersion), reduced intersubject variability in terms of gastrointestinal fluid volume and longer shelf life (Shafiq-un-Nabi et al., 2002), toxicological safety, a high content of the lipid phase and the possibility of large scale production by high pressure homogenization (Mehnert and Mader, 2001). The decrease in the rate of dissolution of the self emulsifying drug delivery system may lower the potential of the developed

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system as compared to a liquid micro/nanoemulsion, administration of a poorly water-soluble compound in a dissolved state and in a liquid formulation can reduce the energy associated with the solid–liquid transition (Bali et al., 2010). It has been revealed from literature that the studies conducted on formulation based drug delivery systems of silymarin are restricted to either in *in vitro* dissolution studies/optimization studies or pharmacokinetic studies of the developed system. But in the present study, along with the pharmacokinetics, biochemical estimation was carried out to support the pharmacokinetic data and the results are better than the reported systems.

In the present investigation, an attempt has been made to develop a thermodynamically stable nanoemulsion formulation of silymarin to increase its aqueous solubility, stability and thereby increasing the oral bioavailability, which has not been attempted till date. The nanoemulsion of silymarin was prepared using oil, surfactant and co-surfactant by titration method. This method is easy to carry out in the normal laboratory conditions and without use of any sophisticated instrument. Thermodynamic stability studies and dispersibility test were carried out and formulation was characterized for size, surface morphology, viscosity, conductivity and refractive index to select the stable and best formulation. *In vitro* drug release was carried out by dialysis membrane method and compared with conventional dosage form. Pharmacokinetic study was compared with the drug suspension and conventional marketed formulation. Hepatoprotective potential of silymarin nanoemulsion, drug suspension and conventional marketed formulation was also evaluated against CCl_4-induced intoxication and the activity of serum enzymes [aspartate transaminase (SGOT), alanine transaminase (SGPT) and alkaline phosphatase (ALP)] was measured.

2. Material and methods

2.1. Materials

Silymarin was provided from Ranbaxy (Gurgaon, India) and Sefsol 218 (Propylene glycol-monocaprylic ester) from Nikko Chemicals (Tokoyo, Japan) as a gift samples. Tween 80 (Polyoxyethylene sorbitan monooleate) was purchased from Merck (Schuchardh, Hokenbrunn, Germany). Water was taken from Milli-Q water purification system (Millipore, Billerica, MA). All other chemicals and reagents used were of analytical (AR) grade and procured from Merck (India) and S.D. Fine, Chem. (India). All components used for the formulation of nanoemulsion were pharmaceutically acceptable for oral administration.

2.2. Formulation development and optimization

2.2.1. Screening of components

Phase solubility studies were done to determine the most suitable oil for the preparation of nanoemulsion for silymarin. Three mL of selected oils [Oleic acid, isopropyl myristate (IPM), glycerol triacetate (Triacetin), caproyl 90, propylene glycol monocaprylic ester (Sefsol 218), propylene glycol laurate (Lauroglycol), labrafac] were taken in small vials (5.0 mL capacity) and excess amount of drug was added in the oils and kept in biological shaker (Nirmal International, Delhi, India) for 72 h at a constant temperature (25 ± 1.0 °C) to reach to an equilibrium (Shafiq et al., 2007; Shakeel et al., 2007). The samples were removed from shaker and centrifuged at 3000 rpm for 15 min. The supernatant was filtered through a 0.45 μm membrane filter and the concentration of drug was determined by taking absorbance using UV at λ_{max} of 288 nm after dilution.

Table 1
Observation for dispersibility test.

S. No.	Grade	Observations
1	A	Rapidly forming (within one min) nanoemulsion, having a clear or bluish appearance.
2	B	Rapidly forming, slightly less clear nanoemulsion, having a bluish white appearance.
3	C	Fine milky emulsion that formed within 2 min.
4	D	Dull grayish white emulsion having slightly oily appearance that is slow to emulsify (longer than 2 min).
5	E	Formulation exhibiting either poor or minimal emulsification with large oil globules present on the surface.

2.2.2. Phase diagram construction

Different volume ratios (1:0, 1:1, 1:2, 1:3, 2; 1, 3:1) of surfactant (Tween 80) and co-surfactant (Ethyl alcohol) (Chennamsetty et al., 2005) mixture (Smix) were made and stocks of 100 mL from each group were prepared. For each phase diagram, sixteen different combinations of oil (Sefsol 218) and Smix [1:9, 1:8, 1:7, 1:6, 1:5, 2:8 (1:4), 1:3.5, 1:3, 3:7 (1:2.3), 1:2, 4:6 (1:1.5), 5:5 (1:1), 6:4 (1:0.7), 7:3 (1:0.43), 8:2 (1:0.25), 9:1 (1:0.1)] were made in different volume ratios from 1:9 to 9:1 so that maximum ratios were covered for the study (Lawrence and Rees, 2000). The mixture of selected oil and Smix were titrated against distilled water. After every 5% addition of aqueous phase to the oil and Smix mixture, visual observation was made and recorded. The percentage of water, oil and Smix in which nanoemulsion forms were selected and plotted on ternary phase diagrams with one axis represents the aqueous phase, the other representing the oil and the third representing the Smix. These observations were made for each Smix ratio in each group separately.

2.2.3. Selection of formulation from phase diagram

Different formulations were selected from each phase diagram plotted for different Smix ratios on the basis of (Shafiq et al., 2007):

- The oil concentration is such that it dissolves single dose of (140 mg) of silymarin easily.
- Oil concentration from each phase diagram was selected as a multiple of five, i.e. 5%, 10% 15% and 20%.
- For each oil percentage selected, the concentration of surfactant should be minimum for nanoemulsion preparation.

2.2.4. Thermodynamic stability studies

Selected formulations were subjected to thermodynamic stability stress tests as heating cooling cycle, centrifugation and freeze–thaw cycle: Heating–cooling cycles between 45 °C temperature and room temperature (25 ± 2 °C) with storage time of 24 h at each temperature (six cycles each) followed by centrifugation (5000 rpm for 30 min) and then

Freeze–thaw cycles at −20 °C in a deep freezer (Vest frost, Hyderabad, India) and room temperature (25 ± 2 °C) for 24 h were carried out six times (six cycles each).

2.2.5. Dispersibility test

The efficiency of self emulsification of oral nanoemulsion was assessed using a standard USP XXII dissolution apparatus. One mL of nanoemulsion was mixed with 500 mL of media (distilled water and 0.1N HCl, seperately) maintained at 37 ± 0.5 °C. The dissolution paddle rotated at a speed of 50 rpm to provide gentle mixing. The *in vitro* performance of the formulations was visually assessed using the grading system given in Table 1 (Ping et al., 2005). Formulations that passed the thermodynamic stability as well as the dispersibility test in Grade A were selected for further studies.

2.2.6. Formulation of drug containing nanoemulsion

Drug containing nanoemulsion formulations were prepared by dissolving 20 mg/kg body weight of drug in 5%, 10%, 15% and 20% of oil and respective Smix ratios on vortex mixer and added required quantity of aqueous phase. The resulting mixture gave nanoemulsion.

2.3. Characterization of silymarin nanoemulsion

2.3.1. Visual observation

Visual observation was done to differentiate between nanoemulsion and macroemulsion.

2.3.2. Surface morphology

Surface morphology of nanoemulsion was studied by Transmission Electron Microscopy (TEM) TOPCON 002B (Topcon, USA) (Shafiq et al., 2007; Shakeel et al., 2007). A drop of nanoemulsion was diluted with distilled water (1:100), filtered (0.22 μm) and applied on carbon coated grid with 2% phosphotungestic acid and kept it for 30 s. The dried coated grid was taken on a slide and covered with a cover slip. The slide was observed under the light microscope operating at 200 KV.

2.3.3. Droplet size analysis

Droplet size of the nanoemulsion was determined by photon correlation spectroscopy using Zetasizer 1000 HS (Malvern Instruments, Worcestershire, UK). The formulation was diluted with distilled water and filtered through 0.22 μm membrane filter in order to eliminate multiscattering phenomena and experimental errors. Light scattering was monitored at 25 °C at a scattering angle of 90°.

2.3.4. Viscosity determination

Brookfield DV III ultra V6.0 RV cone and plate rheometer (Brookfield Engineering Laboratories, Inc, Middleboro, MA) with spindle # CPE40 at 25 ± 0.5 °C was used for the determination of viscosity of the formulations. The optimized parameters used were: Sample size/wt: 0.5 g, Speed: 30 rpm, Data interval: 1.0, Loop start: 1, Wait time: 30 min, Temperature: 25 ± 0.3 °C, Share rate: 60 s^{-1}.

2.3.5. Refractive index

Refractive index of formulation was determined using an Abbes type of refractrometer (Precision Standard Testing Equipment Corporation, India), which was calibrated using castor oil prior to use.

2.3.6. Electrical conductivity

The conductivity (σ) of nanoemulsion was determined by using conductometer, CDM 230 (Radiometer, Copenhagen, Denmark). The reading was taken at the frequency of 94 Hz, having a cell constant of 0.11 cm^{-1}. The measurements were performed at 25 ± 1 °C. It is determined to check the type of nanoemulsion, whether it is oil-in-water (o/w) or water-in-oil (w/o). If the formulation is o/w, then the current will pass through the water and the deflection will be seen. But if the formulation is w/o, then current will not pass through oil and no deflection will be seen.

2.4. In vitro drug release

Dissolution studies were performed to compare the release of drug (20 mg silymarin per kg body weight) from six different formulations (TF13, TF14, TF18, TF19, TF20, TF21) and marketed formulation i.e. silymarin suspension (SILYBON®) manufactured by Microlabs, Bangalore (Mfg. Lic. No. NB-31/62).

In vitro release test was performed in 500 mL of distilled water and simulated gastric fluid using dissolution apparatus # 2, at 50 rpm and 37± 0.5 °C (Hanson Research SR8 plus, California, United States). One millilitre of nanoemulsion formulation was placed in treated dialysis bag (MWCO 1200 g/mole, Sigma Aldrich, USA). One millilitres samples were withdrawn at regular time intervals (0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 24 h) and aliquot amount of distilled water/simulated gastric fluid was replaced. The samples were analysed for the drug content using UV spectrophotometer (UV-1601 Shimadzu, Japan) at 288 nm. The release of the drug from nanoemulsion formulations was compared with the marketed formulation i.e. SILYBON® suspension.

2.5. Animal handling and care

Approval to carry out *in vivo* study was obtained from Jamia Hamdard, Institutional Animal Ethics Committee, New Delhi and their guidelines were adhered for the complete study (Registration No. 173/CPCSEA, 2008). The animals used for *in vivo* experiments were adult Wistar female albino rats (150–200 g) obtained from Central Animal House of Hamdard University, New Delhi, India.

The *in vivo* study was performed to carry out pharmacokinetic studies of silymarin after oral administration of silymarin formulations. The rats were divided into three groups, each containing six animals. The plasma profiles were compared in adult female albino Wistar rats after oral administration of the nanoemulsion (TF14) formulation, marketed suspension (SILYBON®) and drug suspension (in 10% gum acacia).

2.6. Pharmacokinetic study

The animals were kept under standard laboratory conditions, at 25 ± 2 °C temperature and 55 ± 5% relative humidity, which were housed in polypropylene cages, six per cage, with free access to standard laboratory diet (Lipton feed, Mumbai, India) and water *ad libitum*. Formulations were administered orally using oral feeding needle. The rats were anesthetized using ether and blood samples (0.5 mL) were withdrawn from the tail vein of rat at 0 (pre-dose), 0.5, 1, 1.5, 2, 4, 8, 12, 24, 36, 48 and 72 h in microcentrifuge tubes containing eight mg of EDTA as an anticoagulant. The blood collected was mixed with the anticoagulant properly and centrifuged at 5000 rpm for 20 min. Plasma was separated and stored at -21 °C until analysis using HPTLC method reported by author (Parveen et al., 2010).

2.7. Hepatoprotective activity

2.7.1. Dosing schedule

Carbon tetrachloride was used as toxicant for hepatotoxicity in experimental animal models (Racknagel et al., 1989). Animals were divided into five groups of six each and treated as per the schedule given in Table 2.

2.7.2. Serum biochemical estimation

Blood was collected (1.5–2.0 mL) in a sterile centrifuge tube from tail vein of all the groups of overnight fasted rats using microcapillary tube on sixth day and left undisturbed at 37 °C for 45 min to exude serum and clot formation. The serum was aspirated using a sterile pipette after centrifugation at 3000 rpm for 15 min and used for biochemical estimation like SGOT, SGPT (Reitman and Frankel, 1957) and ALP (Bessey et al., 1964).

2.8. Statistical analysis

The pharmacokinetic data among different formulations were compared for statistical significance by the one-way ANOVA followed by Tukey-Kramer multiple comparisons test using Graph Pad Instat software (Graphpad Software Inc., CA, USA).

Table 2
Dosing schedule of silymarin formulations for hepatoprotective activity in wistar albino rats.

Group No.	Group	Drug	Dosing schedule
I	Control	Aqueous 2% gum acacia solution	1 mL/kg (oral) gum acacia solution for five days, daily
II	Toxic control	Aqueous 2% gum acacia solution and carbon tetrachloride	1 mL/kg (oral) gum acacia solution, daily and single dose of CCl_4 (1 mL/kg, s.c.) on day 2 and 3
III	Standard	Bulk drug suspension of silymarin and carbon tetrachloride	Silymarin equivalent to 42 mg/kg/mL on all five days and CCl_4 (one mL/kg, s.c.) on day 2nd and 3rd, one hour after the administration of standard drug
IV	Marketed	Marketed conventional formulation and carbon tetrachloride	Silymarin (equivalent to 35 mg/kg/mL) on all five days and CCl_4 1 mL/kg, s.c. on day 2 and 3, 1 h after the administration of suspension.
V	Test	Optimized nanoemulsion (TF14) and carbon tetrachloride	Silymarin equivalent to 20 mg/kg/mL) on all five days and CCl_4 1 mL/kg, s.c. on day 2 and 3, 1 h after the administration of nanoemulsion.

The results of biochemical estimation are expressed as mean ± SEM of six animals from each group. The data was analysed by one-way ANOVA followed by Dunnnett's post hoc test. p Values < 0.05 were considered as statistically significant.

3. Results and discussion

3.1. Formulation development and optimization

3.1.1. Screening of components

Oil represents one of the most important excipients in the nanoemulsion formulation, which can solubilize marked amounts of the lipophilic drug and also because it can increase the amount of lipophilic drug transportation (Holm et al., 2002). Sefsol 218 was found to solubilize maximum quantity of silymarin i.e. 183.375 ± 0.0036 mg/mL for the preparation of nanoemulsion (Fig. 1). Therefore, it was selected as the oil phase for the development of nanoemulsion. Higher oil solubility of a poorly aqueous soluble drug will favour an overall stability of the formulation with effective dose optimization leading to cost effective delivery system for silymarin. Tween 80 was selected as the surfactant and ethyl alcohol as the co-surfactant. Surfactant lowers the interfacial tension to a very small value to aid dispersion process and provide a flexible film that can readily deform around the droplets. The presence of co-surfactants allows the interfacial film sufficient flexibility to take up different curvatures required to form nanoemulsion over a wide range of composition (Gosh and Murthy, 2006). Milli-Q water was taken as the aqueous phase. All the selected excipients for the preparation of formulation were under the GRAS (Generally Regarded As Safe) category.

3.1.2. Phase diagram construction

Pseudoternary phase diagrams were developed using the aqueous titration method. Slow titration with the aqueous phase was performed for each combination of oil and Smix, separately. The amount of aqueous phase added was varied to produce a water

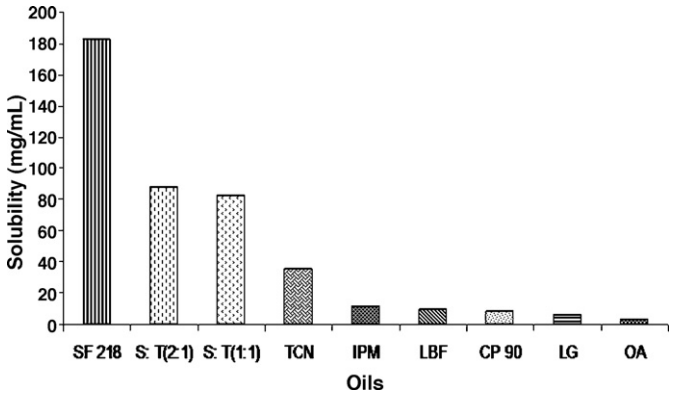

Fig. 1. Bar diagram showing the highest solubility of silymarin in sefsol 218. OA—Oleic Acid; IPM—Isoprpyl Myristate; TCN—Triacetin; CP90—Caproyl 90; SF218—Sefsol 218; LG—Lauroglycol; LBF—Labrafac; S:T(1:1)—Sefsol 218:Triacetin (1:1); S:T(2:1)—Sefsol 218:Triacetin (2:1).

concentration in the range of 5% to 95% of total volume at around 5% intervals. The phase behaviour of nanoemulsion system comprising oil, water and Smix ratio can be studied with the aid of ternary phase diagram in which each corner of the diagram represents 100% of that particular component. Special care was taken to ensure that observations are not made on metastable systems (Gosh and Murthy, 2006). The pseudoternary phase diagrams were constructed using sefsol-218 as oily phase, Smix ratio (Tween 80 as a surfactant and ethanol as a co-surfactant) and water. In the phase diagrams, only o/w nanoemulsion region is shown, other phases are not shown due to overcrowding of the diagrams. Pseudo ternary phase diagrams were constructed separately for each Smix ratio (Fig. 2a–f). In Fig. 2a, (Smix ratio 1:0) surfactant was used alone without co-surfactant and observed that a low amount of oil (25%, w/w) was solubilized at higher concentration of surfactant (45% w/w). Oil solubilization was decreased as the concentration of surfactant was increased. On addition of co-surfactant, solubilization of oil was increased at lower concentration of Smix (1:1) and the region for nanoemulsion in phase diagram was increased, as shown in Fig. 2b. With slight increase in the concentration of co-surfcatant (Smix ratio 1:2), no marked difference in nanoemulsion region in phase diagram, Fig. 2c, was observed. In Smix ratio 1:3, (Fig. 2d), there was an increrement in the nanoemulsion region with increasing concentration of co-surfactant. But as the concentration of surfactant increasing in Smix 2:1 and 3:1, the region for nanoemulsion was decreasing due to decreasing oil solubilization. It has been depicted from Fig. 2e and f, as the surfactant concentration increased (Smix 2:1 and 3:1), the region for nanoemulsion in phase diagram was remarkably decreased. This indicates that the proper ratio of Smix is important for a wide range of nanoemulsion region in phase diagram. Different formulations having less than 25% of the oily phase and minimum quantity of Smix were selected from phase diagrams for further studies. This may be attributed to the fact that the addition of co-surfactant may lead to greater penetration of the oil phase in the hydrophobic region of the surfactant monomers thereby further decreasing the interfacial tension, which will lead to increase in the fluidity of the interface and thus increasing the entropy of the system (Gosh and Murthy, 2006). While studying the phase diagrams (Fig. 2a–f), it can be seen that transient negative interfacial tension is rarely achieved by the use of single surfactant, usually necessitating the addition of a co-surfactant. Fluid interfacial film is again achieved by the addition of a co-surfactant. In the absence of co-surfactant, a highly rigid film is formed by the surfactant and thus produces nanoemulsion over only a very limited range of concentration (Lawrence and Rees, 2000).

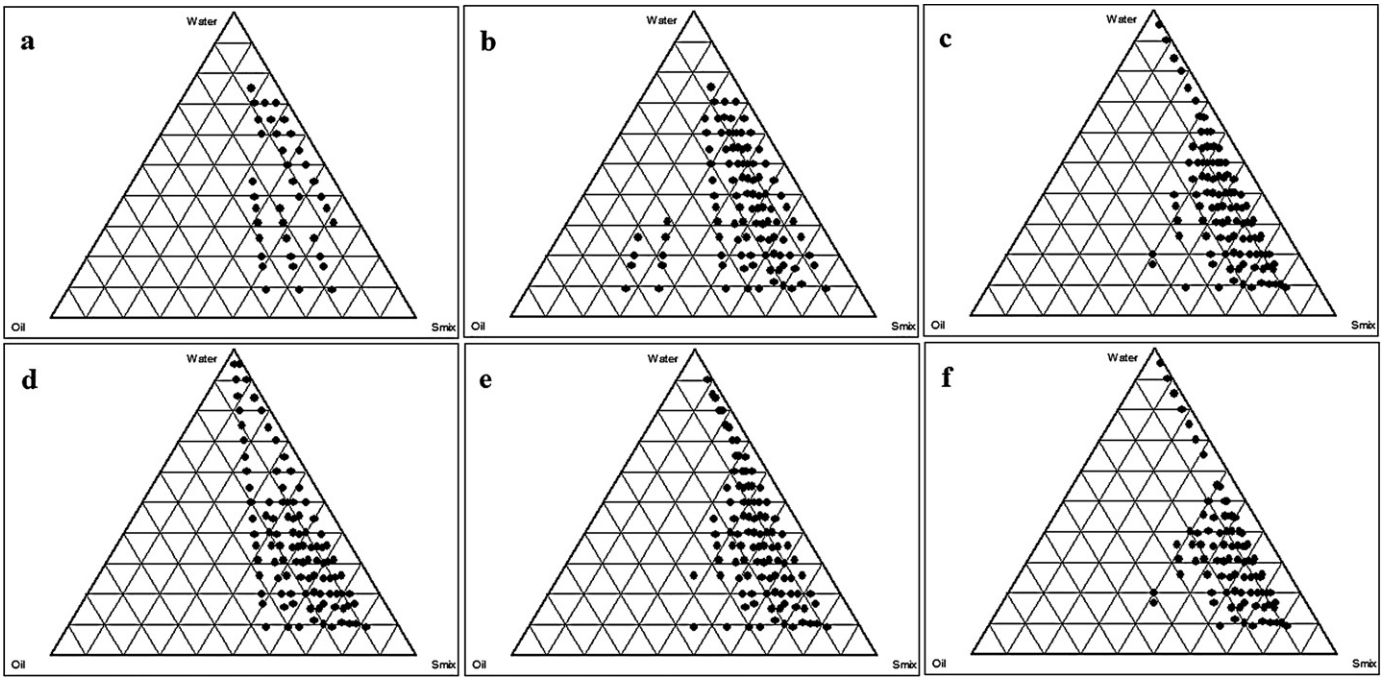

Fig. 2. Pseudo ternary phase diagrams for nanoemulsioin using sefsol 218 as an oily phase, Tween 80 as a surfactant and ethanol as a co-surfactant. (a) Smix ratio 1:0, (b) 1:1, (c) 1:2, (d) 1:3, (e) 2:1 and (f) 3:1. Dotted area indicates nanoemulsion region.

3.1.3. Thermodynamic stability studies

Nanoemulsions are thermodynamically stable systems with no phase separation, creaming or cracking. Therefore, the selected formulations were subjected to thermodynamic studies (i.e. heating cooling cycle, centrifugation and freeze–thaw cycle). The observation for thermodynamic stability studies are given in Table 3. Formulations, which did not pass the thermodynamic tests were dropped out and the remaining were subjected to dispersibility test. In case of macroemulsions, the interfacial energy is much larger than the entropy and hence the process of emulsification is non-spontaneous i.e. energy is needed to produce the emulsion by the use of high-speed mixture, whereas in case of nanoemulsion the interfacial tension is made sufficiently low so that interfacial energy become comparable or even lower than the entropy of dispersion, and hence the free energy of the system becomes zero or negative. This explains the thermodynamic stability of nanoemulsion (Razdan and Deverajan, 2003).

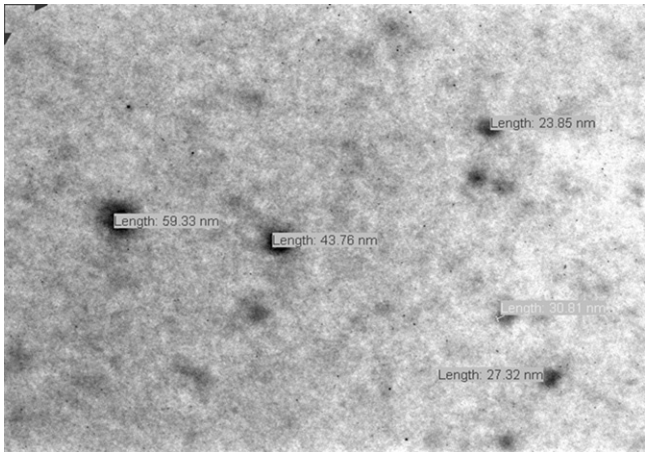

Fig. 3. Statistics graph showing particle size analysis of formulation TF14 by dynamic laser light scattering technique.

3.1.4. Dispersibility tests

The use of gastro-intestinal fluids for dilution of nanoemulsion may result in the gradual desorption of surfactant located at the globule interface leading to precipitation of the drug or phase separation of the nanoemulsion making the formulation useless. The dispersibility test was carried out to assess the efficiency of nanoemulsion (Table 1) and the results are demonstrated in Table 3. Formulations, which failed (grade C, D and E) dispersibility test, were discarded for further studies (Table 3).

3.1.5. Formulation of drug containing nanoemulsion

Six formulations (TF13, TF14, TF18, TF19, TF20 and TF21) were selected on the basis of above studies, which were subjected to further studies after addition of drug. The composition of selected formulations is given in Table 4.

3.2. Characterization of Silymarin Nanoemulsion

3.2.1. Visual observation

The nanoemulsion was clear transparent, easily flowable liquid whereas the macroemulsion was opaque and milky/cloudy white in appearance.

3.2.2. Surface morphology

Morphology and structure of the nanoemulsion droplets were determined by Transmission electron microscopy (TEM). The surrounding was bright and the nanoemulsion appeared dark (Fig. 3). A "positive" image was seen using TEM. It is capable of point-to-point resolution; therefore, droplet sizes were measured using TEM.

3.2.3. Droplet size analysis

Droplet size measurement is the important parameter to optimize the nanoemulsion formulation as well as to distinguish between the nanoemulsion from microemulsion. The TF14 formulation was showing the minimum droplet size 41.22 ± 0.00314 nm and TF19 and TF20 showed increase in the droplet size due to increased concentration of oil (Table 5). In formulation TF14, the distribution of droplets was in the range of 63–89 nm and the

Table 3
Observation for thermodynamic stability study and dispersibility test of silymarin nanoemulsion formulations.

Formulation	Smix ratio	%Smix	%Oil	%Water	H/C[a]	Centr.[b]	F/T[c]	Dispersibility		Inference
								Distilled H_2O	0.1N HCl	
TF1	1:1	20	5	75	×	–	–	–	–	Failed
TF2		25	10	65	×	–	–	–	–	Failed
TF3		25	5	70	×	–	–	–	–	Failed
TF4		30	20	50	×	–	–	–	–	Failed
TF5		30	10	60	×	–	–	–	–	Failed
TF6		30	5	65	√	√	√	A	A	Passed
TF7		35	15	50	√	√	×	–	–	Failed
TF8		35	10	55	√	√	×	–	–	Failed
TF9		35	5	60	√	√	×	–	–	Failed
TF10		40	10	50	√	√	√	B	B	Failed
TF11		40	5	55	√	√	√	B	B	Failed
TF12		45	5	50	√	√	√	A	A	Passed
TF13	2:1	30	5	65	√	√	√	A	A	Passed
TF14		35	5	60	√	√	√	A	A	Passed
TF15		40	10	50	√	√	√	B	B	Failed
TF16		40	5	55	√	√	√	A	A	Passed
TF17		45	5	50	√	√	√	C	C	Passed
TF18	3:1	40	5	55	√	√	√	A	A	Passed
TF19		40	10	50	√	√	√	A	A	Passed
TF20		40	10	50	√	√	√	A	A	Passed
TF21	1:2	30	5	65	√	√	√	A	A	Passed
TF22		30	10	60	√	√	×	–	–	Passed
TF23		35	5	60	√	√	×	–	–	Passed
TF24		40	5	55	√	√	√	B	B	Failed
TF25		40	10	50	√	√	√	C	C	Failed
TF26		45	5	50	√	√	√	A	A	Passed
TF27	1:3	30	20	50	×	–	–	–	–	Failed
TF28		35	15	50	×	–	–	–	–	Failed
TF29		40	10	50	√	√	√	A	A	Passed
TF30		45	5	50	√	√	√	A	A	Passed

[a] Heating–cooling cycle.
[b] Centrifugation.
[c] Freeze–thaw cycle.

Table 4
Selected nanoemulsion formulations of silymarin for *in vitro* studies.

S. No.	Smix Ratio	Formulation Code	%Smix	%Oil	%Water
1	2:1	TF13	30	5	65
2	2:1	TF14	35	5	60
3	3:1	TF18	40	5	55
4	3:1	TF19	40	20	40
5	3:1	TF20	40	10	50
6	1:2	TF21	30	5	65

maximum droplets (78%) were below a size of 70 nm (Fig. 4). The formulation showed nano droplets with low values of polydispersity indicating uniformity in the nanoemulsion formulation. The polydispersity values were 0.216, 0.165, 0.193, 0.327, 0.403 and 0.146 for different formulations TF13, TF14, TF18, TF19, TF20 and TF21, respectively (Table 5). Polydispersity is the ratio of standard deviation to the mean droplet size and denotes the uniformity of droplet size within the formulation. The lower the polydispersity value, higher is the uniformity of the droplet size in the formulation.

3.2.4. Viscosity determination

The viscosity of the nanoemulsions (TF13, TF14 TF18, TF19, TF20 and TF21) was given in Table 3. The viscosity of nanoemulsion formulation was very low as expected as one of the characteristic. It was observed from the Table 5 that viscosity of all the formulations was less than 24 cps. Formumlation TF14 has the minimum viscosity i.e. 21.213 ± 0.235 cps. Results also revealed that the viscosity is directly proportional to the concentration of oils and surfactants used in the formulation. It can be observed that, in general, viscosity of all formulations was very low.

Table 5
Droplet size, polydispersity index, viscosity, refractive index and electrical conductivity of the selected nanoemulsion formulations.

Formulation Code	Droplet Size ± S.D. (nm)[a]	Polydispersity Index	Viscosity ± S.D. (cps)[a]	Refractive Index ± S.D.[a]	Conductivity (μS/cm) ± S.D.[a]
TF13	97.74 ± 0.00176	0.216	23.667 ± 0.314	1.6706 ± 0.000577	531.333 ± 3.152
TF14	68.22 ± 0.00314	0.165	21.213 ± 0.235	1.6733 ± 0.001732	527.106 ± 2.082
TF18	87.43 ± 0.00219	0.193	21.612 ± 0.439	1.6821 ± 0.000713	501.365 ± 4.577
TF19	76.14 ± 0.00284	0.327	22.984 ± 0.209	1.6962 ± 0.00149	397.236 ± 1.193
TF20	92.18 ± 0.00119	0.403	23.047 ± 0.180	1.691 ± 0.001	402.482 ± 1.709
TF21	89.77 ± 0.00351	0.146	21.837 ± 0.441	1.6803 ± 0.000915	504.193 ± 5.648

[a] Mean ± S.D., $n = 3$.

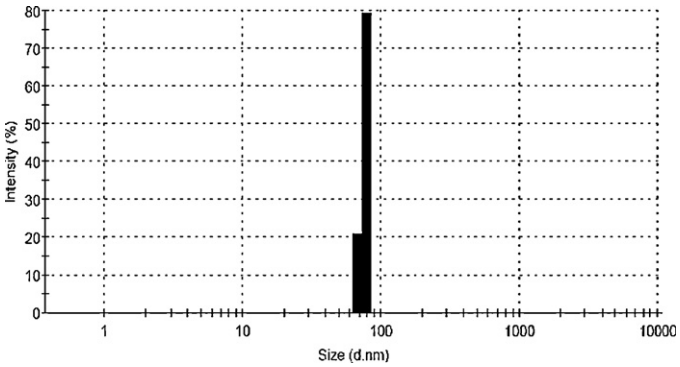

Fig. 4. Transmission electron microscopic positive image of optimized silymarin nanoemulsion.

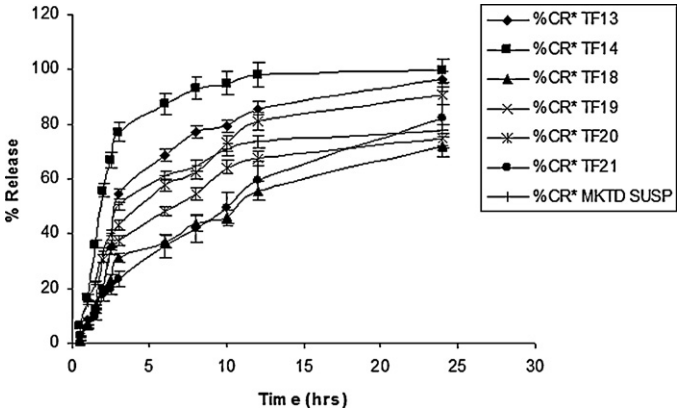

Fig. 5. Comparative *in vitro* release profile of different formulations of silymarin.

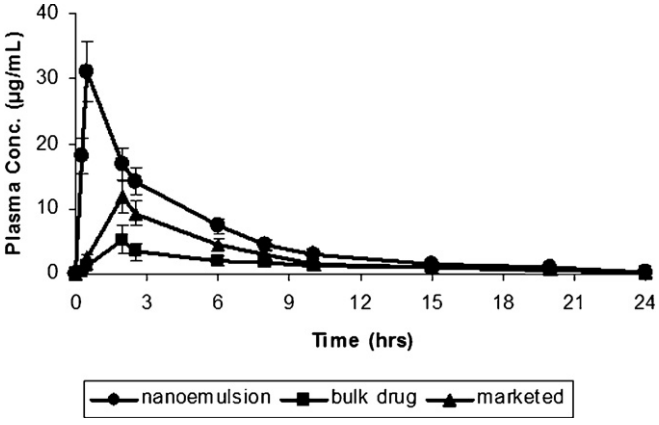

Fig. 6. Plasma concentration profile of silymarin after oral administration of different formulation to adult wistar albino rats. Data is expressed as mean ± SD ($n = 6$).

3.2.5. Refractive index

Refractive index (RI) being an optical property is used to characterize the isotropic nature of the nanoemulsion. It was observed from the Table 5 that the selected nanoemulsion formulations were chemically stable and remained isotropic in nature, thus having no drug excipient interactions. The refractive index of all the formulations was in the range of 1.6 ± 0.05. The observation table shows that as the concentration of the oils increases in the formulation, the RI increases (TF19 and TF20). In formulation TF21, as the amount of the co-surfactants increases, the rigidity of the structure decreases and so ultimately the RI decreases. RI is also affected by the size of the oils globules, as the globules size increases the RI increase as observed for the formulation TF19 and TF20 (Table 5).

3.2.6. Electrical conductivity

Electrical conductivity (σ) was determined to check not only the type of nanoemulsion (o/w or w/o) but also the stability of the nanoemulsion (phase inversion on storage). The conductivity of the formulations is given in the Table 5. The lowest σ was found 397.236 ± 1.193 μS/cm for TF19 and highest conductivity was 531.333 ± 3.152 μS/cm for TF13. This indicated that the formulation was o/w type. Because the current was passed through the water and the diffraction was seen. Electrical conductivity is directly proportional to the percentage of water. Higher the electrical conductivity more will be the percentage of water, which allows more freedom for mobility of ions.

3.3. In vitro drug release

The composition of selected formulations used for *in vitro* release was given in Table 4. Dissolution studies were performed to compare the release of drug from six different formulations (TF13, TF14, TF18, TF19, TF20, TF21) and marketed formulation i.e. silymarin suspension (SILYBON®). The concentration was determined by extrapolation of calibration curve and graph was plotted between time and percent cumulative release (Fig. 5). The pattern of drug release in distilled water and simulated gastric fluid was found very similar to each other in all formulations. The highest release i.e. 99.713% was obtained in case of TF14. The minimum release was observed in TF18 formulation, this may be due to bigger globule size, which may slow down the release of the drug from nanoemulsion formulation. All the nanoemulsion formulations showed better results as compared to conventional marketed formulation, i.e. suspension because of small globule size, low viscosity and low polydispersity values. Release of drug from TF19 (20% w/w, oil) and TF20 (10% w/w, oil) was lower than that from TF13 and TF14 (5% w/w, oil) because of higher oil concentration and bigger droplet size. In addition to this, the higher oil concentration may restrain the release of the drug into the medium due to lipophilic character of silymarin as the partitioning of drug will be more towards the oil. The complete dissolution of silymarin in oily phase showed maximum release because of small droplet size, and eventually higher surface area, which permit faster rate of drug release. The TF14 formulation was selected for *in vivo* studies because it was having higher drug release (99.713%), optimum globule size (68.22 nm), minimum polydispersity value (0.165), lower viscosity (21.213 cps), stability of nanoemulsion and drug and above all, lower surfactant concentration (35%) was selected for the *in vivo* study.

3.4. Comparative pharmacokinetic studies of silymarin formulations in wistar rats

The *in vivo* study was performed to quantify silymarin after oral administration of silymarin formulations. The plasma profiles in adult female albino wistar rats following oral administration of the nanoemulsion (TF14) formulation, marketed suspension (SILYBON®) and drug suspension of silymarin were compared (Fig. 6). It was seen from Fig. 6 that the plasma concentration profile of silymarin for nanoemulsion represents greater improvement of drug absorption than the marketed formulation or simple drug suspension. Pharmacokinetic parameters were calculated by non-compartmental analysis also called as Model independent analysis. All pharmacokinetic parameters (t_{max}, C_{max}, AUC_{0-t}) were calculated individually for each subject in the group and the values were expressed as mean ± SD (n = 6) (Table 6).

AUC of nanoemulsion (TF14), marketed formulation and bulk drug suspension was found to be 199.45 ± 56.07, 101.01 ± 89.11

Table 6
Pharmacokinetic parameters (mean ± S.D.) after oral administration of different silymarin formulations in Wistar albino rats ($n = 6$).

Formulation	t_{max}[a] (h)	C_{max}[b] (μg/mL)	AUC_{0-t} (μg h/mL)[c]
Optimized Nanoemulsion (TF14)	0.5 ± 0.091	31.168 ± 7.56	199.45 ± 56.07
Marketed	2.0 ± 1.48	11.865 ± 5.901	101.012 ± 89.11
Standard	2.0 ± 1.16	5.243 ± 1.83	49.588 ± 4.34

[a] Time of peak concentration.
[b] Peak of maximum concentration.
[c] Area under the concentration time profile curve.

and 49.58 ± 4.34 μg h/mL, respectively. C_{max} of TF14 formulation was significantly higher ($p < 0.01$) than the marketed conventional formulation and bulk drug suspension but it was insignificant when marketed formulation was compared with the bulk drug suspension ($p > 0.05$). The AUC and C_{max} of TF14 formulation after oral administration were 4-fold and 6-fold higher than those of drug suspension of silymarin, respectively. When compared with the marketed formulation, there were 2-fold and 2.6-fold increased in the AUC and C_{max} of TF14 formulation, respectively. However, the t_{max} was shorter than that of drug suspension of silymarin and marketed conventional formulation, indicating the influence of the nanosizing of the oil droplets on the bioavailability. The high value of AUC and C_{max} in case of TF14 formulation ensured higher drug availability at the site of action over a prolonged period of time. The quick onset of the drug action in the body is attributed to the presence of a low t_{max} value of TF14 formulation (0.5 ± 0.091 h) as compared to the conventional marketed formulation (2.0 ± 1.48 h) and bulk drug suspension (2.0 ± 1.16 h). Statistically, the difference in t_{max} of TF14 formulation was significant when compared to t_{max} of marketed formulation and bulk drug suspension ($p < 0.01$) where as the same was insignificant between bulk drug suspension and marketed formulation ($p > 0.05$).

Lorenz et al. (1984) reported that the plasma level of silybin was very low in the conventional formulation. After a single oral dose of silymarin (200 mg/kg) in rats, the AUC and C_{max} values were 77.1 μg h/mL and 6.7 μg/mL, respectively (Morazzoni et al., 1993). Similar data was obtained in the present study.

As discussed above, the nanoemulsion approach appears to be an alternative drug delivery system, which increases the solubility and bioavailability of silymarin. As mentioned earlier, the increase in the bioavailability of silymarin using a nanoemulsion might be due to the higher solubilization of drug in oil and the improved release rate. Morever, the presence of a surfactant and cosurfactant in the nanoemulsion system might have caused changes in the membrane permeability (Chi, 1999), and was able to reach a maximum concentration in minimum possible time while having an increased extent of bioavailability. As a result, nanoemulsions appear to be an effective approach for rapid onset and increased absorption after oral administration of silymarin in comparison to earlier reported results (Arcari et al., 1992; Yanyu et al., 2006; Chen et al., 2005; He et al., 2007; Woo et al., 2007; Wei et al., 2006).

3.5. Biochemical evaluation

The administration of CCl_4 to the animals resulted in production of trichloromethyl free radicle, which causes lipoperoxidation. This in turn increases the levels of SGOT, SGPT and ALP indicating the induction of hepatotoxicity. Administration of silymarin, marketed formulation and optimized nanoemulsion (TF14) reversed the CCl_4-induced toxic effects but in different proportions. Significant hepatoprotective activity was observed in all the three i.e. bulk drug, marketed silymarin formulation and TF14.

There was an extremely significant rise in the mean SGOT level in carbon tetrachloride treated group i.e. the (toxic control, group II) as compared to normal control (group I) rats ($p < 0.01$). It is evident from Fig. 7, that the bulk drug suspension, TF14 formulation and marketed formulation treatment along with carbon tetrachloride administration, significantly ($p < 0.01$) reduced this increase in serum SGOT level when compared to pathogenic control group. The percent fall was 96.4, 97.1 and 97.7% in SGOT, 98.3, 96.7 and 97.5% in SGPT and 82.0, 86.3 and 78.7% in ALP in groups III (bulk drug), IV (marketed) and V (test), respectively on sixth day.

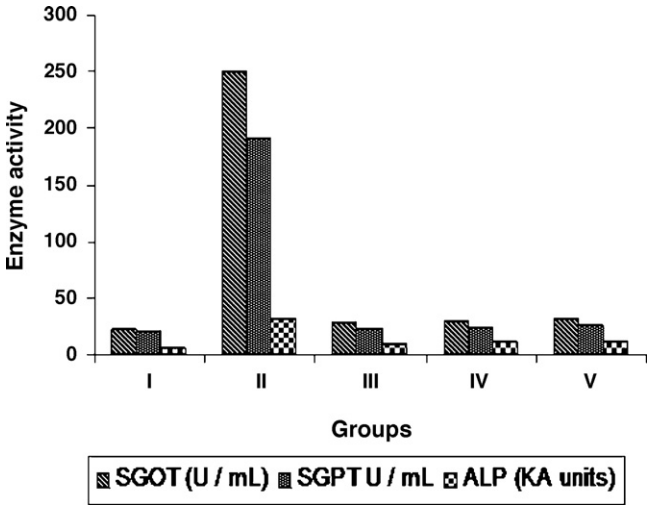

Fig. 7. Statistical graph of hepatoprotective activity of bulk drug suspension, marketed conventional formulation and optimized TF14 nanoemulsion formulation in terms of enzyme activity (SGOT, SGPT and ALP).

Liver is rich in serum enzymes i.e. SGPT, SGOT and ALP, which increase in patients with acute hepatic diseases. These enzymes are the specific markers to assess hepato-cellular damage. Estimation of transaminases and alkaline phosphatase activity is one of the most widely used means of measuring hepato-cellular injury (Hall et al., 1991). In this study, a significant increase in the levels of SGOT, SGPT and serum alkaline was observed. Due to increased levels, cellular leakage and loss of functional integrity of cell membrane occurred. Significant hepatoprotective effect was observed after oral administration of bulk drug suspension, marketed formulation and TF14 formulation. The improved performance of silymarin may be well assigned to the oil. This is known to be taken up passively by the liver and thus could carry the drug molecules along with to the hepatic site. Thus, it serves as a vector for silymarin molecules to target them passively at the hepatic site.

4. Conclusion

The present study on silymarin nanoemulsion revealed successful preparation with efficient solubilization of silymarin. Nanoemulsion approach was used in an attempt to increase its release rate and bioavailability. Different process and formulation variables were evaluated and thermodynamic stability studies were carried out to find out the optimized thermodynamically stable and characterized formulation. In the present study, an optimized silymarin nanoemulsion was prepared using 5% w/w of

sefsol 218 as the oily phase, 35% w/w of Smix (tween 80 as surfactant, ethyl alcohol as the co-surfactant, 2:1) and 60% w/w of distilled water as an aqueous phase. This formulation was optimized on the basis of optimum globule size, minimum polydispersity index, higher drug release, lower viscosity, lower surfactant concentration, higher solubilization of drug in minimum amount of oil as well as higher bioavailability. The AUC and C_{max} of TF14 formulation after oral administration were 4-fold and 6-fold higher than those of drug suspension of silymarin, respectively. The results of pharmacokinetic study were supported by the estimation of enzyme activity in serum, which again proved that administration of less than half dose of silymarin in nanoemulsion form produces similar protection against CCl_4 induced toxicity in rats as compared to more than double dose of silymarin in solution and suspension form. Hence, it can be concluded from present investigations that the nanoemulsion approach developed for silymarin will provide better biopharmaceutic properties as compared to the lipid based systems (Abrol et al., 2005), in which similar and higher dose of silymarin has been used in all formulations. It may be due to its nano size and higher surface area, which permits faster rate of drug release, improved bioavailability and better absorption followed by better bioactivity in lesser dose of drug.

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