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PROCESS OPTIMIZATION FOR PREPARATION OF LEFLUNOMIDE LOADED NANOSTRUCTURED LIPID CARRIERS FOR TOPICAL DELIVERY

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ABSTRACT

Rheumatoid arthritis is a progressive and disabling autoimmune disease. It causes inflammation, swelling, and pain in and around the joints and other body organs. Leflunomide is one of the diseases modifying anti-rheumatic drugs that is considered effective in preventing irreversible damage associated with Rheumatoid arthritis. It is highly lipophilic drug falling in class-II of Biopharmaceutical Classification System. Leflunomide containing Nanostructured lipid carriers were proposed with a view to increase solubility and permeability leading to increased bioavailability. There are different methods listed in literature to prepare NLC. This study was conducted to develop simple single step method for development of Leflunomide containing NLC. Various approaches of hot melt emulsification were tested. The hot melt emulsification technique employing high-speed homogeniser proved to be the most capable one. It was further optimised for stirring speed, stirring time, order of addition and cooling temperature. The dispersion was assessed for particle size, particle size distribution, %entrapment efficiency, morphology using transmission electron microscope, and stability. The optimised batch presented spherical particles with particle size of 155±11.393 nm with tight particle size distribution (PDI: 0.110±0.014) and stability of 6 months. The %Entrapment efficiency was found to be 83.95±3.13%.

KEYWORDS: Nanostructured lipid carriers, NLC, lipidic Nanoparticles, ophthalmic drug delivery, hot melt emulsification, Leflunomide, process optimization.

1. INTRODUCTION

Rheumatoid arthritis (RA) is one of the common and severe autoimmune diseases related to joints. This chronic autoimmune inflammatory disease leads to functional limitation and reduced quality of life, since as there is bone and cartilage destruction, joint swelling and pain. Current advances and new treatment approaches have considerably postponed disease progression and improved the quality of life for many patients. Despite major advances in therapeutic options, restrictions on the routes of administration and the necessity for frequent and long-term dosing often result in systemic adverse effects and patient non-compliance. Unlike usual drugs, nanoparticle systems are planned to deliver therapeutic agents especially to inflamed synovium, so avoiding systemic and unpleasant effects.[1]

Management of RA include four main drug classes; non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids (GCs), disease modifying anti-rheumatic drug (DMARDs) and biological Leflunomide (LEF) is one of the DMARDs that is considered effective in preventing irreversible damage associated with RA.LEF, chemically-named as N-(4-trifluoromethylphenyl)-5-methylisoxazol-4-carboxazide, is a highly lipophilic drug that belongs to Class II Biopharmaceutical Classification System. This isooxazole derivative is considered a prodrug; following its oral administration, the isooxazole ring is opened and LEF is non-enzymatically converted to its active metabolite (teriflunomide) in the intestinal mucosa and plasma. LEF and its metabolite act as immunomodulators by inhibiting dihydroorotate dehydrogenase enzyme, which is responsible for *de novo* pyrimidine biosynthesis. Reduction of pyrimidine nucleotides production will directly influence the proliferation of activated autoimmune T cells and hence the RA autoimmune responses will be regulated.

The most prominent field of formulation science research is solubility or dissolving enhancement strategies. To improve the solubility and dissolution of pharmaceuticals, a variety of techniques have been investigated, with varied degrees of success, including salt creation, pH modification, the use of polymorphs,

solid dispersion, self-emulsifying systems, inclusion complexes, and liposomes.[2] In order to improve solubility and absorption, which in turn improve the bioavailability, particle size reduction is frequently used.[3] Lipidic formulations are one of the most often used methods for improving bioavailability among many strategies. Because they suggest both kinetic (dissolution) and thermodynamic (micro emulsification) improvement of the drug absorption, lipid-based systems are of particular interest. Drug compounds added to oily compositions show increased solubility in addition to facilitating penetration at the desired site.[4] Anatomical barriers and short residence times are drawbacks of topical ocular delivery. The tear film and corneal barrier are the two main obstacles to topical ocular administration. A bionic tear film is created by lipidic composition. The tear film has three levels by nature. These are the lipid layer, aqueous layer, and mucous layer, arranged from outside to within. Lipid-based nanocarriers resemble the tear film in its characteristics. The nanocarriers' lipidic nature makes it simple for them to diffuse into the lipidic layer.[5],[6]

In the early 1990s, professors R.H. Müller and M. Gasco began investigating the potential of a novel nanoparticulated system and gave it the term solid lipid nanoparticles (SLN). In addition to being free of organic solvents, biocompatible, biodegradable, and having a high in vivo stability, lipid formulations have several other benefits. Although SLN has the potential to be employed as a delivery method in many commercial goods, it has some drawbacks, such as low stability during storage and a restricted drug payload. It also has poor trapping and drug expulsion during storage. To regulate medication ejection during storage, a combination of lipids that will form an erroneous and disorganised arrangement can be utilised instead of a single lipid.[7],[8][9]

Several methods have been reported, including the high-pressure homogenization technique, which includes hot and cold homogenization techniques, microemulsion technique, solvent emulsification diffusion, phase inversion temperature method, melting dispersion method, ultrasonication technique, and solvent injection (solvent displacement) technique. The most well-known of these is the hot melt emulsification process, which involves emulsifying a lipid drug melt with an aqueous phase that contains surfactants.[10] Cunha et al performed double optimization Rivastigmine NLC for nose to brain delivery using hot melt emulsification technique with use of high pressure homogenizer and ultra-sonicator.[11] Zhang K *et al* Nanostructured Lipid Carriers for intravenous delivery that were concurrently loaded with oleanolic acid and gentiopicrin using the film-ultrasonication approach.[12]

In this study, various approaches of hot melt emulsification were screened to prepare Leflunomide containing NLC.

1. MATERIALS AND METHODS

1.1 Materials: Leflunomide was kindly gifted by alembic pharmaceutical limited (Vadodara, India). Stearic acid and Tween 80 were purchased from Atur instruchem, Vadodara, India. All other reagents used in the study were of analytical reagent grade.

1.2 Preparation of LEF-NLC using hot melt emulsification approach

1.2.1 Hot melt emulsification involving primary emulsion

A 80 mg of stearic acid, 20 µl of Capmul MCM were heated together at 75^0 C to make the lipid phase. After the homogenous melt was obtained, 20 mg of LEF was added to the lipidic phase. A 200 mg of Tween 80 was added to 20 ml of distilled water and was heated at 75 ^{0}C. Primary emulsion was prepared by adding aqueous phase to lipid phase dropwise using magnetic stirrer(Remi, India) at 2000 rpm and the primary emulsion was stirred for 30 min on magnetic stirrer at same speed. Then primary emulsion was treated under high-speed homogenizer (Inkarp, Germany) at 10,000 rpm for 20 min. The temperature was maintained throughout the process. The prepared dispersion was checked for particle size, PDI and stability.

1.2.2 Hot melt emulsification involving High speed homogenizer

The aqueous phase and lipidic phase were prepared as mentioned in 2.2.1 The aqueous phase was added to the lipidic phase dropwise under high-speed homogenization and stirred at different levels such as 5000, 7500, 10000 rpm. Two different levels of stirring 10 min and 20 min were tested. The temperature was kept constant throughout the process. The prepared dispersion was assessed for stability.[13]

A modification was made in above mentioned method. Initially the dispersion was stirred at high speed at 22000 rpm so that both phases get mixed properly and then stirring at 10000 rpm was performed. The prepared dispersion was assessed for particle size, PDI and stability.

1.2.3 Hot melt emulsification involving High pressure homogenizer

The aqueous phase and lipidic phase were prepared as mentioned in 2.2.1 The aqueous phase was added to the lipidic phase dropwise under high-speed homogenization and stirred at 10000 rpm. Two different levels of stirring 10 min and 20 min were tested. After high-speed homogenization, the dispersion was subjected high pressure homogenization (Panda Plus, GEA, Germany) at two different pressures viz. 600 bar and 800 bar and treated for different number of cycles viz. 6 and 8 cycles. The temperature was maintained throughout the process. The temperature was kept constant throughout the process. The prepared dispersion was assessed for particle size, PDI and stability.[14]

1.2.4 Hot melt emulsification involving High speed homogenizer with change in order of addition

The aqueous phase and lipidic phase were prepared as mentioned in 2.2.1 The lipidic phase was added to the aqueous phase dropwise and stirred under high-speed homogenizer. Temperature was maintained throughout the stirring. After stirring the dispersion was immediately brough to the temperature of 4-6^{0}C. The process mentioned in the 2.2.4 was optimized for process parameters such as stirring speed, stirring time, order of addition and cooling temperature.

1.3 Characterization of NLC

1.3.1 *Particle size, Particle size distribution*

Particle size of NLC dispersion was determined using a Zetasizer (Nano ZS 90, Malvern Instruments Ltd., Malvern, UK) based on the dynamic light scattering phenomenon By illuminating the nanoparticles with a laser beam and examining the intensity variations in the scattered light, a technique known as dynamic light scattering (also known as photon correlation spectroscopy) first determines the Brownian motion of the particles and then correlates this to the size of the nanoparticles using the Stokes-Einstein equation. Prior to the measurement all samples were diluted with double-distilled water to have a suitable scattering intensity, in a disposable polystyrene cell. All measurements were performed at 25°C, at a scattering angle of 90°. The z-average diameter value of the particles provided the intensity mean diameter of the bulk population of particles, and the polydispersity index was used to describe the width of the size distribution (PDI).[15],[16],[17]

1.3.2 *% Drug Entrapment Efficiency (%EE) and %Drug loading(%DL)*

Entrapment efficiency (EE) and drug loading of NLCs were determined by measuring the concentration of free drug (unentrapped) in the external aqueous phase using the Protamine sulfate conjugation method. The NLC in dispersion were aggregated by adding 0.1 ml of 10 mg/ml Protamine sulfate solution overnight and centrifuged at 15000 rpm for 30 min at 15 ^{0}C to obtain a pellet. The supernatant was suitably diluted to determine the free drug using HPLC method. The % EE and %DL of the NLC were calculated by equation-2 and equation-3, respectively.[18]

$$\%EE = \{(Wtotal - Wfree)/Wtotal\} * 100 \text{ ----------(2)}$$

$$\%DL = \{(Wtotal - Wfree)/Wlipid\} * 100\text{----------(3)}$$

W_{total}, the total amount of drug used in the formulation; W_{free}, the analyzed amount of drug in the supernatant; *Wlipid* total amount of lipid in the formulation.

1.3.3 *Transmission Electron Microscopy (TEM)*

The TEM was employed to determine the morphology and shape of the NLC dispersion. The samples were prepared by placing a 5 µl droplet of the NLC dispersion onto a 300- mesh copper grid which is carbon-coated and allowing it to settle NLCs for 3–5 min. Afterwards, the excess fluid was removed, and the grid was dried carefully in the air. Analysis of the samples was performed by using a Tecnai 20 Transmission Electron Microscope (Tecnai 20, Philips, Holland).[19]

1.3.4 Stability studies

The stability studies were performed to determine the physical stability of the prepared formulation under recommended temperature and relative humidity (RH) conditions to check the effects of storage conditions and effect of the presence of formulation components. The stability studies of the formulation were carried out under different storage conditions as per ICH guidelines, specifically 30 ± 2°C/60 ± 5% RH. The samples of stability studies were evaluated at 0, 1, 3and 6 for physical appearance, particle size, PDI and %EE. Each measurement was done in triplicate.[20]

2. RESULT AND DISCUSSION

2.1 The results obtained from the screening of various methods are discussed in the following section

2.1.1 Hot melt emulsification involving primary emulsion: The NLC were formulated using the method stated in 2.2.1. The stated method did not produce any dispersion and the phase separation was observed. This may be due to inadequacy of the magnetic stirring to disrupt the larger globules and make stable nano dispersion. The aggregation of the larger globules eventually results into coalescence and phase separation. The dispersion could not yield particles in nanorange and was not stable due to phase separation.

2.1.2 Hot melt emulsification involving High pressure homogenizer: The 6 trials were taken as per the method stated in 2.2.2. The results are summarized in the Table 1.

Table 1: Optimization of Hot Melt Emulsification Involving High Pressure Homogenizer.

Sr No	Stirring speed(rpm)	Stirring time(min)	Pressure bar	No of cycles	Stability
1	10000	10	800	5	Phase separation
2	10,000	20	800	7	Phase separation
3	10,000	10	900	5	Phase separation
4	10,000	20	900	7	Phase separation

The process could not produce stable dispersion with any of the process parameters.

2.1.3 Hot melt emulsification involving High speed homogenizer: As none of the methods could produce stable dispersion, the method was modified. The lipidic phase was added to the aqueous phase under continuous stirring at constant temperature of 75^{0}C and after completion of stirring the dispersion was immediately

brough to 4-6^0 C. Both the modifications viz. addition of lipidic phase to the aqueous phase and storing dispersion at 4-6 ^{0}C were essential for the stable dispersion. Either of them did not work in single capacity. Addition of lipidic phase to aqueous phase allowed the homogenization force to disseminate the particles while cooling at lower temperature allowed solidification of the oil droplets which further stabilized the dispersion. The optimization of stirring speed and stirring time was carried out at three levels of stirring speed (5000, 7500 and 10000 rpm) and two levels of stirring time (10 and 20 minutes). The results of optimization of process parameters are presented in Table 2. The stirring at 5000 rpm and 7500 rpm produced stable dispersions while stirring at 10000 rpm resulted into aggregation and phase separation. This may be explained by the smaller particles produced by higher shear forces that lack the surfactant concentration necessary to form stable dispersions. Due to the hydrolysis of the liquid lipid and surfactant, over stirring will cause the particles to acquire a charge, while under stirring will result in insufficient surfactant coverage on the particle surface. As a result, the right balance between stirring speed and time is crucial. Both will result in instability of the dispersion. Stirring at 5000 rpm for 20 min produced stable dispersion. Moreover, desired results were obtained at lower energy output (at 5000 rpm as compared to 7500 rpm). So, stirring speed of 5000 rpm and stirring time of 20 min were considered as optimized process parameters.

Table 2: Results of Optimization of Hot Melt Emulsification Involving High Speed Homogenizer.

Sr No	Stirring speed (rpm)	Stirring time (min)	Particle size(nm)	PDI	Stability
1	5000	10	-	-	Phase separation
2	5000	20	155±11.393	0.110±0.014	stable
3	7500	10	135±65.56	0.285±0.032	stable
4	7500	20	-	-	Phase separation
5	10000	10	-	-	Phase separation
6	10000	20	-	-	Aggregation upon storage

(Mean ±S.D., n=3)

2.2 Characterization of NLC

2.2.1 ***Particle size and Particle size distribution:*** Particle size of the optimized batch was found to be 155±11.393with narrow distribution (PDI 0.110±0.014). Narrow particle size is essential for the stability of the dispersion. The dispersion with broad particle size distribution tends to aggregate and results in instability.

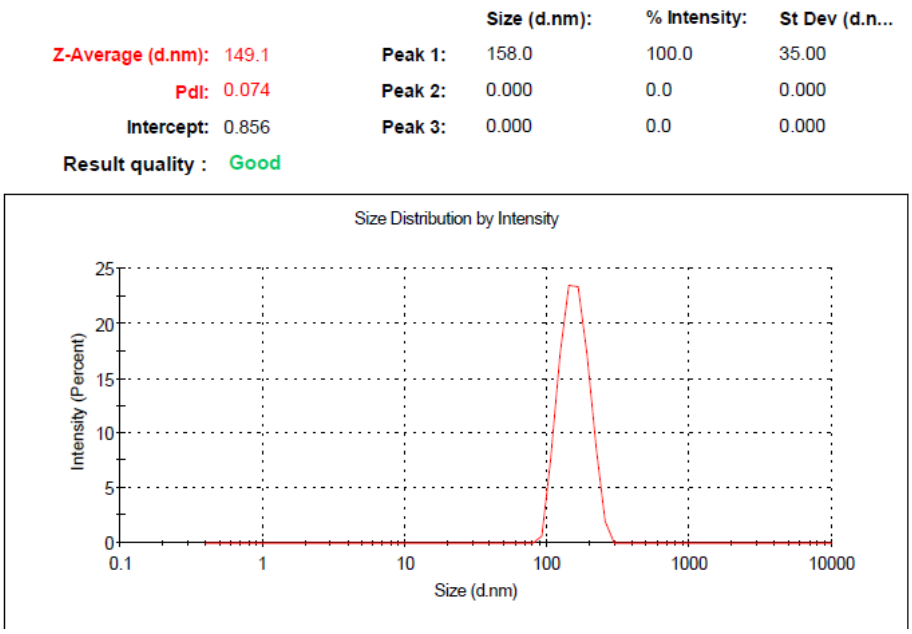

Figure 1: Particle size report of NLC.

2.2.2 ***% Drug Entrapment Efficiency (%EE) and %Drug loading(%DL):*** % Drug entrapment efficiency is indicator of capacity of dispersion to carry the drug load. Solubility of drug in lipid phase and surfactant are the decisive factors of %EE. Higher solubility of drug into lipidic phase leads to higher %Entrapment efficiency while higher solubility of drug in surfactant leads poor %EE. The %EE of the prepared dispersion was found to

be 83.95±3.13%. The %drug loading was found to be 8.395±0.313%.

2.2.3 ***Transmission Electron Microscopy (TEM):*** The TEM was employed to determine the morphology and shape of the NLC dispersion. The shape of the particles of the lipidic core was nearly spherical and the size of particles was found within nanometer range. The particles did not show a sticking tendency with each other. The particle size obtained from TEM analysis was in line with the particle size measured by dynamic light scattering (DLS). TEM presents the particle size of a single particle while DLS presents the average particle size of dispersion.

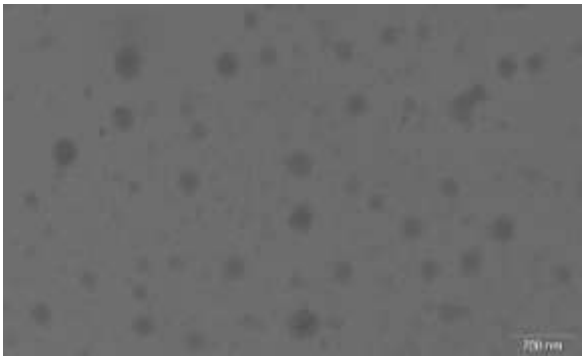

Figure 2: Transmission Electron micrograph of NLC.

2.2.4 Stability studies: The particle size, PDI and %EE were found to be 175±13.265, 0.129±0.034 and 79.62± 2.764. There was no significant change in the appearance, particle size, PDI and %EE of the dispersion after the storage period. Hence, it was concluded that the formulated NLC were stable.

3. CONCLUSION

Using a modified hot melt emulsification approach and a high-speed homogenizer, second generation lipidic nanocarriers, or NLC, were effectively created. The particles' narrow particle size distribution was found to be below 100 nm. They provided high entrapment efficiency, an important factor in carrying the medication dose. Particles in transmission electron microscopy had a spherical form and were nonorange in colour. They demonstrated good physical stability over a six-month period at 30± 2 °C and 60± 5% RH. The updated approach has the potential to be investigated further for NLC development. To offer dispersion with higher qualities, the formulation parameters can also be improved.

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