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Potential of nanoemulsions for intravenous delivery of rifampicin

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The aim of the present study was to develop, characterize and evaluate nanoemulsion formulations for intravenous delivery of rifampicin (RIF). Different oil-in-water (o/w) nanoemulsions were prepared by the aqueous phase titration method. Prepared nanoemulsions were subjected to thermodynamic stability tests for phase separation, creaming, cracking, coalescence or phase inversion and dispersibility test for dilution capacity. Nanoemulsion formulations which passed these tests were characterized in terms of droplet size, viscosity, entrapment efficiency, homogeneity and pH. The selected formulations were subjected to *in vitro* dissolution studies using a dissolution apparatus-XXIII in dialysis bag. Best results were obtained with the formulation which consisted of 150 mg of RIF, 15% w/w of Sefsol 218, 18.75% w/w of Tween 80, 6.25% w/w of Tween 85 and 60% w/w of normal saline. The optimized formulation was also subjected to stability studies according to the ICH guidelines. The formulation was found to be stable for more than 19 months. These results indicated the potential of nanoemulsions for intravenous delivery of RIF.

1. Introduction

Rifampicin is the most widely used antitubercular drug (Ali et al. 2007). It is used in combination with isoniazid for the treatment of tuberculosis caused by *Mycobacterium tuberculosis* (Martindale 1989; Rang et al. 2003). It has been reported that RIF degrades in the presence of acids which means that at acidic pH in stomach its bioavailability is very poor (Shishoo et al. 1999; Singh et al. 2000; Shishoo et al. 2001). Therefore the aim of the present study was to develop, characterize and evaluate intravenous (IV) nanoemulsions of RIF in order to improve its solubility, stability and bioavailability. Moreover there is no marketed nanoemulsion formulation for parenteral delivery. In recent years much attention has been focused on lipid based formulations to improve solubility and bioavailability of poorly soluble drugs either by the oral route or the parenteral route (Shafiq et al. 2007a). The most popular approach is the entrapement of an active drug moeity into inert lipid vehicles such as oils, microemulsions, nanoemulsions, surfactant dispersions, emulsions, liposomes, self-emulsifying drug delivery systems (SEDDS), self-microemulsifying drug delivery systems (SMEDDS) and self-nanoemulsifying drug delivery systems (SNEDDS) (Shafiq et al. 2007a). All these systems improve solubility as well as bioavailability by increasing surface area and reducing droplet size. Nanoemulsions are thermodynamically stable transparent (translucent) dispersions of oil and water stabilized by an interfacial film of surfactant and cosurfactant molecules having the droplet size 10–100 nm (Baboota et al. 2007; Shakeel et al. 2007; Shafiq et al. 2007b). Nanoemulsions are one of the promising systems applied to increase bioavailability of poorly soluble drugs (Shafiq et al. 2007c). Nanoemulsions have shown potential for delivery of any drug (hydrophilic, lipophilic and amphiphilic) through many routes like transdermal, oral, ophthalmic and parenteral route (Lawrence and Rees 2000). They are produced on large scale without utilizing high energy homozenization (Shafiq et al. 2007a, b, c). They are formed spontaneously and can be sterilized by filtration due to very low droplet size. O/w nanoemulsions are most suitable for parenteral delivery of poorly soluble drugs where emulsions or suspensions are not desirable because nanoemulsions increase solubility of such drugs. Several poorly soluble drugs have been formulated successfully into o/w nanoemulsions for parenteral delivery (Voncorswant et al. 1998; Park and Kim 1999; Lee et al. 2002; Zhao et al. 2005; Rhee et al. 2007). Nanoemulsions do not induce pain at the site of injection (Lee et al. 2002). O/w emulsions have shown to be effective in reducing the pain at the site of injection by encapsulating some drugs like diazepam and propofol (Vondardel et al. 1983; Doenicke et al. 1996). The aim of the present study was to investigate the potential of nanoemulsions for IV delivery of RIF using nonirritant, pharmaceutically and parenterally acceptable ingredients. These nanoemulsions were prepared using Sefsol 218 as an oil phase, Tween 80, Tween 85 and normal saline as surfactant, cosurfactant and aqueous phase respectively. All these chemicals are nonirritant, parenterally safe and falling under generally regarded as safe (GRAS) category.

2. Investigations, results and discussion

2.1. Materials for component selection

The important criterion for selection of the materials was that the components are pharmaceutically acceptable, non-irritant and fall under GRAS category.

Safety is a major determining factor in choosing a surfactant as large amount of surfactants may cause gastrointestinal (GI) irritation (Shafiq et al. 2007b). 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 o/w nanoemulsion must be greater than 10 (Craig et al. 1995). The right blend of low and high HLB surfactants leads to the formation of a stable nanoemulsion formulation (Craig et al. 1995). In this study, we selected Tween 80 as a surfactant having HLB value of 15.0. Transient negative interfacial tension and fluid interfacial film is rarely achieved by the use of single surfactant, usually requiring the addition of a cosurfactant. The presence of cosurfactant decreases the bending stress of interface and provides the interfacial film sufficiently flexibility having different curvatures required to form nanoemulsion over a wide range of composition (Kawakami et al. 2002). Thus, the cosurfactant selected for the study was Tween 85 that again is nonionic surfactant but having HLB value of 11.0.

2.2. Screening of components

The most important criterion for the screening of components is the solubility of poorly soluble drug in oils. Both long and medium chain triglyceride oils with different degrees of saturation have been used for the design of nanoemulsions. For the present study, at least one oil from different categories such as long chain triglyceride, medium chain triglyceride as well as synthetic monoglyceride oils was selected, so that highest solubility of RIF could be achieved.

The solubility of drug in surfactants and cosurfactants is also important for oral and parenteral drug delivery. Thus, study was done to check the solubility of drug in all these components. The solubility of RIF was found to be highest in Sefsol 218 (124.53 ± 4.52 mg/ml) as compared to other oils, thus Sefsol 218 was selected as the oil phase for the development of the optimal formulation (Table 1). High solubility of drug was seen in Tween 80 and Tween 85. Therefore, Tween 80 and Tween 85 were selected as surfactant and cosurfactant respectively for the phase study. Normal saline was selected as aqueous phase because it is isotonic with body fluids.

Table 1: Solubility of RIF in various oils, surfactants and cosurfactants

Components	Solubility mean ± SD (mg/ml)[a]	Components	Solubility mean ± SD (mg/ml)[a]
Sefsol 218	124.53 ± 4.52	Labrafil	41.231 ± 3.41
IPP	68.40 ± 2.75	Cremophor-EL	105.41 ± 5.22
IPM	62.31 ± 2.69	Labrasol	113.21 ± 4.32
Triacetin	92.40 ± 1.89	Tween 80	222.32 ± 6.99
Olive oil	27.31 ± 1.79	Tween 85	192.42 ± 5.79
Caster oil	21.21 ± 2.11	Ethanol	8.24 ± 1.21
Labrafac	35.21 ± 3.13	Propylene glycol	32.14 ± 3.14

[a] Mean ± SD, n = 3

2.3. Pseudo-ternary phase diagram study

The relationship between the phase behavior of a nanoemulsion and its composition can be captured with the aid of a pseudo-ternary phase diagram (Lawrence and Rees 2000). Pseudo-ternary phase diagrams were constructed separately for each ratio of surfactant and cosurfactant (S_{mix} ratio), so that o/w nanoemulsion regions could be identified and nanoemulsion formulations could be optimized (Fig. 1).

In Fig. 1a, it was observed that when Tween 80 was used alone without cosurfactant i.e. S_{mix} ratio was 1 : 0, very low amount of oil (13% w/w) was solubilized by using surfactant as high as 62% w/w. When cosurfactant was added and S_{mix} ratio was 1 : 1 [Fig. 1(b)], it was observed that 25% w/w of oil was solubilized but at higher percentage of S_{mix} (50% w/w). As we further increased the surfactant concentration in S_{mix}, S_{mix} ratio 2 : 1 (Fig. 1c), further higher nanoemulsion region was observed. The maximum concentration of oil that was solubilized by this ratio was 28% w/w utilizing 41% w/w of S_{mix}. As we moved on S_{mix} 3 : 1 (Fig. 1d), nanoemulsion region was further increased. The maximum concentration of oil that was solubilized by this ratio was 32% w/w utilizing 32% w/w of S_{mix}. When the surfactant concentration was increased to 4 parts to 1 part of cosurfactant [Fig. 1(e)], the nanoemulsion area decreased as compared to 2 : 1 and 3 : 1. The maximum amount of oil that could be solubilized was 20% w/w with higher concentration of S_{mix} (60% w/w). There was no point in going for 5 : 1 S_{mix} ratio which would have further resulted in decreased nanoemulsion area.

2.4. Thermodynamic stability studies

It is well known that large amount of surfactant causes GI irritation (Lawrence and Rees 2000; Ping et al. 2005) therefore, it is important to determine the surfactant concentration properly and to use the lowest concentration of surfactant in the formulation. Nanoemulsions are thermodynamically stable systems and are formed at a particular concentration of oil, surfactant and water. It is the thermostability which differentiates nano or microemulsions from emulsions that have kinetic stability and will eventually phase separate (Shinoda and Kunieda 1983; Lawrence and Rees 2000). Thus, selected formulations were tested for thermodynamic stability by using centrifugation, heating and cooling cycles and freeze thaw cycles. Only those formulations, which showed no phase separation, creaming, cracking, coalescence or phase inversion etc upon these tests, were selected for further study. The compositions of selected formulations are given in Table 2.

2.5. Dispersibility tests

When a nanoemulsion formulation is diluted infinitely with GI fluids, there is every possibility of phase separation, leading to precipitation of a poorly soluble drug as nanoemulsions are formed at a particular concentration of oil, surfactant, cosurfactant and water. For parenteral and oral nanoemulsions the process of dilution by the GI fluids will result in the gradual desorption of surfactant located at the globule interface. In the present study, distilled water was used as a dispersion medium because it is well reported that there is no significant difference in the nanoemulsions prepared using nonionic surfactants, dispersed in either water or simulated gastric or intestinal

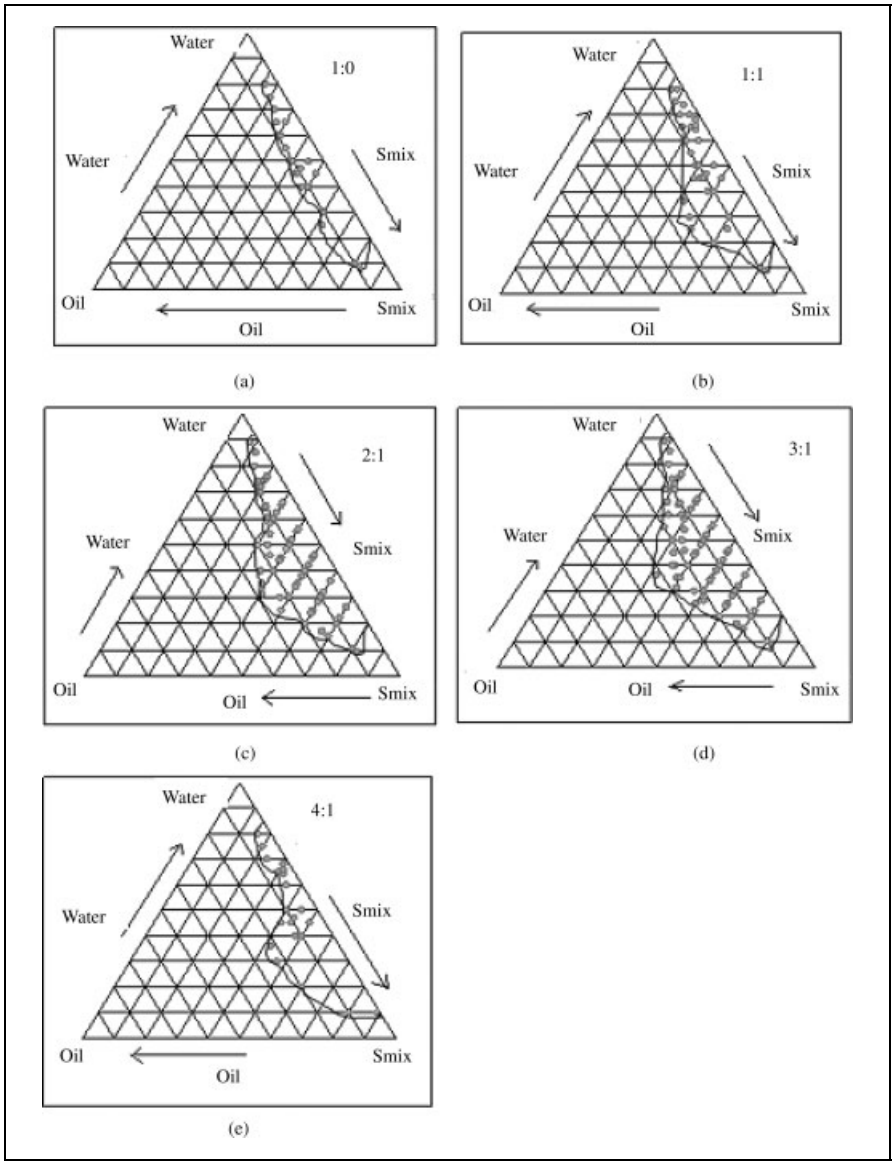

Fig. 1:
Pseudo-ternary phase diagrams indicating o/w nanoemulsion region of Sefsol 218 (oil), Tween 80 (surfactant) and Tween 85 (cosurfactant) at different Smix ratios indicated in Fig. a to e

fluid (Khoo et al. 1998; Lawrence and Rees 2000; Ping et al. 2005). Nanoemulsion formulations that passed dispersibility test in grade A and B were taken for further study, as grade A and B formulations will remain as nanoemulsions when dispersed in GIT. All the formulation, which fell in grade C, D and E of dispersibility tests were discarded for further studies. Optimized formulations (Table 2) were taken for characterization, *in vitro* release studies, osmotic pressure determination and stability studies.

2.6. Characterization of nanoemulsions

The mean droplet size of nanoemulsion formulations ranged from 47.41–115.40 nm (Table 3). The droplet size of the formulation F4, containing 15% of oil was significantly lower (47.41 ± 4.36 nm) as compared to other formulations ($p < 0.05$) that could be due to lowest oil concentration. It is difficult to describe the effects of S_{mix} concentration on droplet size because concentration of S_{mix} was

Table 2: Composition of selected nanoemulsion formulations

S_{mix} Ratio	% w/w of components			S_{mix}: Oil Ratio	Dispersibility test grade	Code
	Oil*	S_{mix}	Normal Saline			
2 : 1	15	25	60	1.67	A	F1
2 : 1	20	25	55	1.25	A	F2
2 : 1	25	25	50	1.00	A	F3
3 : 1	15	25	60	1.67	A	F4
3 : 1	20	25	55	1.25	A	F5
3 : 1	25	25	50	1.00	A	F6

* Oil phase containing 150 mg of RIF

Table 3: Mean droplet size, polydispersity and viscosity of the nanoemulsion formulations

Formulation Code	Droplet size mean ± SD (nm)[a]	Polydispersity	Viscosity mean ± SD (cps)[a]
F1	60.41 ± 6.76	0.112	21.21 ± 1.09
F2	89.21 ± 14.18	0.159	36.41 ± 2.11
F3	115.40 ± 24.46	0.212	51.21 ± 3.24
F4	47.41 ± 4.36	0.092	15.11 ± 1.51
F5	82.25 ± 13.32	0.162	27.22 ± 1.24
F6	104.12 ± 21.76	0.209	43.14 ± 1.59

[a] Mean ± SD, n = 3

Table 4: Entrapment efficiency, homogeneity and pH of nanoemulsion formulations

Formulation Code	Entrapment efficiency	Homogeneity	pH
F1	100.00	Excellent	7.20
F2	99.86	Excellent	6.99
F3	99.74	Excellent	7.10
F4	100.00	Excellent	7.30
F5	99.64	Excellent	6.89
F6	99.54	Excellent	6.74

constant in all selected formulations. When the ratio of S_{mix} to oil was increased, it was found that droplet size was significantly decreased ($p < 0.05$). Droplet size of nanoemulsion formulation was acceptable for IV delivery of RIF. All the formulations had low values of polydispersity (0.092–0.209) which indicates uniformity of droplet size within the formulation. The polydispersity was lowest for formulation F4 (0.092).

The viscosity of nanoemulsion formulations was found to be 15.11–51.21 Cps. Formulation F4 had the lowest viscosity as compared to other formulations (Table 3). Viscosity was decreased when concentration of oil was increased. It was also observed that the viscosity of all nanoemulsion formulations was very low which is one of the characteristics of the nanoemulsion formulation. The viscosity of optimized nanoemulsion formulation (F4) was found to be 15.11 Cps which is acceptable for IV delivery.

The entrapment efficiency was found to be more than 99% in all nanoemulsion formulation which indicated excellence of nanoemulsion formulations for IV drug delivery (Table 4). The entrapment efficiency of formulation F4 was found to be 100%.

The homogeneity of nanoemulsion formulations was assessed visually in terms of appearance and clarity. Homogeneity was found to be excellent in all formulations (Table 4).

The pH of nanoemulsion formulations was found to be 6.74–7.30 (Table 4). This pH was within the pH range of lipid injectable formulations.

2.7. In vitro drug release studies

In vitro drug release studies were performed to compare the release of RIF from six nanoemulsion formulations (F1–F6). The release of drug was highest (99.4%) in formulation F4 and lowest for F3 (Fig. 2). In the first 2 h of study, around 70% of drug release from formulation F4 was obtained. The drug release profile of F4 was significant as compared to other formulations ($p < 0.05$). This could be due to smallest droplet size, lowest polydispersity and lowest viscosity in formulation F4. It was also found that when oil concentration in nanoemulsions was decreased, drug release was significantly increased ($p < 0.05$). The formulation F4 containing 15% w/w of oil, having highest drug release (99.4%), lowest droplet size (47.41 nm), lowest polydispersity (0.092) and lowest viscosity (15.11 Cps) was optimized for further studies.

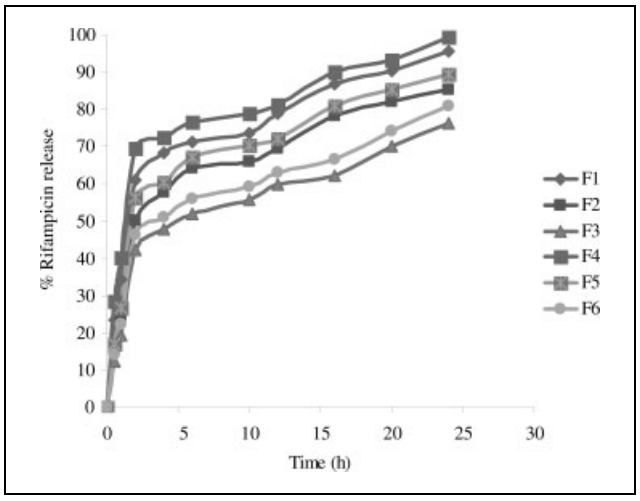

Fig. 2: *In vitro* drug release profile of RIF from six different nanoemulsion formulations (F1 to F6)

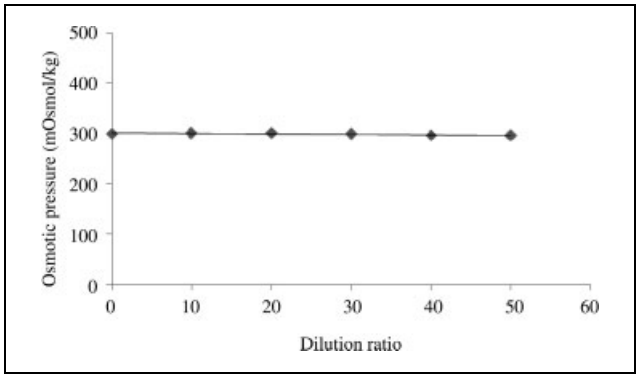

Fig. 3: Changes in osmotic pressure of optimized nanoemulsion formulation F4 upon dilution with normal saline

2.8. Osmotic pressure measurement

Osmotic pressure of optimized nanoemulsion formulation (F4) was measured to determine isotonicity which is essential for IV preparations. Hypertonicity or hypotonicity of IV preparations is known to cause pain, tissue damage at the injection site and morphological changes of erythrocytes (Klement and Arndt 1991; Kim et al. 1997). The osmotic pressure of normal saline solution and blood plasma is approximately 308 and 306 mOsm/kg respectively. The osmotic pressure of nanoemulsion was found to be around 300 mOsm/kg at all dilution ratio. When nanoemulsion was diluted with normal saline there were no significant changes in the value of osmotic pressure (Fig. 3). These results indicated the potential of nanoemulsion formulation for IV delivery of RIF.

Table 5: Droplet size, viscosity, entrapment efficiency and pH of optimized nanoemulsion formulation during storage

Time (months)	Temp (°C)	Droplet size mean ± SD (nm)[a]	Viscosity mean ± SD (cps)[a]	Entrapment efficiency	pH
0	4.0 ± 0.5	48.11 ± 4.48	15.11 ± 1.51	100.00	7.30
1	4.0 ± 0.5	48.53 ± 4.95	15.52 ± 1.87	99.79	7.20
2	4.0 ± 0.5	48.97 ± 5.16	15.59 ± 1.91	99.48	7.10
3	4.0 ± 0.5	49.12 ± 5.23	15.98 ± 2.43	99.29	7.10
0	25 ± 0.5	47.41 ± 4.36	15.11 ± 1.51	100.00	7.30
1	25 ± 0.5	48.59 ± 4.98	15.56 ± 1.91	99.77	7.10
2	25 ± 0.5	49.07 ± 5.11	15.88 ± 2.10	99.41	7.00
3	25 ± 0.5	49.23 ± 5.85	16.12 ± 2.67	99.22	7.00

[a] Mean ± SD, n = 3

2.9. Stability studies

During stability studies droplet size, pH, viscosity and entrapment efficiency were determined at temperature of 4 °C and 25 °C. These parameters were determined at 0, 1, 2 and 3 months. It was found that droplet size and viscosity were slightly increased at both temperatures (Table 5). The drug entrapment efficiency and pH were also slightly increased at both temperatures. The changes in these parameters were not significant ($p \geq 0.05$). These results indicated that the optimized formulation is stable and suitable for IV delivery of RIF. The effect of temperature on the degradation was studied by plotting log K v/s 1/T. (Fig. 4). The value of K at 25 °C (K_{25}) was obtained by extrapolation of the plot and shelf life was then calculated. The shelf life of RIF in formulation F4 was found to be 19.2 months.

3. Experimental

3.1. Materials for component selection

RIF was a kind gift sample from Sandoz Pharmaceutical (Germany). Propylene glycol monocaprylic ester (Sefsol 218) was gifted from Nikko chemicals (Japan). Labrafac, Labrafil and Labrasol were obtained from Gattefossé (France) as gift samples. Isopropyl myristate (IPM), Isopropyl palmitate (IPP), castor oil, olive oil and Triacetin were purchased from E-Merck, France. Tween 80, Tween 85, and Cremophor-EL were purchased form Sigma Aldrich, USA. All other chemicals used in the study were of analytical reagent (AR) grade.

3.2. Screening of components

The solubility of RIF in various oils (Triacetin, IPM, IPP, caster oil, Labrafac, Labrafil, olive oil and Sefsol 218), surfactants (Labrasol, Tween 80 and Cremophor-EL) and cosurfactants (Ethanol, Propylene glycol and Tween 85) was determined by taking excess amount of RIF in each oil, surfactant and cosurfactant separately. Excess amount of RIF was added to each 5 ml capacity stoppered vial and mixed for 10 min using a vortex mixer. The mixture vials were then kept at 37 ± 1.0 °C in an isothermal shaker (Memmert, Germany) for 72 h to get equilibrium. The equilibrated samples were removed from shaker and centrifuged at 3000 rpm for 15 min. The supernatant was taken and filtered through a 0.45 μm membrane filter. The concentration of RIF was determined in each oil, surfactant, cosurfactant by HPTLC at 254 nm (Ali et al. 2007).

3.3. Preparation of nanoemulsions

On the basis of the solubility studies Sefsol 218 was selected as the oil phase. Tween 80 and Tween 85 were selected as surfactant and cosurfactant respectively. Normal saline was used as an aqueous phase because it is isotonic with body fluids. Surfactant and cosurfactant (S_{mix}) were mixed in different weight ratios (1 : 0, 1 : 2, 1 : 1, 2 : 1, 3 : 1, 1 : 3 and 4 : 1). For each phase diagram, oil and specific S_{mix} ratio were mixed well in different weight ratios from 1 : 9 to 9. Pseudo ternary phase diagrams of oil, S_{mix} and aqueous phase were developed using aqueous titration method (Shakeel et al. 2007; Baboota et al. 2007; Shafiq et al. 2007c). Slow titration with aqueous phase was done to each weight ratio of oil and S_{mix} and visual observations were made for transparent and easily flowable o/w nanoemulsions. The physical state of the nanoemulsion was marked on a pseudo-three-component phase diagrams with one axis representing aqueous phase, one representing oil and the third representing a mixture of surfactant and cosurfactant at fixed weight ratios.

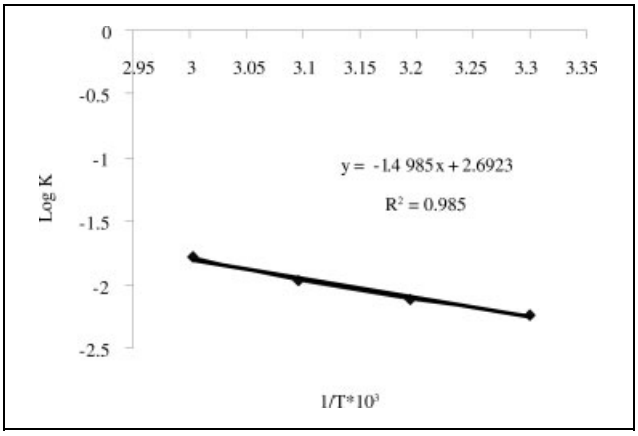

Fig. 4: Arrhenius plot between Log K and 1/T for formulation F4

Following criteria were set up for selection of nanoemulsions from phase diagram

- Drug should be freely soluble in oil phase.
- The concentration of oil phase should be very low in order to get lowest viscosity and to reduce pain at the site of injection.
- The concentration of aqueous phase should be significantly higher than oil phase and surfactant mixture.
- The concentration of S_{mix} should be very low, because higher concentration of S_{mix} may cause gastrointestinal (GI) irritation.

From each phase diagram constructed, different formulas were selected from the nanoemulsion region based on above selection criteria. 150 mg of RIF was loaded in each selected nanoemulsion formulations. Selected nanoemulsion formulations were subjected to different thermodynamic stability tests.

3.4. Thermodynamic stability tests

To overcome the problem of metastable formulation, thermodynamic stability tests were performed on selected nanoemulsion formulations. Selected nanoemulsions were centrifuged at 4000 rpm for 25 min. Those formulations that did not show any phase separation were taken for the heating and cooling cycle. Six cycles between refrigerator temperature (4 °C) and 45 °C with storage for 48 h at each temperature was done. The formulations, which were stable at these temperatures, were subjected to freeze thaw cycle test. Three freeze-thaw cycles were done for the formulation between −21 °C and +25 °C. The formulations which survived these tests were selected for dispersibility tests for assessing the efficiency of self-emulsification.

3.5. Dispersibility tests

The efficiency of self-emulsification of injectable nanoemulsions was evaluated using a standard USP XXII dissolution apparatus 2 (Pouton 1997; Khoo et al. 1998). Each selected formulation (3 ml) was added to 900 ml of distilled water at 37 ± 1 °C. The mixture was stirred with a stainless steel paddle at 50 rpm. The self-emulsification efficiency of each formulation was visually assessed using the following grading system:

Grade A: Rapidly forming nanoemulsion (within 1 min), having a clear or bluish appearance.

Grade B: Rapidly forming, slightly a less clear emulsion, having a bluish white appearance.

Grade C: Fine milky emulsion that formed within 2 min.

Grade D: Dull, grayish white emulsion having slightly oily appearance that is slow to emulsify (longer than 2 min).

Grade E: Formulation exhibiting either poor or minimal emulsification with large oil globules present on the surface.

Formulations that passed dispersibility tests in Grade A and B were selected for further studies.

3.6. Characterization of nanoemulsions

Droplet size and size distribution of the nanoemulsion was determined by photon correlation spectroscopy using a Zetasizer 1000 HS (Malvern Instruments, UK). Light scattering was monitored at 25 °C at a scattering angle of 90°. A solid state laser diode was used as light source. Third order cumulant fitting analysis was applied to obtain mean droplet size and polydispersity as a correlation function. The samples of nanoemulsions were suitably diluted with distilled water and filtered through 0.22 μm membrane filter in order to eliminate mutiscattering phenomena and experimental errors. The diluted samples were then placed in quartz couvettes and subjected to droplet size analysis.

The viscosity of the formulations was determined using Brookfield DV III ultra V6.0 RV cone and plate rheometer (Brookfield Engineering Laboratories, Inc, Middleboro, MA) using spindle # CPE40 at 25 ± 0.3 °C.

For determination of entrapement efficiency of RIF, nanoemulsions were filtered through 0.45 μm filter paper in order to remove any traces of precipitated or unentraped drug. The RIF was extracted from nanoemulsions using methanol and chloroform (50 : 50% v/v) followed by sonication for 20 min. The samples were diluted to 100 ml and solutions were applied to TLC plates followed by development and scanning as described by Ali et al. (2007). The amount of RIF was determined at 254 nm using calibration curve.

Homogenecity of selected nanoemulsions was determined by visual inspection.

The pH of selected nanoemulsions was determined using digital pH meter.

3.7. In vitro drug release studies

In vitro drug release studies were performed in 900 ml of distilled water containing 1% sodium lauryl sulphate (SLS), which was based on USP

XXIV dissolution apparatus 2 at 50 rpm. 3 ml of each nanoemulsion formulation (single dose containing 150 mg of RIF) was placed in dialysis bag (MWCO 12000 g/mol, Sigma Aldrich, USA). Samples (3 ml) were withdrawn at regular intervals (0, 0.5, 1, 2, 4, 6, 8, 10, 12, 16, 20 and 24 h) and replaced with drug free distilled water containing 1% SLS. The drug content in *in vitro* samples was quantified by HPTLC at 254 nm. The same procedure was used for extraction of RIF from *in vitro* samples as described above. *In vitro* drug release data of different nanoemulsions was compared by applying Dunnet test of one-way analysis of variance (ANOVA).

3.8. Osmotic pressure measurement

Osmotic pressure of nanoemulsions was measured using precision system Inc, Natick, MA by diluting nanoemulsions with normal saline.

3.9. Stability studies

Stability studies on optimized nanoemulsion were performed by keeping them at refrigerator temperature (4 °C) and room temperature (25 °C). These studies were performed for a period of 3 months. Droplet size, pH, viscosity and entrapement efficiency were determined during storage. The shelf life of RIF was also determined by accelerated stability method using Arrhenius plot. For determination of shelf life, formulation F4 was taken in glass vials and was kept at accelerated temperature of 30 °C, 40 °C, 50 °C and 60 °C at ambient humidity. The samples were withdrawn at regular intervals of 0, 1, 2 and 3 months and were analyzed for drug content by HPTLC method at a wavelength of 254 nm. Arrhenius plot was constructed between log K and 1/T to determine the shelf life of optimized nanoemulsion formulation. The degradation rate constant at 25 °C (K_{25}) was determined by extrapolating the value of 25 °C from Arrhenius plot. The shelf life ($T_{0.9}$) was determined by using the formula:

$$T_{0.9} = \frac{0.1052}{K_{25}}$$

3.10. Analytical methods

The concentration of RIF was quantified by HPTLC at 254 nm (Ali et al. 2007). HPTLC analysis was performed on aluminium plates coated with 0.2 mm layers of silica gel 60 F264 (E. Merck, Germany). Samples were applied to plates using Camag Linomat V sample applicator fitted with a Camag microlitre syringe. Plates were developed using *n*-hexane:2-propanol : acetone : ammonia : formic acid (3 : 3.8 : 2.8 : 0.3 : 0.1% v/v) as mobile phase in a twin through glass chamber. After development, plates were scanned at 254 nm by means of a Camag TLC scanner in absorbance mode using the deuterium lamp.

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