Flufenamic Acid-Loaded Self-Nanoemulsifying Drug Delivery System for Oral Delivery: From Formulation Statistical Optimization to Preclinical Anti-Inflammatory Assessment

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Abstract: This research work aimed to prepare and optimize “self-nanoemulsifying drug delivery system (SNEDDS)” by applying full factorial design (FFD) to improve solubilization and subsequently anti-inflammatory efficacy of flufenamic acid (FLF). Suitable excipients were screened out based on the maximum solubility of FLF. FFD was applied using lipid (X1) and surfactant (X2) as independent variables against droplet size (Y1, nm), zeta potential (Y2, mV) and polydispersity index (PDI, Y3). Desirability function identified the main factors influencing the responses and possible interactions. Moreover, the optimized formulation (OFS1) was characterized and compared with pure FLF suspension. The prepared formulations (FS1–FS9) showed the size, PDI and zeta potential of 14.2–110.7 nm, 0.29–0.62 and –15.1 to –28.6 mV, respectively. The dispersion and emulsification of all formulations meted out within 2 min suggesting immediate release and successful solubilization. The optimized formulation OFS1 demonstrated ~ 85% drug release within 1 h which was significantly higher (p < 0.05) than FLF suspension. The hemolysis study negated the probable interaction with blood cells. Eventually, improved anti-inflammatory efficacy was envisaged which might be attributed to increased drug solubility and absorption. The present nanocarrier could be a promising approach and alternative to conventional dosage form.

Key words: flufenamic acid, experimental design, in vitro release, hemolysis, anti-inflammatory activity

1 Introduction

Flufenamic acid (FLF) is chemically anthranilic acid derivative which possessing analgesic, anti-inflammatory and antipyretic properties and recommended for oral or topical administrations1. It belongs to biopharmaceutical classification system (BCS) class II with low aqueous solubility (9.09 mg/L) and high permeability (log P = 5.25)2,3. High octanol/water partition coefficient, poor aqueous solubility and gastric disturbances are the prime deciding factors for alternate formulation development intended for oral or topical administration4. Several approaches have been explored to enhance the solubility and dissolution of FLF including solid dispersion5-6, inclusion complex7,8 and co-crystal9. However, lipid based nanocarrier could be a promising technique for improved solubilization, increased oral absorption, enhanced therapeutic efficacy and high patient compliance when fabricated into “self-nanoemulsifying drug delivery system (SNEDDS)”. The lipid-based drug delivery system has gained increasing attention for oral delivery as the most preferred route of administration. Lipid nanocarriers offer several advantages over conventional dosage forms such as (a) prolonged residence time in the lumen for increased absorption available to the mucosal membrane, (b) increased access of nanocarrier to the M-cells (Peyer’s patches) for maximized absorption through the hepatic portal vein and (c) small size particles bypass hepatic circulation and thus prevents from its degradation10,11. This hypothesis achieved by lymphatic transport via Peyer’s patches along the gastrointestinal tract (GIT)12. SNEDDS belongs to the lipid-based formulation

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and shown improve drug solubility, dissolution, therapeutic efficacy and facilitate drug absorption.\(^{5,10}\) It can easily be filled in the hard and soft gelatin capsules due to their anhydrous nature.\(^{31}\) It is an isotropic mixture of an active drug compound in a combination of lipids, surfactant, and water-soluble co-surfactant which can help to formulate ultrafine emulsion upon gentle agitation in the aqueous media such as GIT fluids.\(^{5,14}\) It can encapsulate hydrophobic solutes in the molecular state with relatively smaller droplet size. The nano-size range and higher solubility gives a larger surface area for the drug absorption and lead to enhanced oral bioavailability.\(^{30}\) It also provides better enzymatic and chemical stability.\(^{10}\) The lipid-based drug delivery system such as SNEDDS is easily taken up by the body and its digestion involves the dispersion of oil droplets into fine emulsion of high surface area, enzymatic hydrolysis of ester compounds at the oil/water interface and dispersion of the lipid formulation into an absorbable form.\(^{10}\) The resemblance of their degradation product with the wide acceptance for SNEDDS has contributed to their wide acceptance for SNEDDS.\(^{17}\) The systemic optimization of drug delivery system employing the design of experiment approach has been popular in the research.\(^{28}\) It provides a comprehensive understanding of the formulation system identifying interactions among the formulation composition to select the best possible formulation.\(^{10}\) The formulation optimization was carried out with the help of suitable experimental design for a critical understanding of the formulation and process parameters.\(^{20}\) The full factorial designs are the most powerful screening designs, once they allow estimating the main effects of input factors and their interactions on output responses.

The present study is aimed to formulate FLF-SNEDDS (FS1-FS9) with the objective of increasing the solubility and therapeutic efficacy. To accomplish this, the formulation was optimized using full factorial design (FFD) to select the optimized formulation (OF91) based on minimum size, optimal polydispersity index (PDI) and surface charge. Finally, the optimized formulation (OF91) was further evaluated for morphology study, encapsulation efficiency, drug release and in vivo anti-inflammatory activity.

2 Experimental

2.1 Materials

FLF (purity 99.5% by HPLC) was purchased from Sigma Aldrich (St. Louis, MO, USA). Labrasol and Cremophor EL (CEL) were obtained from Gattefosse (Lyon, France) and BASF India Ltd. (Mumbai, India), respectively. Miglyol 812 (MIG) was obtained from Sesol (Tucson, AZ, USA). Mill-Q water was used as diluent in the experiment. All chemical reagents used in the study were of AR grade.

2.2 Solubility study

The solubility of FLF was performed in various lipids, surfactants and co-surfactants to select the excipients for formulation preparation following the method reported earlier.\(^{12}\) An accurately weighed amount of the drug was added to a glass vial containing excipient (2 mL) individually. The vials were shaken for 72 h in mechanical shaker and addition of drug continued till saturation. Finally, samples were centrifuged to separate the supernatant and diluted with methanol to quantify the dissolved amount of FLF using UV-Vis spectrophotometer at 286 nm. All measurements were carried out in triplicates.

2.3 Construction of phase diagram

All formulations were prepared by slow and spontaneous titration/emulsification method following the reported method.\(^{2,22}\) Excipients possessing maximum drug solubility were screened for formulation preparation with varied ratio of surfactants and co-surfactants (Smix) such as \((2:1, 1:2, 3:1, 1:3, 1:1 \text{ and } 1:4)\). Thus, various combinations of oil and Smix were tried to delineate precise boundary of phase diagram to obtain stable and clear nanoemulsion after slow titration with water (aqueous phase).

2.4 Pre-optimization

Pre-optimization is the computational-based technique for the identification of the factors and their levels involved in the optimization process. Such computational-based techniques help in selecting the important factors which affects the measured responses in the study and dropping the non-significant factors.\(^{23}\) Moreover, it is also useful in assessing the possible interactions between the factors.\(^{24}\) The formulation was optimized by full factorial design as \(3^2\) (level independent variables) using Design Expert (Design Expert 10.0.1, Stat Ease. Inc., USA) at two factors and three different levels. The design predicted nine different compositions and their effect observed on the droplet size \((Y_1)\), PDI \((Y_2)\) and zeta potential \((Y_3)\). The variables screened for the optimization were MIG (lipid as \(X_1\)) and Labrasol (surfactant as \(X_2\)) at low (-), medium (0) and high (+) levels. The lower and higher level of MIG (75 mg and 150 mg) and Labrasol (200 mg and 400 mg) were taken for the experimental design. The \(\text{linear and quadratic equations}\) generated by Design Expert software are expressed as \(Y = b_0 + b_1 X_1 + b_2 X_2 + b_{11} X_1^2 + b_{22} X_2^2\) respectively, in which \(Y\) = measured response, \(b_0 = \) intercept, \(b_1\) and \(b_2 = \) linear coefficients, \(b_{11}\) and \(b_{22} = \) the coefficients, and \(b_{12} = \) interaction coefficient between \(X_1\) and \(X_2\) independent variables. The positive and negative sign of the term in the polynomial equation signify the synergistic and the antagonistic influence of each factor on the response, respectively.\(^{25,26}\)
2.5 Optimization and application of desirability function

The computational-based desirability function technique was developed by Derringer and Suich in order to find out the optimized composition\(^27\). The application of desirability function is the identification and evaluation of optimized formulations using computational-based techniques. The objective of desirability function is to optimize the prepared formulations by measuring the level of variables in order to get the most desirable and robust formulation.

2.6 Formulation of SNEDDS

The screened excipients were employed to prepare FLF SNEDDS (FS1–FS9) using MIG, Labrasol and CEL as lipid, surfactant and co-surfactant, respectively as per reported method\(^29\). The SNEDDS pre-concentrate (i.e., anhydrous SNEDDS) was prepared by varying the ratio of lipid and surfactant as per the FFD design. FLF and excipients were precisely weighed and mixed manually for sufficient time to get an isotropic blend (clear and homogeneous). Finally, the obtained pre-concentrate of SNEDDS was reconstituted using Milli-Q water \((100 \, \text{mL})\) under mild agitation in order to obtain a homogenous SNEDDS. The samples were stored at room temperature \((22^\circ \text{C})\) for bench top stability \((24 \, \text{h})\). Stable formulations were used for further evaluations and studies. Pre-concentrate exhibiting any sign of phase separation or physical changes (color, texture or smell) and drug precipitation before and after reconstitution were omitted for further studies.

2.7 Characterization of SNEDDS

2.7.1 Droplet size and PDI

The droplet size and PDI were assessed using "Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK)" which is based on the dynamic light scattering (DLS) technique. The freshly prepared SNEDDS (1 mL, FS1–FS9) were diluted using Milli-Q water \((100 \, \text{fold})\) and sonicated well before DLS measurements. The droplet size and PDI were measured at \(25^\circ \text{C}\) temperature and a scattering angle of \(90^\circ\) \(^28\). The size distribution was expressed in term of PDI and measured concurrently at the same experimental conditions.

2.7.2 Zeta potential

Zeta potential is the most vindicated marker for stability of the nanoemulsion. Therefore, it was prerequisite to determine the surface charge distribution after reconstitution. The zeta potential of prepared SNEDDS (FS1–FS9) was assessed using "Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK)". The sample (1 mL) was diluted using Milli-Q water \((100 \, \text{mL})\) and sonicated well before zeta potential measurement. Finally, the sample (1 mL) was taken and their surface charge was measured at a temperature of \(25^\circ \text{C}\) at a \(90^\circ\) angle.

2.8 Post-optimization

The data were processed using "Design Expert software" and two best optimized SNEDDS were selected for further investigation. After post-optimization, the optimized SNEDDS (OFS1 and OFS2) were characterized for morphological assessments using transmission electron microscopy (TEM), dispersibility and emulsification efficiency, percent drug entrapment, drug release, \textit{in vitro} hemocompatibility (hemolysis) study and anti-inflammatory activity.

2.9 Evaluation of optimized SNEDDS

2.9.1 Dispersibility and emulsification efficiency

The dispersibility test on SNEDDS was performed in order to evaluate the "emulsification efficiency and emulsification time". The sample was observed for the time required to get a homogeneous mixture upon dilution and observed visually\(^29\). The dispersibility and emulsification efficiency of SNEDDS were measured using Milli-Q water. The sample (1 mL) was dispersed in a beaker containing water \((100 \, \text{mL})\) with stirring at the temperature of \(37 \pm 1^\circ \text{C}\). The formulations were observed visually for uniform dispersibility and the presence of any visible droplets. The sample was assessed for complete dispersion and self-emulsification time as per the following grading system. The formulations in grade A and grade B were selected for further studies\(^30\).

- Grade A: Rapidly forming a clear or slight bluish color within a minute.
- Grade B: Rapidly forming a slightly clear bluish white color.
- Group C: Milky emulsion formed within two minutes.
- Group D: Greyish white emulsion having oily appearance, slow emulsification (more than two minutes).
- Group E: Poor emulsification with large globules.

2.9.2 Cloud point determination

The cloud point was assessed to find out the influence of temperature on the phase behavior of prepared samples\(^30\). The formulations (FS1–FS9) were diluted with Milli-Q water \((100 \, \text{fold})\) and transferred to a water bath with gradual increase in temperature \((5^\circ \text{C})\). The sample was equilibrated at every temperature for at least 2 min and visually evaluated for "optical transparency, sings of phase separation or drug precipitation"\(^29\).

2.9.3 Optical clarity

The optical clarity is a measure of the isotropic condition of sample. The prepared formulations (FS1–FS9) were subjected for optical clarity upon dilution \((50 \, \text{fold})\) using spectrophotometer at 638 nm against distilled water as a blank\(^31\). The study was carried out in triplicate to obtain mean values.

2.9.4 Entrapment efficiency

The entrapment efficiency (EE) of formulations (FS1-
FS9) was determined by dispersing the sample in methanol (100 mL). It was mixed and sonicated to extract the FLF completely from the excipients. The insoluble excipients were removed by the centrifugation of samples at 5000 rpm for about 15 min. The supernatant was taken carefully and filtered using a membrane filter and further diluted with methanol. The amount of FLF was determined in diluted samples using “UV-Vis spectrophotometer” at 286 nm. The % EE was obtained using the following equation:

\[
\% \text{ Entrapment efficiency} = \left( \frac{E_d}{E_i} \right) \times 100
\]

where \(E_d\) is the amount of drug estimated from the pre-concentrate (mg) of SNEDDS and \(E_i\) indicates the total amount of drug added initially.

2.9.5 In vitro diffusion study

The in vitro diffusion study was performed for the optimized formulation (OFS1) and pure FLF suspension using the dialysis membrane with some modifications. The sample (equivalent to 5 mg of FLF) was filled to the "dialysis bag" (MWCO 12-14 KDa; Sigma Aldrich, St. Louis, MO, USA) and both the end was sealed. The bag was placed in the glass beaker filled with 500 mL release media (phosphate buffer pH 6.8) and study was performed at 37 ± 1°C with continuous stirring (100 rpm). The released content (2 mL) were withdrawn at predetermined time intervals and replenished with the same volume of fresh release medium. The samples were filtered through a syringe filter (0.22 µm) and assayed to obtain the released amount of the drug using spectrophotometer at 286 nm.

2.9.6 Transmission electron microscope (TEM)

The most sophisticated instrument to visualize nanocarrier is TEM which accorded precise shape and size. The morphological study was investigated for the optimized formulation (OFS1). A drop of the sample was placed over the carbon-coated copper grid and kept aside for air drying. The negative staining was performed using phosphotungstic acid (0.1% w/v) for 5 min and allowed to dry at ambient temperature (25°C). Finally, the images were captured and viewed under the electron microscope at varied magnification and resolution.

2.9.7 Hemolysis study

Erythrocytes lysis study was performed to negate the possible hemolysis caused by the formulation and its components at explored concentration. The red blood cells (RBCs) from rat blood were collected in Eppendorf tube containing anticoagulant. The sample was centrifuged at 1000 g for 15 min at 4°C and the supernatant were discarded. The pellet containing RBCs was washed and diluted with isotonic saline solution to obtained RBCs suspension (4%). The test sample (0.5 mL, 10% v/v) and 0.5 mL of RBC suspension were mixed followed with an addition of 3 mL phosphate buffer saline solution (PBS). Then, the samples were incubated at 37°C for 1 h without shaking. Similarly, 0.5 mL of PBS and 0.5 mL of Triton-X 100 were used as negative and positive control, respectively. After incubation, the released hemoglobin (Hb) was estimated by UV spectrophotometer at 576 nm.

2.9.8 Anti-inflammatory activity

The evaluation of anti-inflammatory activity of pure FLF and OFS1 was performed using Carrageenan-induced rat paw edema method. The protocol for these studies was reviewed and approved by “Animal Ethics Committee of King Saud University, Riyadh, Saudi Arabia (Approval number KSU-SE-19-19)”. Eighteen male Wistar rats (weight 220–250 g) were taken from the "Pharmacy Experimental Animal Care Center (King Saud University, Riyadh, Saudi Arabia)". All the animals were fasted overnight before giving any treatment. Both the samples were prepared in sodium carboxymethyl cellulose suspension (0.1% w/v) and administered orally using oral gavage. The animals were divided into three different groups, which includes group-I (control), group-II (OFS1) and group-III (pure FLF). Each group contained six rats (n = 6). All the rats were housed together in a standard animal room with free access to “Lipton feed and water ad libitum”. Paw edema was induced using (0.1 mL) carrageenan injection (1.0% w/v) which was injected subcutaneously into the left hind paw. The paw edema was evaluated at different time intervals for the investigation of anti-inflammatory efficacy. After 30 min of carrageenan injection, pure FLF and OFS1 (10 mg/kg) were administered orally. The paw volume before (0 h) and after carrageenan injection (1, 2, 4 and 6 h) was measured using a “Digital Plethysmometer (Ugo, Italy)”. The activity for pure FLF and OFS1 was determined as per reported formula in the literature.

2.10 Statistical analysis

The experiments were carried out in triplicates and expressed as mean ± SD (standard deviation). The variation was considered as significant (p < 0.05) as compared to control group. The statistical comparison was performed by student t-test using MS Excel 2016 program and analysis of variance (ANOVA) was applied using Origin 8.0 and Design Expert software.

3 Results

3.1 Solubility study

The mean solubility of FLF in various lipids, surfactant and co-surfactant was determined. With respect to lipids, the maximum solubility was obtained in Mig (249.73 ± 23.94 mg/mL) followed by Captex 355 (64.83 ± 10.52 mg/mL). Among different surfactants studied, the maximum solubility was obtained in Labrasol (212.62 ± 19.27 mg/mL) followed by Gelucire 44/14 (119.38 ± 14.70 mg/mL) and Tween 80 (18.09 ± 0.80 mg/mL). Among different cosurfactants studied, the maximum solubility was found in CEL.
(198.53 ± 15.74 mg/mL) followed by polyethylene glycol 400 (179.11 ± 7.91 mg/mL), glycerol (176.31 ± 17.53 mg/mL), Transcutol P (170.61 ± 8.65 mg/mL), Cremophor RH40 (154.15 ± 16.91 mg/mL), dimethyl sulfoxide (139.04 ± 11.88 mg/mL), and Span 80 (74.93 ± 12.19 mg/mL). Based on these results, Miglyol, Labrasol, and CEL were selected as the lipid, surfactant, and cosurfactant, respectively.

3.2 Pseudo-ternary phase diagram construction

Based on solubility study, Miglyol, Labrasol, and CEL were used as a lipid/oil phase, surfactant and cosurfactant, respectively for the construction of pseudo-ternary phase diagrams. Initially, various Smix ratios were screened for the construction of pseudo-ternary phase diagrams. Finally, the 1:2 and 1:3 ratio of oil to Smix were found to show maximum SNEDDS zones. The representative phase diagrams for 1:2 and 1:3 ratio of oil to Smix are presented in Fig. 1. It was noticed that both phase diagrams showed good SNEDDS zones (Fig. 1A-B). However, Fig. 1B showed maximum SNEDDS zones compared with Fig. 1A. In addition, the optimized formulations with oil to S mix ratio of 1:2 and 1:3 were found to be stable and apparently clear without the sign of any physical instability. OFS1 (1:3) showed maximum delineated area as shown in Fig. 1B.

3.3 Formulation optimization

Several FLF-SNEDDS formulations (FS1-FS9) were prepared and optimized using FFD. The lower and higher levels of independent variables and responses for FLF SNEDDS are summarized in Table 1. A total of nine formulations were prepared with different composition as shown in Table 2. The independent variables along with their levels were selected and evaluated for all measured responses (Y1-Y3) in order to obtain the optimized formulations (Table 2).

3.4 Effect of independent variables on size (Y1)

Based on the present data set, the relationship between independent and dependent variables (particle size, Y1) is expressed using the linear equation: $Y_1 = 548.2 - 3.8X_1 + 1.6X_2$. The symbols in this equation have already been explained in experimental section. The higher F-value (F = 43.07) and lower P-value (p < 0.001) obtained in this study suggested the reliability and the best fit of the model (Table 3). The adjusted and predicted r^2 values were also obtained very close to each other (Fig. 2A), suggested the reliability of the model to select the most robust SNEDDS.

![Fig. 1](image_url)  

Fig. 1 Pseudo-ternary phase diagrams of Miglyol (lipid/oil), Labrasol (surfactant), CEL (cosurfactant) and water (aqueous phase) showing nanoemulsion sizes at (A) 1:2 ratio of oil:Smix and (B) 1:3 ratio of oil:Smix.

### Table 1

| Independent variables | Levels | Constraints |
|-----------------------|--------|-------------|
| Coded (mg)            | Actual (mg) | Low | High | Target |
| X1: Miglyol           | (-1)   | 75.0 | (0)  | 112.5 | (1)  | 150.0 | Minimum |
| X2: Labrasol          | (-1)   | 200.0| (0)  | 300.0 | (1)  | 400.0 | In range |
| Y1: Vesicle size (nm) |        | 14.2 |      | 110.7 |      | Minimum |
| Y2: Zeta potential (mV)|       | -28.6|      | -15.1 |      | In range |
| Y3: PDI               |        | 0.29 |      | 0.62  |      | Minimum |

*Note: All values in bracket are observed value and the values outside the bracket are predicted values.
Table 2  Formulation composition of FLF SNEDDS with their observed responses.

| Formulation code | X₁  | X₂  | Y₁ (Size) | Y₂ (Zeta potential) | Y₃ (PDI) |
|------------------|-----|-----|-----------|---------------------|---------|
| FS1              | (-1)| (-1)| 36.8      | -16.3               | 0.32    |
| FS2              | (-1)| (0) | 23.5      | -15.1               | 0.31    |
| FS3              | (-1)| (+1)| 14.2      | -16.8               | 0.29    |
| FS4              | (0) | (-1)| 61.5      | -22.7               | 0.41    |
| FS5              | (0) | (0) | 43.9      | -22.1               | 0.48    |
| FS6              | (0) | (+1)| 28.2      | -24.8               | 0.38    |
| FS7              | (+1)| (-1)| 110.7     | -28.3               | 0.48    |
| FS8              | (+1)| (0) | 71.1      | -28.6               | 0.62    |
| FS9              | (+1)| (+1)| 56.03     | -27.9               | 0.41    |

Composition optimized (OFS1) Y₁ Y₂ Y₃
X₁: 150 mg 57.91 -29.14 0.411
X₂: 300 mg (61.12)* (-25.53)* (0.432)*
Desirability: 0.992

Composition optimized (OFS2) Y₁ Y₂ Y₃
X₁: 150 mg 49.41 -25.03 0.459
X₂: 242 mg (53.06)* (23.53)* (39.51)*
Desirability: 0.981

*Note: All values in bracket are observed value and the values outside the bracket are predicted values.

Table 3  Model summary statistics of responses with best fit model equations.

| Regression equations with best fitted model |
|--------------------------------------------|
| Y₁ = 548.2 - 3.8X₁ + 1.6X₂ |
| Y₂ = 44.33 - 0.24X₁ + 0.10X₂ |
| Y₃ = 21.35 + 1.22X₁ + 0.173X₂ |

Statistical parameters

| Vesicle size (Y₁, nm) | Zeta potential (Y₂, mV) | PDI (Y₃) |
|-----------------------|-------------------------|---------|
| r²                    | 0.9248                  | 0.9743  | 0.7477 |
| Adjusted r²           | 0.9033                  | 0.9670  | 0.6751 |
| Predicted r²          | 0.7929                  | 0.9439  | 0.4243 |
| Model f value         | 43.07                   | 132.94  | 10.37  |
| p value               | 0.0001                  | 0.002   | 0.002  |
| Model                 | Linear                  | Linear  | Linear |
| SD                    | 8.64                    | 0.905   | 0.05   |
| Mean value            | 49.05                   | -22.71  | 0.403  |
| % CV                  | 17.62                   | 3.98    | 13.65  |
| PRESS value           | 1440.9                  | 12.55   | 0.0483 |

Value of regression coefficient represented as r², SD = Standard deviation, % CV = Coefficient of variation, PRESS = Prediction residual sum of squares.
3.5 Effect of independent variables on zeta potential ($Y_2$)

The zeta potential is the deciding parameters to obtain stable formulation. Based on the present data set, the mathematical relationship of $Y_2$ with independent variables is expressed using the polynomial equation $Y_2 = 44.33 - 0.24X_1 + 0.10X_2$ (Table 3). From response surface plot $Y_2$ for $X_1$ and $X_2$, it was found that zeta potential was increased significantly with decrease in the amount of $X_1$ and $X_2$ as shown in the Fig. 2B.

3.6 Effect of independent variables on PDI ($Y_3$)

The polynomial equation for the response $Y_3$ (PDI) was found to be $Y_3 = 21.35 + 1.22X_1 + 0.173X_2$ (Table 3). Generally, an equation having coefficient with greater than one-factor terms as well as higher-order terms suggests the interaction terms and quadratic relationship, respectively. ANOVA report showed $F$-value $= 10.37 (p < 0.0081)$ for measured response $Y_3$, suggested the suitability of the equation (predicted $r^2 = 0.4243$; adjusted $r^2 = 0.6756$) (Fig. 2C). The polynomial equations illustrated interaction curves for responses $Y_1$, $Y_2$ and $Y_3$ as shown in Fig. 3.

3.7 Desirability function for optimization

Figure 4 illustrates the 3D response surface plots which are representing the different regions of desirability at different levels of variables. Red zones of 3D plots suggested maximum values of desirability of all set of measured responses and the blue zones suggested minimum values. However, the corresponding observed mean values of the responses $Y_1$, $Y_2$ and $Y_3$ were obtained as 61.12 nm, $-25.53$ mV and 0.432 for OFS1, respectively (Table 2).

3.8 Droplet size and PDI

The mean droplet size of prepared FLF-SNEDDS was studied using DLS technique and found in the range of 14.2-110.7 nm (Table 2). The large difference in the droplet size among different SNEDDS was due to difference in the lipid content and surfactant concentration in the formulations. With respect to lipid concentration in the formulation, the droplet size of SNEDDS was found to be reduced with decrease in the lipid concentration. However, with respect to surfactants concentrations, the droplet size of SNEDDS was found to be reduced with increase in the surfactant concentration. The PDI is important parameter as it predicts the uniformity and stability of FLF-SNEDDS. The FLF-SNEDDS showed the PDI in the range of 0.29 to 0.62 (Table 2).

3.9 Zeta potential

The surface charges for SNEDDS FS1-FS9 were found in the range of $-28.6$ to $-15.1$ mV (Table 2). The values of surface charges suggested negative charge distribution over nano-sized globules. In addition, the hydrophilic nature of nonionic surfactant i.e., Labrasol is required for...
Fig. 3 Interaction plots between independent variables for the measured responses (droplet size, zeta potential and PDI).

Fig. 4 3D response surface plots for the influence of the concentrations of surfactant and lipid on droplet size, zeta potential and PDI.
the rapid emulsification of oil-in-water SNEDDS in an aqueous medium such as GI fluids.

3.10 Dispersibility and emulsification efficiency

SNEDDSs formulation released in the lumen of the GIT and dispersed into form of a fine oil-in-water nanoemulsion in the presence of GI fluid. It should be dispersed completely and quickly when subjected to dilution under mild GIT agitation due to peristaltic activity. The prepared formulations (FS1–FS9) evaluated for dispersibility and emulsification. The result showed eight formulations (FS1–FS8) passed this test with grade A. However, the formulation (FS9) showed the slight slower emulsification time with bluish appearance (grade B).

3.11 Cloud point determination

The nanoemulsions prepared with non-ionic surfactant have the problem of temperature dependent phase behavior. The cloud point defined as the temperature above which the clear formulation turns into turbid (cloudiness). The phase separation takes place at high temperatures due to the reduction in water solubility of the surfactant and phase separation takes place. The cloud points of different formulations were found in the range of 56-67°C. The cloud point of an optimized SNEDDS OFS1 was obtained as 58°C. The lower values of cloud points obtained for all formulations suggested that all formulation were remained clear and there was no phase separation.

3.12 Optical clarity

The blank water is considered to have 100% transmittance vindicating minimum absorbance and vice versa. The diluted formulations (FS1-FS9) showed the absorbance values in the range of 0.098–0.412 (Table 4).

3.13 Entrapment efficiency (EE%)

OFS1 showed the high% EE of 98.23 ± 4.67% of FLF in the formulation with obvious loss during processing. High% EE may be attributed to the improved solubility of FLF in the explored lipid and surfactant.

3.14 In vitro diffusion study

The results of in vitro diffusion study of pure FLF and OFS1 are portrayed in the Fig. 5. OFS1 showed significantly better and faster release than FLF suspension. The result exhibited 91.38 ± 5.34% and 11.38 ± 1.12% drug release from OFS1 and FLF suspension, respectively within first 2 h.

3.15 Transmission electron microscopy

The sophisticated technique assured the shape and the microstructure of droplet after reconstitution. The morphology and structure of the reconstituted OFS1 formulation was observed using an electron microscope (Fig. 6). The spherical images of dark droplet found with bright surroundings.

3.16 Hemolysis study

The effect of pure drug, excipients, Triton X-100 and OFS1 to an erythrocyte was studied by in vitro RBC lysis (hemolysis) test (Fig. 7). The positive control Triton X-100 showed 100% lysis of RBC, whereas pure FLF showed about 16.4 ± 1.92%. OFS1 showed the hemolysis of 11.2 ± 1.98% which is lower than the pure FLF. However, these excipients and OFS1 showed minimum hemolysis close to that of negative saline.

3.17 Anti-inflammatory activity

The results of in vivo anti-inflammatory activity of OFS1 in comparison with pure FLF are presented in Fig. 8. The
activity of pure FLF as well as OFS1 was found to be increased for all time points. The % inhibition for OFS1 was found as 25.68 ± 2.68% compared with pure FLF 6.42 ± 0.78% after 1 h of oral administration. There was a highly significant difference in the % inhibition values with pure FLF \((p<0.05)\) at all-time points. After 6 h of oral administration, the anti-inflammatory activity of FLF-SNEDDSopt was also found significant compared to the pure FLF.

4 Discussion

In spite of good therapeutic efficacy of several drugs, poor-solubility in water and inadequate absorption of these drugs limited their clinical applications. SNEDDS is well studied in enhancing the in vitro and in vivo performance of poorly soluble drugs and thus serves as an ideal carrier for the delivery of BCS classes II and IV drugs. The present research work was carried out to study the role of SNEDDS in order to enhance the dissolution rate and therapeutic efficacy of FLF.

The solubility of the drug in excipients plays a vital role in the formulation stability. There are number of formulations undergo precipitation before undergoing in situ solubilization. For an ideal formulation, the entire amount of drug should be solubilized in SNEDDS composition (oils, surfactant and co-surfactant). The drug must be soluble in explored composition and the formulation should be clear, isotropic and monophasic liquid at room temperature. The composition must have the good solubilizing capacity to incorporate the drug in minimum volume intended for oral administration. In order to select different components for the preparation of FLF SNEDDS, the solubility of FLF was determined in various lipids, surfactants and co-surfactants. Based on the maximum solubility of FLF in different components, MIG (as a lipid), Labrasol (as a surfactant) and CEL (as a co-surfactant) were selected for the formulation of FLF SNEDDS. The selected excipients exhibited thermodynamically stable and isotropic SNEDDS pre-concentrate with complete emulsification in water.

Phase titration methods are used for the construction of pseudo-ternary phase diagrams in order to obtain optimum SNEDDS formulation. For the construction of phase dia-
Flufenamic Acid-Loaded SNEDDS

J. Oleo Sci.

grams, MIG (oil phase), Labrasol (surfactant) and CEL (cosurfactant) were utilized and titrated with water. Various pseudo-ternary phase diagrams were constructed against different ratio of oil to S mix in order to get maximized area covered with stable nanoemulsions. The maximum nanoemulsion area was recorded in 1:3 ratio of oil: S mix. Optimized formulation was also selected from 1:3 ratio of oil: S mix. The objective of the present optimized SNEDDS formulation was to evaluate their anti-inflammatory activities which could lead to producing enhanced therapeutic efficacy than pure FLF suspension. The prepared FLF SNEDDS and placebo formulations were found to be isotropic, clear and stable without the sign of precipitation or phase separation. Finally, the optimized SNEDDS formulations were taken for further studies.

The droplet size of prepared SNEDDS was obtained in the range of 14.7-110.7 nm. In addition, formulation FS3 showed the lowest droplet size amongst other SNEDDS which could be due to low level of lipid (X1) and increased amount of Labrasol (X2).

Fig. 7 In vitro hemolysis study of FLF, excipients and optimized FLF SNEDDS (OFS1); values are presented as mean ± SD, n = 3.0.

Fig. 8 Comparative anti-inflammatory activity of pure FLF and optimized FLF SNEDDS (OFS1); values are presented as mean ± SD, n = 6.0.
The ANOVA report indicated that the measured response \( Y_2 \) was well described by this polynomial mathematical model as evidenced by high F-value (132.94).

Generally, an equation having coefficient with greater than one-factor terms as well as higher-order terms suggests the interaction terms and quadratic relationship, respectively. An optimum concentration of \( X_1 \) and \( X_2 \) need to be taken into account for minimum PDI \( (F = 113.18) \). The synergistic activity of linear term \( X_i^2 \) was significant \( (p < 0.01) \). Based on these results, it can be suggested that the optimum content of both components has to be implemented to get an optimized formulation which is ostensible from the plots. There are no interactions between independent variables \( (X_1 \) and \( X_2 \)) against explored responses based on polynomial equation.

The aim of experimental design was to get the most robust SNEDDS by optimizing the levels of each independent variable. The desirability function is being used to get the formulations within the expected constraints. Desirability function (i.e., level zero to one) is a quantitative optimization method in which each measured response is assigned a goal to optimize the experimental conditions. Using this approach, the most robust formulation is optimized within the desired experimental conditions \(^{41} \). In this research work, the measured responses \( Y_1 \) and \( Y_2 \) were assigned minimum and \( Y_2 \) was set in the desired range \( Y_3 \) for optimization. The main aim was to obtain an optimized SNEDDS with minimum size \( (Y_1) \) and PDI \( (Y_2) \) with the zeta potential in the desired range \( (Y_3) \). In the given set of experimental conditions, the overall desirability was observed as maximum at the medium levels of \( X_1 \) and \( X_2 \) of optimized SNEDDS. Between optimized SNEDDS (OFS1 and OFS2), OFS1 was selected as the most optimum one based on the maximum overall desirability value \( (0.992) \) compared with OFS2 \( (0.981) \). The statistical analysis indicated that the predicted values of OFS1 at \( X_1 \) (150 mg) and \( X_2 \) (300 mg) for the responses \( Y_1 \), \( Y_2 \) and \( Y_3 \) were 57.91 nm, \( -29.14 \) mV and 0.48, respectively. In addition, the observed values fall within the two-sided 95 percent prediction interval and confidence interval of predicted responses. This observation indicated the accuracy of the proposed model. The good agreement between the predicted and observed values suggested the reliability of the optimization process with the highest desirability function.

The minimum size (14.7 nm) shown by FS3 may be due to the presence of a higher amount of surfactant (400 mg, Labrasol) as compared to other formulations. The droplet size is a very important factor for the efficient emulsification of SNEDDS in order to enhance the \( \text{in vivo} \) performance. In general, the smaller droplet size had several advantages over larger one including "efficient emulsification, increased surface area, increased dissolution in the hydrophilic mucus layer, increased drug absorption and increased intraenterocyte endoplasmic reticulum processing for chylomicron formation " \(^{22-25} \). In the present research, we prepared SNEDDS with the addition of a fixed concentration of co-surfactant (CEL, 200 mg) which played a greater role in efficient emulsification of SNEDDS. The SNEDDS formulations containing a mixture of oil and a single surfactant are not capable of forming a stable SNEDDS \(^{42} \). Most of the particles were homogeneously distributed within the standard range which reflects a good sign of emulsification of stable product.

The values of zeta potential suggested negative charge distribution over nano-sized globules \(^{40} \). The negative zeta potential values of SNEDDS formulations were possible due to the presence of various fatty acid esters in studied lipid i.e., MIG (caprylic/capric triglycerides). Fatty acid esters are negative charged and hence they produced negative zeta potential of SNEDDS \(^{42-45} \). In addition, the hydrophilic nature of nonionic surfactant i.e., Labrasol is required for the rapid emulsification of oil-in-water SNEDDS in an aqueous medium such as GI fluids. MIG is lipophilic in nature which may lead to phase separation with an inefficient concentration of Labrasol. In the same way, hydrophobic drug (FLF) could be precipitated in the GIT after oral application. Hence, the amphiphilic Labrasol can solubilize relatively high concentrations of lipid and prevents the precipitation of FLF in the GIT lumen after oral application. Therefore, drug-loaded lipid prolonged the existence of the drug in molecular state which results in an effective absorption \(^{45} \). Because all prepared SNEDDSs passed the test either in grade A or B, these formulations were taken for further evaluation \(^{40} \). The drug solubility and stability decreased due to the phase separation, and ideally the cloud point should be over 37°C \(^{25} \). The cloud point for all SNEDDS formulations was found significantly higher than 37°C, indicating a stable and transparent system. The optically clear and small droplet size formulations showed the lower absorbance value (high% transmittance) due to nanization of lipidic carrier via self-emulsification \(^{40} \). It was observed that the optical absorbance was inversely proportional to the amount of Labrasol (surfactant). At higher surfactant concentration, the droplet size may decrease due to sufficient surfactants available to stabilize the o/w interface \(^{40} \). The higher EE of optimized formulation might be prudent to correlate augmented emulsification and subsequent absorption at the absorption site in GIT lumen. The significantly higher drug release shown by SNEDDS due to higher solubility of FLF in used lipid, small size attributed to the greater surface area and thus promoting faster release rate. The sophisticated technique assured the shape and the microstructure of droplet after reconstitution. The size and surface morphology of the internal oil droplets of an optimized SNEDDS was evaluated using sophisticated technique i.e., TEM. The droplet size of SNEDDS is generally very low (less than 100 nm). The ultra-low droplet size and surface morphology of SNEDDS

\[ \text{SNEDDS} \]
Flufenamic Acid-Loaded SNEDDS

5 Conclusion

FLF is a well-established anti-inflammatory drug prescribed in conventional dosage form. However, oral delivery possessed limited absorption and dose-related side effects. In this context, SNEDDS formulation was investigated as a promising approach exploiting GRAS excipients intended for oral administration. Based on the in vitro findings, the optimized FLF-loaded SNEDDS was suitable in term of globular size, PDI, zeta potential, and % EE. Proficient emulsification and drug release within 2 h (immediate release) concluded successful formulation intended for oral delivery. Hemolysis study negates the probable chances of surfactants triggered hemolysis which ensured hemocompatibility of the product within explored concentration. Eventually, anti-inflammatory efficacy of FLF-SNEDDS was relatively higher than pure FLF suspension as evidenced with in vivo finding which might be owing to poor solubility of FLF in suspension. Improved pharmacokinetic parameters still need to be investigated to generate preclinical data using rat model. Conclusively, the present formulation could be a suitable alternative to conventional dosage form with enhanced efficacy.

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Conflict of Interest

Authors report no conflict of interest associated with this manuscript.

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