RESEARCH ARTICLE

Nanoemulsion Formulation of Leflunomide for Transdermal Delivery: Preparation and Characterization

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ABSTRACT

Leflunomide (LEF) is an antirheumatic drug belonging to a class of drugs known as disease modifying antirheumatic drugs, indicated for moderate to severe psoriatic arthritis and rheumatoid arthritis. Orally administrated LEF causes many side effects that could result in treatment failure. Hence, in this study, leflunomide was formulated as a nanoemulsion to develop a transdermal dosage form that can circumvent such side effects and increase the rate of successful treatment by improving patient compliance with the medication. The nanoemulsion area was constructed by using pseudo-ternary phase diagrams. A total of 20 nanoemulsion formulas were prepared. These formulas' mean particle size, zeta potential, and droplet morphology were studied. Thermodynamic stability studies were performed to test the stability of the prepared formulations. Also, the *in-vitro* release of the drug from the formulations was evaluated using phosphate buffer saline pH 7.4 as the release medium. According to the saturation solubility study results, capryol TM 90 was selected as oil, labrasol and Tween 20 as surfactants for formulation coded A1 and A2, respectively, and transcutol p as cosurfactant together with deionized water as the aqueous phase for the preparation of nanoemulsions. The LEF-nanoemulsion formulations were in the nanosize range (from 54.7–210.2 nm). Images for the chosen formulas revealed dark spherical nanoemulsion droplets against bright surroundings. All of the preparation showed a complete drug release by the end of 220 minutes and the release was highly dependent on the particle size. Based on the result of this study, the prepared LEF-nanoemulsion is successfully prepared and can be considered a promising approach to developing a transdermal preparation.

Keywords: Capryol 90, Leflunomide, Nanoemulsion, Transdermal, Transcutol P, Tween 20.

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INTRODUCTION

Rheumatoid arthritis (RA) is a systemic, chronic autoimmune disease that affects up to 1% of the population. It is accompanied by moderate and severe impairment, lowering a patient's quality of life. Many RA therapies aim to manage pain and inflammation, maintain the patient's functional abilities, and prevent the illness from progressing and damaging the joints. The treatment is complicated and entails the use of a variety of different pharmacological classes through a variety of different modes of administration, including oral, intramuscular, and subcutaneous. Glucocorticoids (GC) are extensively utilized in acute illness flares to rapidly reduce pain and swelling and seize control of the inflammation, either orally or intraarticular injections. Oral GC is intended for short-term usage (up to 3–4 months) and must be tapered as quickly as possible to prevent negative effects. To maintain long-term management of inflammation, disease-modifying antirheumatic drugs (DMARD) that spare GC are required. Altogether, the transdermal drug delivery route offers attractive potential for addressing various oral drugs' low bioavailability, the difficulty and discomfort associated with injections, and the poorly regulated release concerns associated with both. Transdermal medication delivery has developed significant translational applications as a viable method for the treatment of RA. The fast growth of enhancing technologies has accelerated stratum corneum disruption and increased the number of therapeutic compounds available for the effective treatment of RA. Additionally, the use of passive targeted delivery techniques through the transdermal channel has emerged as a potential strategy for the effective treatment of RA.[1,2]

During the last years, the development of the drug delivery system has been changed from the use of oral drug delivery system to the transdermal route of administration. Therapeutic benefits and patient compliance are improved by using the

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transdermal route of administration.[1] Gastrointestinal, renal, and cardiovascular adverse reactions are minimized by the use of transdermal drug delivery. Compared to intravenous (IV) and intramuscular (IM) routes of administration, the transdermal route is considered as a pain-free and non-invasive drug delivery route, also, patients' compliance is greatly improved as it is a self-administrated route of drug delivery.[3] Many factors affect drug penetration through the skin, a complex organ characterized by multiple layers, some of these factors are the molecule mass, partition coefficient, and molecule charge. The skin can be utilized as a delivery site for systemic and local drug delivery. Molecules with a small molecular weight of less than 500 Daltons and moderate lipophilicity with (log P of 1 to 3) can pass through the stratum corneum passively.[4]

Nanoemulsions are kinetically stable colloidal dispersion of biphasic, isotropic, kinetically stable, and transparent nature consisting of two liquids immiscible in each other with a vesicular size of lower than 200 nm.[5] Because the skin is considered as a complex organ with a challenging layer to pass through that is stratum corneum, a nanoemulsion system can be utilized as a vehicle for the delivery of drugs through this barrier as it is a highly stable system characterized by its quick permeation through the skin.[6] The side effects associated with routes other than the transdermal route of administration are minimized by using nanoemulsions because it provides a profound increase in the passage of drugs across the skin layers. Low surface tension and large surface area are two essential features of nanoemulsions that enable these systems to provide improved penetration of drugs through the skin.[7]

Nanoemulsions are stabilized by using both emulsifiers and co-emulsifiers as an interfacial layer. Oil and aqueous phases, when mixed, form an emulsion of coarse nature, but the use of an emulsifier changes the nature of coarse emulsion to nanoemulsion either spontaneously or by using high-energy methods. Drugs of low water solubility are the best choices for forming o/w nanoemulsion, as this system improves the poor solubility of these drugs. Because of their safety, non-ionic emulsifiers are preferred to prepare nanoemulsions as these emulsifiers have a lower toxicity profile and are less irritant than cationic and anionic emulsifiers. Also, the best emulsifiers are chosen on the basis of their emulsification ability and their solubility. Hydrophilic-lipophilic balance value of eight to sixteen is an essential feature in surfactants used in oil preparation in water nanoemulsion. Generally, C3 to C8 alcohols such as glycerin, Transcutol p, ethylene glycol, ethanol, propylene glycol, and propanol are used as co-emulsifiers. The use of co-emulsifiers helps reduce the fluidity of the interface and thereby increases the system's entropy.[8] Nanoemulsions are better formulated using surfactants, cosurfactants, and oils that are non-irritant, non-toxic, and approved by the FDA to be used as a safe system for human consumption.[9]

Leflunomide (LEF) is an antirheumatic drug belonging to a class of drugs known as disease-modifying antirheumatic drugs, it is indicated for the treatment of moderate to severe psoriatic arthritis and RA. LEF is a class II drug with low solubility in water (lower than 40 mg/L). LEF side effects cause patients incompliance, mainly gastrointestinal side effects like abdominal pain and oral ulceration.[10] Despite being the most preferred route of drug administration, the oral route of LEF is associated with systemic side effects. LEF's most known side effect is diarrhea. There are also other undesirable side effects combined with LEF, including colitis, cholelithiasis, esophagitis, and aphthous stomatitis. LEF has a low molecular weight, favoring its formulation in a transdermal dosage form. However, its low aqueous solubility and poor lipophilicity make it a challenging route. The utilization of nanoemulsion as a carrier system will provide a high surface area due to the nanosized particles. Therefore, there will be an improvement in the aqueous solubility, and this formulation will provide deep skin penetration. Hence, the objectives of this study are the preparation and evaluation of a nanoemulsion for transdermal delivery of LEF in order to circumvent some of the side effects associated with the oral route of delivery of this drug and to improve patient compliance with the medicine.

MATERIALS AND METHODS

Materials

Leflunomide powder, Tween 20, Tween 80, and oleic acid were obtained from Hyper-Chem LTD CO, China. Ethanol and methanol lab-grade solvents, PEG-200, and PEG-400, were purchased from Sigma Aldrich, USA. Labrasol, transcutol p, capryol ™ 90, and Labrafil M 1944 were purchased from gattefosse (Saint-Priest, Cedex, France). Anise oil, olive oil, castor oil, almond oil, avocado oil, coconut oil, and sesame oil were purchased from NOW® CO., USA. All of the substances and reagents used in this work were of analytical grade.

Methods

Differential Scanning Calorimeter (DSC)

Leflunomide powder was evaluated using a DSC study for its thermal behavior and thermotropic properties by DSC using DSC /TA-60 (Shimadzu, Japan) instrument equipped with the intercooler 2 colling system. Nitrogen was used as a blank gas, and samples (3–5 mg) were heated in opened aluminum pans at a scanning rate of 10°C per minute. The scanning temperatures were between 50–250°C.[11]

Saturated Solubility Study of LEF

The saturated solubility of LEF was measured in different surfactants, cosurfactants, oils, and dissolution media by using the saturation shake-flask method. Measuring solubility was done by adding an excess amount of LEF in a conical flask containing 5 mL of tested surfactants, cosurfactants, and oils. The saturated solution was prepared by shaking each flask in a shaking water bath for 72 hours at 25 ± 1°C. Then when equilibrium was reached, the samples were centrifuged for fifteen minutes at 3000 rpm. After that, filtration of the centrifuged samples was done using a 0.45 µm millipore filter. The filtrated sample solution was prepared for reading in UV-vis spectrophotometer by diluting each sample with

methanol. The reading was done at λ_{max} of LEF. All the measurements were done in triplicate, and the results were represented as mean ± SD.[12]

Construction of Pseudo-ternary Phase Diagrams

The aqueous titration method was used to create the pseudo-ternary phase diagrams to determine the zone of nanoemulsion. A mixture of surfactant and cosurfactant (S_{mix}) was used consisting of either labrasol or Tween 80 as surfactant with transcutol p as cosurfactant. The surfactant: cosurfactant mixture was mixed with the oil phase (Capryol^{TM} 90). The S_{mix} weight ratios that were used for the titration are 1:1, 2:1, 3:1, 4:1, 1:2. Each S_{mix} proportion oil phase was mixed in a range of weight ratios from 1:9 to 9:1, then distilled water was titrated with each proportion at 25°C with continuous stirring with recording the amount of water added until the mixture changed from clear to turbid by visual inspection of each sample. The pseudo-ternary phase diagrams were created in which the oil phase represents one axis, the S_{mix} represents another axis, and distilled water represents the third axis. Origin 7.0 software was used as a drawing tool to construct pseudo-ternary phase diagrams.[13]

Preparation of Nanoemulsion

Tweenty nanoemulsion formulations were made using Capryol TM 90 as an oil ingredient, transcutol p as co-surfactant, labrasol, and Tween 20 as surfactants for A-1 formulations and A-2 formulations, respectively. LEF at 1% (w/w) percentage was used to prepare the nanoemulsion formulation by dissolving it in the oil phase (*i.e.,* Capryol ^{TM} 90). The dissolved drug was mixed with the S_{mix}, then the whole system was mixed using a vortex mixer for 5 minutes. The water phase was added gradually to the mixture at room temperature. The compositions of the 20 formulations are shown in Table 1.[5]

Evaluation of LEF Nanoemulsion Thermodynamic Stability Studies

Thermodynamic stability studies were used to assess the prepared formulation stability and choose the best one to prepare the nanoemulsion. Three types of tests were used, which are centrifugation test, heating-cooling test, and freeze-thaw test. Initially, a 30 minute centrifugation test at 25°C was done for each sample at a centrifugation speed of 5000 rpm. The primary endpoint was the visual inspection of any turbidity, phase separation, or precipitation. After that, six heating and cooling cycles at temperatures between 4 and 40°C were used. The formulations were tested in these temperatures for 48 hours in each of the temperatures mentioned above. Finally, a freeze-thaw test was carried out by storing each formulation for 48 hours at –20 and 25°C. Freeze-thaw stability was observed by visual inspection of each sample for any physical changes.[14]

Droplet Size Measurement and PDI

The prepared formulation average droplet size was measured by first mixing 0.1 mL of each formulation in a 50 mL volumetric

Table 1: Composition of LEF loaded nanoemulsions

Ingredients (%w/w)						
Formula Code	*Formula Number*	*Smix ratio*	*X1*	*X2*	*X3*	*X4*
			(S_{mix})	*(oil)*	*(water)*	*(Drug)*
A1	F-1	1:1	60	7.5	32.5	1
	F-2	1:1	50	15	35	1
	F-3	2:1	60	7.5	32.5	1
	F-4	2:1	50	15	35	1
	F-5	3:1	60	7.5	32.5	1
	F-6	3:1	50	15	35	1
	F-7	4:1	60	7.5	32.5	1
	F-8	4:1	50	15	35	1
	F-9	1:2	60	7.5	32.5	1
	F-10	1:2	50	15	35	1
A2	F11	1:1	60	7.5	32.5	1
	F12	1:1	50	15	35	1
	F13	2:1	60	7.5	32.5	1
	F14	2:1	50	15	35	1
	F15	3:1	60	7.5	32.5	1
	F16	3:1	50	15	35	1
	F17	4:1	60	7.5	32.5	1
	F18	4:1	50	15	35	1
	F19	1:2	60	7.5	32.5	1
	F20	1:2	50	15	35	1

flask containing water. Fluctuations in light scattering caused by Brownian particle motion were analyzed by measuring dynamic light scattering at a temperature of 25°C at an angle of 90° using Brookhaven (Crop 90 plus, NY, USA) as an analyzing tool for droplet size determination. Homogeneity in droplet size was determined by measuring the PDI.[15]

Percent of Light Transmittance Assay

Percentage transmittance of the prepared formulations was measured using a UV-visible spectrophotometer at 650 nm (Pathak *et al.,* 2014). The blank used was distilled water. This measurement gives an insight into the particles' size and the formulation's stability. All the measurements were done in triplicate, and the measurements were represented as mean ± SD for more precise results.[16]

Dye Solubility Test

To confirm the formation of o/w nanoemulsion, a dye test was performed using methyl orange, a water-soluble azo dye, which would disperse homogeneously in the prepared formulations if they are of o/w type.[11]

Measurement of pH

A pH measurement test was utilized. The pH values of all the prepared o/w nanoemulsions were measured using a digital pH meter calibrated prior to use with a standard buffer solution of pH 4.01 and pH 7.01. The test was done in triplicate, and the measurements were represented as mean ± SD for more precise results.[17]

Drug Content Measurement

The drug content of nanoemulsion formulations was done by dissolving 10 g (supposed to contain 10 mg of LEF) of each formulation in 100 methanol. To ensure complete mixing, sonication was done for 15 minutes; the samples were then filtered using a 0.45 µm filter syringe. UV/vis spectrophotometer was used at the λ_{max} of LEF to determine the drug content. The test was done in triplicate and the measurements were represented as mean ± SD for more precise results.[18]

In-vitro Drug Release Study

The dialysis bag technique was used to assess the *in-vitro* drug release by using a semipermeable membrane. The dialysis bag (molecular weight cut off 12000 Dalton) was used with USP dissolution apparatus type II. To create a sink condition, a volume of 500 mL phosphate buffer of pH 7.4 was used and the temperature was set at 37 ± 0.5°C, the stirring was fixed at 50 rpm. All the prepared nanoemulsion formulations containing LEF were evaluated by adding 1-mL (contains 10 mg LEF) of each formula to a separate dialysis bag. Samples (5 mL each) of the release media were withdrawn at fixed time intervals 5, 10, 15, 30, 45, 60, 90, and 120 minutes and were filtrated through a 0.45 um filter syringe. Then each filtrate was analyzed by UV-vis spectrophotometer at the λ_{max} of LEF 258 nm. A freshly prepared medium was used to replace the withdrawn volume. Drug release was compared with a prepared formula containing drug suspension. The test was done in triplicate and the measurements were represented as mean ± SD for more precise results.[19]

Zeta Potential Measurement

The measurement of zeta potential was done for all A1 and A2 formulations. The droplets' charge was detected using Aetasizer Brook Heaven instrument USA. A small amount (0.1 mL) of the samples was dispersed in 50 mL double distilled water with sonication then samples were placed in clear disposable zeta cells for the recording of the results.[20]

Selection of Optimum LEF Nanoemulsion Formula

The optimized formula was chosen based on the evaluation tests, especially particle size analysis, PDI, pH, drug content, and *in-vitro* release study.

Transmission Electron Microscope (TEM)

The structure and size of the nanoemulsion were analyzed with the aid of a TEM. The examination started by applying one drop of diluted nanoemulsion on a carbon-coated copper film grid of 300 mesh, then staining was done using one drop of phosphotungstic acid (2% w/v). The preparation was left to dry for 30 seconds then, the dried coated grid was observed in the electron microscope.[21]

RESULTS AND DISCUSSION

LEF Solubility Study in Different Nanoemulsion-Forming Ingredients

Solubility study is an integral part of nanoemulsion development. Therefore, screening for oils, surfactants, and co-surfactants that show maximum drug solubilizing capacity and show the highest miscibility with each other was carried out. The selection of an appropriate oil is a crucial step in preparing stable nanoemulsion as it is considered as a fundamental ingredient in nanoemulsion, especially in o/w nanoemulsion. The oil content of nanoemulsion highly affects the morphology and size of the particles and significantly affects the release of the drug from the formulation. Choosing oil of maximum drug solubilizing capacity will ensure optimum drug content in the prepared nanoemulsion. Moreover, the oil aids in the solubilization of the lipophilic drug molecule, which is highly important in nanoemulsion preparation. It helps maintain the drug in the solubilized form to avoid precipitation over time and increase drug permeation through the skin.[16] In addition, high drug solubility in the oil phase will avoid using high oil content to ensure proper drug loading, hence there will be less need for the addition of high surfactant concentration and will eventually avoid any instabilities and toxicity of the prepared formulation.[22]

A wide range of oils has been utilized to prepare nanoemulsions, oils of modified origin were widely utilized as oil phase due to their high emulsification capacity. Capryol™ 90 is a light yellow oily liquid consisting of 90% caprylic acid monoester and it is defined as propylene glycol monocaprylate (type II).[23]

LEF exhibited the highest solubility in Capryol™ 90 (119.67 mg/mL) as an oil phase among other oils that have been

tested, as shown in Figure (1a). The high solubilizing capacity of Capryol™ 90 may be due to its low molecular volume, surfactant properties, and ability to easily dissolve lipophilic drugs. Capryol ™ 90 has an HLB value of six. [23] The fact that LEF has a low polar surface area of 53.92 A^2, high solubility in modified oil, as the one with the above-mentioned properties, was expected.[24] Also, based on the available literature reviews, incorporating Capryol ™ 90 as an oil phase in nanoemulsion preparation will improve the penetration of drugs with low water solubility in the transdermal delivery system.[15]

Results of LEF saturated solubility in various oils, surfactants, and co-surfactants are shown in Figure 1. As per the solubility data obtained, Capryol ™ 90 was utilized as an oil phase.

Tween 20 and Labrasol as the surfactants, and transcutol p as the co-surfactant to prepare a stable nanoemulsion formulation. In order to determine the optimum concentration for each nanoemulsion ingredient (oil, surfactant, and cosurfactant), the aqueous titration method (spontaneous emulsification method) was used to construct the pseudo-ternary phase diagrams. These diagrams are a valuable tool in order to determine the zone of nanoemulsion and providing a picture of the system that has the best concentration blends of surfactant/cosurfactant so that there will be a reduction in the free energy of the system and an increment in the dispersion entropy which will eventually lead to spontaneous and thermodynamically stable nanoemulsion. Also, diagram construction is beneficial in determining the association and effect of each component of the nanoemulsion with the phase behavior of the mixture.[25]

Construction of Pseudo-ternary Phase Diagrams

Continuous visual inspection of the mixture was done during the aqueous titration process to illustrate any turbidity of the system that indicates the formation of a biphasic system. In contrast, a clear and transparent appearance indicates the formation of a monophasic system. In the pseudo-ternary phase diagrams, the shaded areas indicate the existence of nanoemulsion. The presence of diagrams with wider regions reflects a better self-nano emulsification ability.[25] According to the rule stated by Bancroft, the phase in which the surfactant is more soluble represents the continuous phase which reflects the type of nanoemulsion prepared to be either oil in water or water in oil nanoemulsion. Based on this rule, Tween 20, which has an HLB value of 16.7, and Labrasol with an HLB value of 14, were used as surfactants.[26]

As shown in Figure 2, ten phase diagrams were constructed using various ratios of Capryol™ 90 as oil phase, Tween 20 and labrasol as surfactants, and Transcutol p as cosurfactant with deionized water aqueous phase. The 1:1, 2:1, 3:1, 4:1, and 1:2 w/w ratios of the S_{mix} component were used to construct the phase diagrams. CHEMEX school software 3.6 was used to build up the diagrams in which the orange area represents the nanoemulsion area.

Selection of the Nanoemulsion Formulas

Many formulations can be made from the nanoemulsion region that appeared in the pseudo-ternary phase diagram. According to the constructed phase diagrams, the w/w percentage of oil phase that could be used is up to 15%, keeping the particle size within the desirable nanoemulsion particle size which is less than 200 nm. Another criteria that was considered in choosing the best concentration of the oil phase is that the oil phase concentration should completely solubilize the dose of the drug used. Furthermore, to avoid any irritation and deliver the

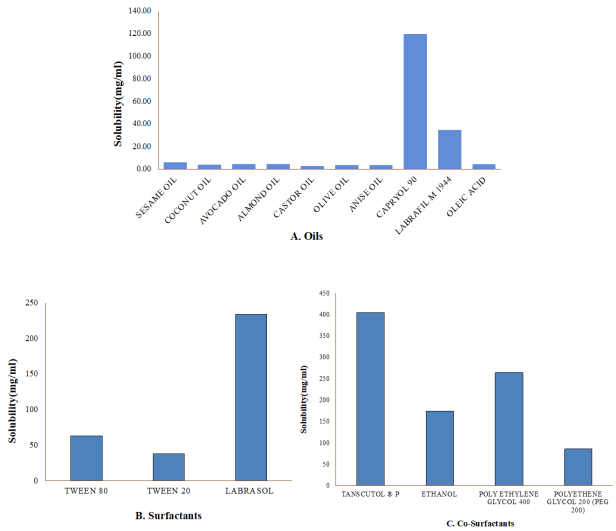

Figure 1: Leflunomide saturation solubility study in different oils (panel A), Surfactants (panel B), and Co-surfactants (panel C).

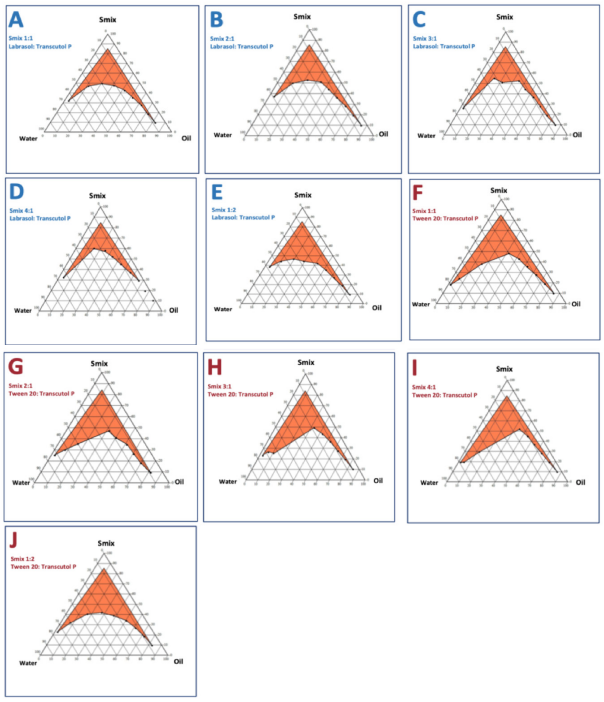

Figure 2: Pseudo-ternary phase diagrams of Capryol TM 90 (oil), Tween 20, Labrasol (surfactants) and Transcutol (cosurfactant) using different ratios of these ingredients as shown in each panel

drug safely, a non-ionic surfactant such as labrasol and Tween 20 in a low concentration was preferred in the formulations.[27]

Thermodynamic Stability Tests

Stress tests were used to prepare a stable nanoemulsion formulation and avoid the possibility of phase separation, drug precipitation, and turbidity of the formulations. All the 20 formulations were subjected to the heating-cooling cycle, centrifugation, and freeze-thaw cycle tests and all the formulas passed the thermodynamic stability tests. These results confirm that all the formulations have optimum stability, which could translate into a long shelf-life.[19]

Droplet Size Measurement and Polydispersity

The average droplet size of the prepared formulas ranged from (54.7–210.2 nm). as shown in Table 2, indicating that all the droplets were nanosized. It was observed that increasing the oil content increased the globule size of the droplets. These results were also reported in previous studies, illustrating that the expansion of oil droplets of nanoemulsion will increase the mean size of the droplets by further adding oil and due to the simultaneous decrease in the concentration of Smix in the formulations.[28] Increasing the surfactant concentration decreases the mean droplet size and this may be attributed to the fact that interfacial tension between water and oil phases will decreases using a surfactant; therefore the amount of free energy required to deform the droplets will decreases resulting in smaller droplet size.[29] The PDI gives an insight into the stability of the nanoemulsion, droplet size homogeneity, and the width of the particle size distribution. It is defined as the ratio of standard deviation to mean droplet size, ranging from 0.0 to 1.0. A high value of PDI indicates an undesirable feature of low homogeneity of the particles. In contrast, low PDI implies good stability of the preparation and uniform droplet size distribution.[30] PDI value near zero reflects monodisperse droplets, while PDI closer to one indicates a wide droplet size range.[31] All the formulations had a PDI lower than 0.5 (Table 2), indicating homogeneity of the particles and narrow globule size distribution.[32,33]

Percent Light Transmittance Measurement

The percentage of light transmittance of the nanoemulsion formulations is illustrated in Table 2. All the formulation has a percentage of light transmittance closer to 100%, indicating that the nanoemulsion's formulations are transparent due to their small droplet size.[34]

pH Measurement

Skin irritation could result from the pH variation between the nanoemulsion's pH and that of the skin. The pH of the developed 20 formulations is shown in Table 2, which ranges from 4.93 to 5.58, indicating compatibility and safety of all the formulations for transdermal delivery.[7,35]

Drug Content in the Prepared Nanoemulsion

The result of the drug content of the 20 formulas complies with united states pharmacopeia 38, 2015, the drug content of LEF was between 97.25 to 99.78. These drug content results are considered within the acceptable range, indicating that LEF

Table 2: Characterization of the prepared nanoemulsion formulations

Formula Code	*Formula Number*	*Mean droplet size (nm)*	*PDI*	*%light transmittance*	*pH*	*Drug Content*	*Zeta potential*
A1	F-1	55.7	0.26	99.84 ± 0.12	5.58 ± 0.02	99.42 ± 0.11	-19.29
	F-2	123.1	0.094	99.27 ± 0.24	5.45 ± 0.03	98.06 ± 0.24	-17.51
	F-3	78.3	0.234	100 ± 0.17	5.56 ± 0.08	97.25 ± 0.16	-13.42
	F-4	157.9	0.147	99.43 ± 0.09	5.37 ± 0.03	97.91 ± 0.18	-15.32
	F-5	82.2	0.214	99.27 ± 0.16	5.07 ± 0.07	98.73 ± 0.23	-18.92
	F-6	156.5	0.533	99.97 ± 0.23	5.36 ± 0.02	99.78 ± 0.28	-12.52
	F-7	80.3	0.163	99.11 ± 0.17	5.15 ± 0.06	99.65 ± 0.27	-15.13
	F-8	110.5	0.334	99.7 ± 0.13	5.54 ± 0.04	99.04 ± 0.18	-14.92
	F-9	68.6	0.263	99.96 ± 0.25	5.29 ± 0.01	99.41 ± 0.16	-16.42
	F-10	70.3	0.298	100 ± 0.16	5.38 ± 0.01	99.32 ± 0.21	-18.42
A2	F11	86.4	0.321	98.43 ± 0.16	5.08 ± 0.04	99.23 ± 0.16	-15.31
	F12	210	0.141	98.63 ± 0.17	5.23 ± 0.03	99.51 ± 0.12	-20.28
	F13	63.3	0.334	98.43 ± 0.19	4.93 ± 0.08	98.93 ± 0.22	-19.42
	F14	139.9	0.251	98.47 ± 0.31	5.12 ± 0.05	99.45 ± 0.15	-18.42
	F15	58	0.335	97.35 ± 0.15	5.26 ± 0.03	99.72 ± 0.23	-16.49
	F16	130.1	0.234	98.45 ± 0.13	5.19 ± 0.03	99.36 ± 0.17	-17.48
	F17	54.7	0.112	99.53 ± 0.32	5.56 ± 0.06	99.45 ± 0.18	-21.58
	F18	110.3	0.159	98.95 ± 0.17	5.38 ± 0.04	98.68 ± 0.17	-18.46
	F19	149.1	0.251	98.45 ± 0.16	5.38 ± 0.01	99.78 ± 0.12	-24.21
	F20	210.2	0.346	98.34 ± 0.28	5.13 ± 0.07	99.59 ± 0.18	-21.9

was successfully loaded in all the prepared formulations with homogeneity and stability without precipitation of the drug.[36] The results are shown in Table 2.

Dye Test

Methyl orange, an azo dye that is miscible with water, was added to all the formulations. It was observed that the dye had been dispersed homogenously with all LEF nanoemulsion formulations. This result confirms the formation of a stable o/w nanoemulsion with no clumps or aggregates.[20] Figure 3 shows the dye solubility test of all the 20 prepared nanoemulsions.

Zeta Potential Measurement

Zeta potential is defined as an indicator for the stability of LEF-loaded nanoemulsions, and its measurement indicates the surface charge of the droplets. Values above thirty mV correspond to the good stability of the nanoemulsion irrespective of the charge. These values mean that the system does not tend to aggregate and have good stability. These measurements indicate that all the formulations have good zeta potential values. The zeta potential of the prepared pure leflunomide dispersion was found to be -3.64 mV, which implies that there was a significant enhancement of the stability after the preparation of nanoemulsion formulation.[37] For A1 formulations, the zeta potential values were ranged from -12.52 to 19.29 mV, while A2 formulations had zeta potential values ranging from -15.31 to 24.21 mV. All the values of zeta potential are illustrated in Table 2.

Drug Release from the Prepared Formulations

In-vitro release study was done using modified USP dissolution apparatus type II using dialysis bag membrane (molecular weight cut off 12000 Dalton) using phosphate buffer saline pH 7.4 as a release medium. The release time duration was different among various formulations, and it was also found that the mean droplet size highly affected the dissolution process and thus drug release. The release of the 20 prepared formulations and the prepared LEF dispersion is shown in Figure 4. All the preparation shows complete release at the end of 220 minutes. Small particles show faster diffusion through the dialysis membrane. The effect of small droplet size on drug release could result from the increased interfacial area of solubilized LEF within the droplets of nanoemulsion exposed to the phosphate buffer saline medium. Hence, a higher rate of dispersibility and the drug will be released faster. The *in-vitro* dissolution profile of pure LEF dispersed in phosphate buffer saline pH 7.4 as control was found to be not more than 39% in 220 minutes. Therefore, faster LEF release was achieved in formulas that have smaller particle sizes. F-1 and F-9 displayed the fastest release among other formulas. Moreover, the release rate was significantly ($p \leq 0.05$) faster in all the prepared o/w nanoemulsions than the prepared pure LEF dispersion. The drug release pattern was comparable in many formulas, which could be attributed to the similarity in particle size. Also, the release of the prepared formulations showed that increasing the surfactant will decrease the release of the drug as there will be an increase in the formula's viscosity.[13,38]

Thermal Analysis by Differential Scanning Calorimeter (DSC)

The DSC thermogram of pure LEF is shown in Figure (5A). Pure LEF thermogram displays a characteristic sharp endothermic peak at 166.5°C. This peak refers to the melting point of LEF, indicating the crystalline state and purity of the drug.[42] Also, the DSC analysis of the prepared F-1 o/w nanoemulsion formula as shown in Figure 5B reveals the absence of the characteristic LEF peak, indicating that LEF has been completely dissolved in the prepared nanoemulsion. Furthermore, the disappearance of the LEF peak confirms that there is no incompatibility between the drug and the ingredients used to prepare the nanoemulsion.[43]

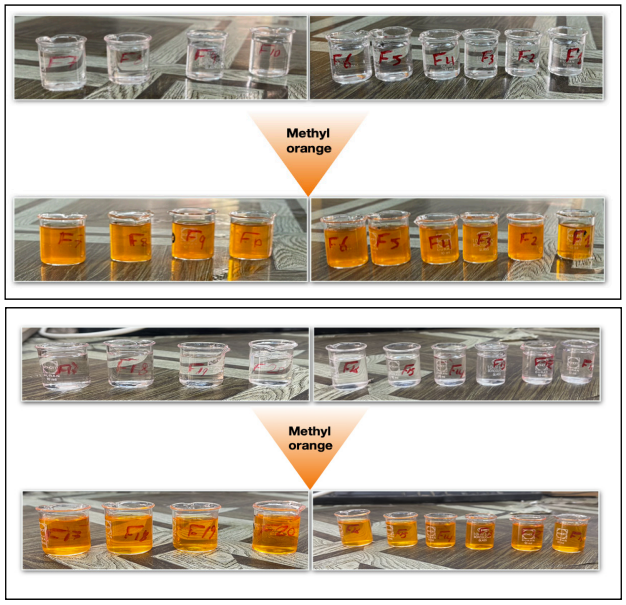

Figure 3: Dye test of the prepared formulations.

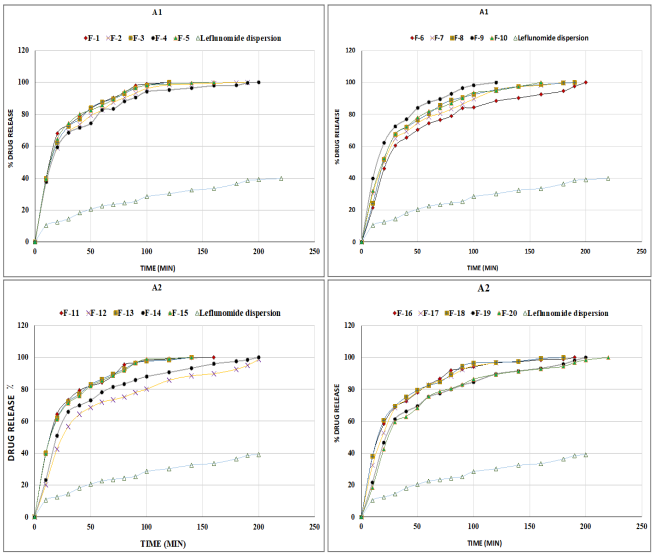

Figure 4: Comparative in vitro release study between pure leflunomide dispersion and nanoemulsion formulations.

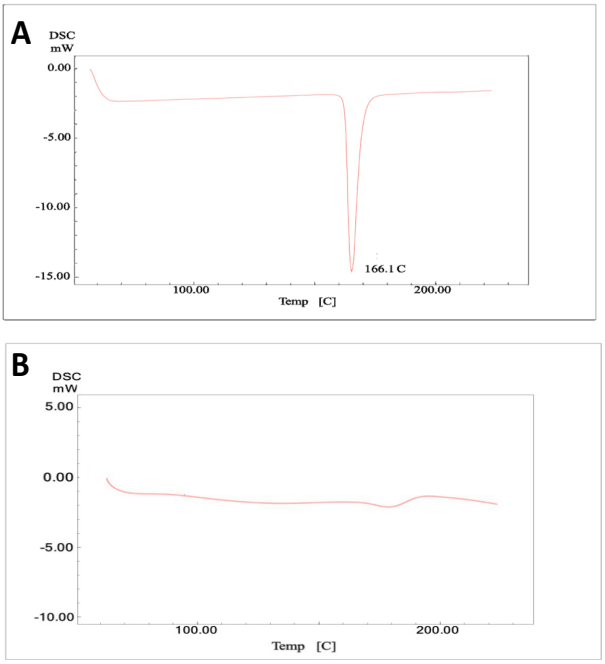

Figure 5: DSC thermogram of (A) pure leflunomide (B) prepared o/w nanoemulsion of F-1 formulation.

TEM

TEM images of F-1 and F-17 revealed dark spherical nanoemulsion droplets against bright surroundings as shown in Figure 6. Also, the TEM image confirms the size of the droplets analyzed using the dynamic light scattering technique (<200 nm), which confirms nanoemulsion formation. Moreover, there was no sign of coalescence or aggregation of the globules of nanoemulsion, which indicates the physical stability of the prepared nanoemulsion formulations.[14]

Selection of the Best Formula

Based on the results of the characterization study that was conducted, F-1 was selected as the optimum formula of A-1 preparations because it is characterized by fine droplet size 55.7 nm, low PDI 0.26, pH 5.58, which is considered acceptable for transdermal application, high drug content 99.42%, good percent light transmittance 99.84%, and the highest release from the prepared A-1 formulations. However, F-17 had the following findings: good droplet size 54.7 nm, low PDI 0.112, pH 5.56, high drug content 99.45%, light transmittance 99.53%, and the highest release from the prepared A-1 formulations, and it was selected as the optimum formula for A-2 preparations that use Tween 20 as a surfactant.

CONCLUSION

Based on the findings of this research, using Capryol ™ 90 as an oil phase together with Tween 20 or labrasol as surfactants and transcutol p as a co-surfactant, LEF was prepared successfully as an o/w nanoemulsion of droplet size lower than 100 nm with proven stability. Based on the fact that treatment of RA implies the long-term usage of LEF medication orally which could be associated with many adverse effects, such as gastric irritation and peptic ulcers. Knowing that a starting LEF dose of 100 mg for three consecutive days is highly intolerable by the patient, and these events may result in treatment failure. In this regard, LEF was successfully formulated as a nanoemulsion, which is considered a novel delivery system for transdermal dosage forms. The transdermal formulation of LEF could potentially improve patient adherence to the therapy by minimizing the side effects of oral administration that decrease patient compliance to the medication. The LEF formulated for transdermal application could be the answer to such intolerability. Such formulation will ultimately improve the quality of life for patients living with RA.

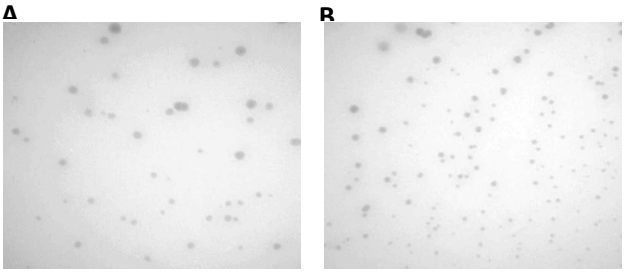

Figure 6: Transmission electron microscopic images of the optimized F-1 (A) and F-17 (B) with mean droplet size lower than 100 nm.

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