A new medium-throughput screening design approach for the development of hydroxymethylnitrofurazone (NFOH) nanostructured lipid carrier for treating leishmaniasis

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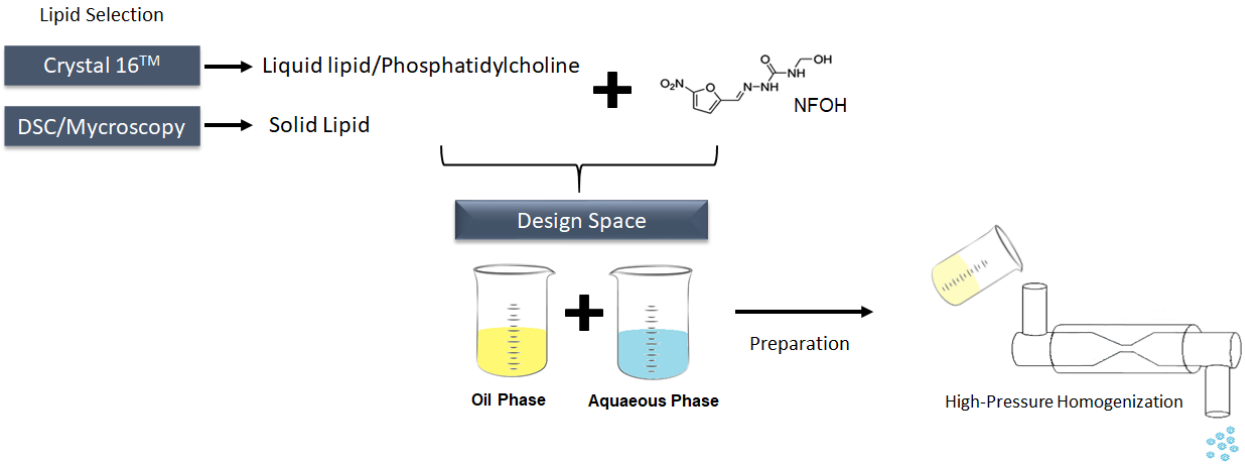
Graphical Abstract



Highlights

- Developing a novel NFOH-loaded nanostructured lipid carrier for oral administration to treat leishmaniasis, using a design space approach
- Selecting liquid and solid lipids using new Crystal 16TM and differential scanning calorimetry, respectively
- High-pressure homogenization used for the preparation of formulations
- Determination of NFOH in plasma rat after oral administration

Abstract

Hydroxymethilnitrofurazone (NFOH) is a nitrofurazone derivative, and has potential use in treating leishmaniasis. However, due to low solubility and bioavailability, NFOH has failed in *in vivo* tests. Nanostructured lipid carrier (NLC) is an alternative to overcome these limitations, by improving pharmacokinetics and modifying drug delivery. This work is focused on developing a novel NFOH-loaded NLC (NLC-NFOH) for oral administration to treat leishmaniasis, using a design space approach. The solubility of NFOH in different liquid lipids (LL) was performed in a Technobis Crystal 16TM (Technobis, Alkmaar, Netherlands) at 80°C, 700 rpm for 1h. Aiming to improve NFOH solubility, 0.2g of phosphatidylcholine from soybean (Lipoid® S100) was added to the LL. The solubility of NFOH in solid lipid (SL) was performed by microscopy and DSC analysis. High-pressure homogenization, at 600 bar, five consecutive cycles, and a D-Optimal mixture statistical design was applied to develop NLC formulation. NLC-NFOH (2.8 mg/kg) was administered to one healthy male Wistar rat (341g) by gavage. Blood from the carotid vein was collected and the sample was analyzed by HPLC. Mygliol® 840 was selected as LL and Gelucire® 50/13 and Precirol® ATO 5 as SL to solubilize NFOH. The optimized NLC-NFOH

presented z-average of 198.6 ± 5.4 nm, PDI of 0.11 ± 0.01 and zeta potential of -13.7 ± 0.7 mV. The plasma concentration of NFOH after 5h of oral administration was 0.22 µg/mL. A rational approach allowed for developing the first NLC-NFOH. Moreover, NLC-NFOH showed the potential development of a new oral medicine for treating leishmaniasis.

Keywords: Leishmaniasis; Nanoparticle(s); Drug delivery system(s), Lipid-based formulation(s); Nanostructured lipid carrier; Hydroxymethylnitrofurazone

Introduction

Leishmaniasis is a neglected tropical disease, in which the parasite infects macrophages in liver, spleen, bone marrow, and lymph nodes [1]. Currently, the first-line treatment for leishmaniasis is pentavalent antimonials administered via parenteral: meglumine antimoniate (Glucantime®, Sanofi Aventis, 81 mg/mL, solution) and sodium stibogluconate (Pentostam™, Glaxo Smith Kline, 100 mg/mL, solution for injection). Other alternative drugs may include pentamidine and amphotericin B, also administered parenterally [2]. However, they require the administration of high and repeated doses and at least 20 days of treatment and hospitalization [1,3]. Additionally, these medicines show side effects such as pancreatitis, cardiotoxicity, nephrotoxicity and hepatotoxicity and induction of drug-resistant strains [4].

Hydroxymethylnitrofural (NFOH) (Figure 1A) has been a promising alternative for treating leishmaniasis. Chung et al [5] first obtained this compound, using a molecular modification of nitrofurazone (NF) (Figure 1B), a topical antimicrobial agent. NFOH showed high activity in cultures of the infected cell of *Trypanosoma cruzi* and lower toxicity compared to NF. It also shows activity against *L. amazonensis*, which was confirmed by *in vitro* test using NFOH-poly n-butyl cyanoacrylate (PBCA) nanoparticles [6]. Despite this promising efficacy, the low water solubility of NFOH has limited its therapeutic use [6].

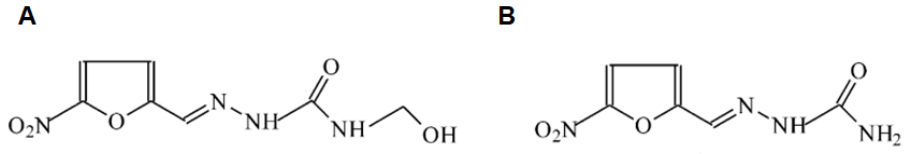


Figure 1. Chemical structure of hydroxymethylnitrofural (A) and nitrofurazone (B) [5].

In search of new approaches to overcome this limitation, nanostructured lipid systems have promising qualities. They have a distinctive record in improving pharmacokinetics, modifying drug delivery, and can be used to target the drug at a specific site [3,7]. Thus, it is possible to increase efficacy, reduce toxicity, and reduce the frequency and duration of treatment [8]. Also, nanostructured preparations can increase the time of drug circulation in the

body and encapsulated lipophilic molecules [9]. Among the alternatives there are nanoemulsion (NE), solid lipid nanoparticles (SLN), and nanostructured lipid carriers (NLC), the second generation of solid lipid nanoparticles.

NLC is comprised of colloidal particles that present a matrix composed of a binary mixture of solid lipid and liquid lipid giving rise to a less ordered structure, solid at room and body temperature [10]. This feature allows higher encapsulation efficiency compared to conventional SLN [11]. NLC can be prepared using high pressure homogenization, a scale up feasible technology [12]. NLC has many advantages, such as low production cost, no need for organic solvents, and high stability. Additionally, Monteiro et al. showed that NLC loaded with buparvaquone improved the in vitro activity against *Leishmania infantum* [13]. Similar results were revealed using amphotericin B-NLC and cedrol-NLC. These nanosystems show better activity against *Leishmania sp* after oral administration in an animal model compared to the free drugs [14,15].

However, a systematic rational approach is required in developing NLC to select its proper components and their concentration. The lipids with higher drug solubility can increase the drug loading, encapsulation efficiency and enhance the stability of nanoparticles [16,17]. Thus, this task is a critical part of the formulation development.

Moreover, the concept of space design in pharmaceutical development has been considered an important stage of quality by design. Design space offers quality assurance and process understanding by a multivariate combination and interaction of input variables [18,19]. It can be achieved using design of experiments (DoE). The applicability of DoE has increased due to its ease in overcoming limitations and time-consuming methodologies [12,18,20]. Therefore, this work is focused on developing a novel NFOH-loaded NLC for oral administration to treat leishmaniasis, using a design space approach.

Material and Method

Material

Hydroxymethylnitrofurazone was synthesized as previously described by Chung et al. (2003). Hydrogenated palm oil (Softisan® 154 and Dynasan® P60), tristearin (Dynasan® 118) and saturated fatty acid triglycerides (Witepsol® E85) were kindly donated by IOI Oleo Chemical (Germany). Lauroyl polyoxyl-32 glycerides (Gelucire® 44/14), stearoyl polyoxyl-32 glycerides (Gelucire® 50/13), glyceryl dibehenate (Compritol® 888) and glyceryl distearate (Precirol® ATO 5) were kindly donated by Gattefossé (France). Oleic acid, glyceryl caprylate/caprate (Capmul® MCM EP), glyceryl tricaprylate/tricaprate (Captex® 300 EP/NF) and triglycerides of oleic acid (Captex® GTO) were kindly donated by Abitec (USA). Super refined cottonseed, safflower, corn, olive, soybean, and sesame oils were kindly donated by Croda (UK). Stearyl alcohol, buriti oil,

wheat germ oil, hazelnut oil, grape seed oil, sunflower oil and isopropyl palmitate were kindly donated by Mapric (Brazil). Propylene glycol dicaprylate/dicaprate (Miglyol® 840) and Capric and caprylic acid esters (Miglyol® 812) were kindly donated by IOI Oleo Chemical. Phosphatidylcholine from soybean (Lipoid® S100) were kindly donated by LIPOID GMBH. Methanol and Acentonitrile HPLC grade were purchased from JTBaker. Purifed water was prepared using Milli-Q quality (Millipore, USA). All other reagents were at least analytical grade and were used without further purification.

Selection of Liquid Lipids

The equipment, Crystal 16™ (Crystal Pharmatech Inc., USA), was used to determine the liquid lipid that solubilized the highest quantity of NFOH. For the test, 5.0 mg of NFOH was added to 1.0 g of liquid lipid in a 2.0 mL vial. The parameters used were: 700 rpm, at 80°C for one h, with a heating and cooling ramp of 2.7°C/min. The second step for selection was performed as follows: 200 mg of Lipoid® S100 were added to the previous vials, under the same conditions described above. According to the equipment manufacturer's guidelines, the lipids presenting turbidity were excluded from the experiment. In the third step, the optimized proportion of Lipoid® S100: liquid lipid was investigated. Therefore, the selected liquid lipids and the suitable proportion of Lipoid®: liquid lipid were tested using different concentrations of NFOH (1.0-12.0 mg/g of lipid). The system with the highest concentration of the solubilization NFOH was selected.

The quantification of NFOH was performed using spectrophotometry UV/vis Evolution series 201 (Thermo Fisher Scientific, Waltham, MA, USA) operated at 370 nm. The sample was prepared with the addition of NFOH in excess to the liquid lipid and placed in a magnetic stirrer at 80°C. After 1 h, aliquots were filtered through a PVDF membrane of 0.22-µm pore size and diluted in acetonitrile.

Selection of Solid Lipid

NFOH solubility in solid lipids was evaluated according to Kasongo et al [21]. NFOH was added to the solid lipid in a concentration of 0.001% (w/w); this preparation was heated 10°C above the melting point of each lipid, under constant shaking. Aliquots were taken and observed by an optical microscope Olympus CK2 (Japan); the NFOH concentration was adjusted until crystals were observed.

Additionally, drug solubility and crystallization behavior in solid lipids were carried out by using differential scanning calorimetry (DSC), according to Monteiro et al [12]. The NFOH, solid lipids, 1:1 physical mixtures (PM) and the mixtures of selected lipids thermal behavior were characterized in a DSC 4,000 Perkin Elmer cell (Perkin Elmer Corp., Norwalk, CT, USA), under a dynamic N_2 atmosphere (50 mL/min), using a sealed aluminum pan with about 2.0 mg of

samples. DSC curves were obtained at a heating rate of 10°C/min, and the temperature ranged from 25 to 290°C. An empty sealed pan was used as a reference.

The onset temperature, maximum peak in the melting range (T_{peak}) and melting enthalpy (ΔH_m) were calculated using the software provided by PerkinElmer. The crystallinity indices (CI) of NFOH were calculated in percentage according to the following Equation (1) [22,23],

$$CI\ (\%) = \frac{\Delta H\ NFOH\ PM * D}{\Delta H\ NFOH\ 100\%} * 100 \qquad \textbf{Equation (1)}$$

where $\Delta H_{NFOH\ PM}$ is the enthalpy of fusion ($J.mg^{-1}$) of NFOH in the binary physical mixture of NFOH and solid lipid, $\Delta H_{NFOH\ 100\%}$ is the enthalpy of fusion ($J.mg^{-1}$) of pure drug. D is the dilution of NFOH in the solid lipids, in this case, 1:1, D=2.

Preparation of NLC-NFOH

A total of 50.0g of the formulation was prepared using the selected liquid lipid, solid lipids and poloxamer 188. The oil and aqueous phases were heated to 80°C using RTC basic IKA® magnetic stirrer at 300 rpm until complete dissolution. Then, the aqueous phase was dispersed in the oil phase, and mixed by a mechanical homogenizer at 8000 rpm (Ultraturrax, IKA) for 5 min. The coarse emulsion was passed through a high-pressure homogenizer using Nano DeBEE® (BEE International, Inc. USA) using five successive cycles at 600 bar [12].

Z-average, polydispersity index and potential zeta analysis

The *z*-average of the nanostructured lipid carriers was assessed by photon correlation spectroscopy (PCS) using Zetasizer ZS90 (Malvern Instruments, Malvern, UK) at 25°C and a 90° detection angle. The equipment was also used to measure the zeta potential (ZP). ZP measurements were carried out in purified water with a conductivity adjusted to 50 $\mu S.cm^{-1}$ by adding NaCl 0.2% (w/v), to avoid ZP fluctuations caused by the difference in conductivity (n = 3). The pH of formulations was determined using a pH measurement system (MP 220, Mettler Toledo® Inc, Columbus, OH, US) previously calibrated at 20°C. The particle size of the optimized NLC-NFOH was also determined by laser-based particle size analyzer CILAS 1090 (Orleans, France) in aqueous medium.

Determination of encapsulation efficiency (EE%) and drug loading (DL%)

NLC-NFOH was ultrafiltrated using Amicon Ultra-0.5 mL Ultracel-10 membrane 50 kDa centrifugal filters (Millipore Merck, USA). Then, the filter was centrifuged at 590 g for 30 min. The supernatant and the filtrate were suitably diluted and analyzed for NFOH in spectrophotometry method as described earlier. The EE% and DL% were determined using the equation (2) and equation (3), respectively.

$$EE\% = \frac{Wtotal - Wfree}{Wtotal}\ x\ 100 \qquad \textbf{Equation (2)}$$

$$DL\% = \frac{Wtotal - Wfree}{Wlipid}\ x\ 100 \qquad \textbf{Equation (3)}$$

Where W_{total} is weight of initial NFOH added, W_{free} is the weight of free drug detected in the filtrate after centrifugation and W_{lipid} is the weight of lipid at the formulation.

NLC development and optimization

An extreme vertex experiment, with five-factor, grade 3, two-level D-Optimal mixture statistical design was used to evaluate the lipids and surfactant concentration effects on NLC *z*-average using Minitab® 18 software (State College PA, USA). To design the mathematical model the following restrictions were used: 2.0% to 5.0% w/w of Gelucire® 50/13 concentration and ≤ 8.0% w/w of Precirol® ATO 5 + Miglyol® 840 concentration. The independent variables are shown in Table 1 and the dependent variable (response) was z-average. A total of 45 experiments were designed by Minitab 18 (Table S2).

Table 1. Variables and level of extreme vertex experiment in developing NLC-NFOH.

Independent Variables	Coded Level	
	-1	+1
Gelucire® 50/13 (Gel)	2.0	5.0
Precirol® ATO 5 (Prec)	0.0	7.0
Miglyol® 840 (Mig)	2.0	9.0
Poloxamer® 188 (Pol)	0.0	3.0
Water	86.0	90.0

Determination of NFOH in plasma

The Ethics Committee approved the animal experiment protocol (protocol n°1155/2019). NLC-NFOH (2.8 mg/kg) was administered to one healthy male Wistar rat (341g) by gavage. After 5 h blood from the carotid vein was collected. A blood sample was collected to heparin tubes (10 µl, 500 IU/mL). Plasma samples were obtained immediately by centrifuging at 8000 rpm for 10 min, and stored at -20°C until further analysis by HPLC [24]. Plasma samples (200 µL) were mixed with acetonitrile (200 µL), to precipitate proteins, then centrifuged at 10000 rpm for 10 min. The supernatant was transferred to a vial insert and analyzed in HPLC.

The quantification of NFOH in plasma was performed according to Monteiro et al [25]. The Shimadzu HPLC-UV system consisted of a CBM-20A controller, LC-20AT-pump, SPD-20A detector, and SIL-20AC sampler LC Solution software (version 1.25SP4) was applied for data collecting and processing. The mobile phase consisted of acetonitrile: water (20:80) at a flow rate of 1.2 mL/min and the UV detector at 265 nm. The volume of injection was 20 µl. The method was linear (r= 0.9997) in the concentration range of 10 - 0.1 µg/mL.

Result

Selection of liquid lipid

Crystal 16™ detects the turbidity of the solution through a laser that passes through the vials. The clearer the solution, the higher the solubility of the drug in the oil. In the first screening, none of the 23 oils tested showed a 100% clear solution. In the second screening, Lipoid® S100, a solubilizer agent, was added to improve the solubility of NFOH in these oils. Buriti oil, grape seed oil, Miglyol® 840, Capmul® MCM, Captex® 300 and Miglyol® 812 showed 100% clear solution. The interaction of Lipoid® S100 and the oils helped solubilize the NFOH. The proportion of Lipoid® S100 and liquid lipid was optimized to 1:9.

To determine if the selected liquid lipids were able to solubilize more than 5.0 mg/g of drug substance, different vials with variable amounts of NFOH (6.0 to 12.0 mg/g) were evaluated in a Crystal 16™ instrument. The maximum amount of NFOH solubilized in Miglyol® 840 was 8.0 mg/g. This solubilized concentration remains after cooling the solution. For the Miglyol® 812, the maximum quantity of solubilized NFOH was 6.0 mg/g. The NFOH quantification by spectroscopy in Miglyol® 840 and Miglyol® 812 were 14.0 mg/g and 10.2 mg/g, respectively. Due to the superior NFOH solubility, Miglyol® 840 was chosen as the liquid lipid for the preparation of nanostructure lipid carrier.

Selection of solid lipids

Eight solid lipids were analyzed by microscopy to evaluate the solubility of NFOH. NFOH was soluble in all the lipids tested at 1.0 mg/g, except for Gelucire 44/10. Microscopy revealed Gelucire® 50/13 was the only solid lipid to solubilize 2.0 mg/g of NFOH. Figure 2 showed the microscopy of NFOH in Gelucire® 50/13.

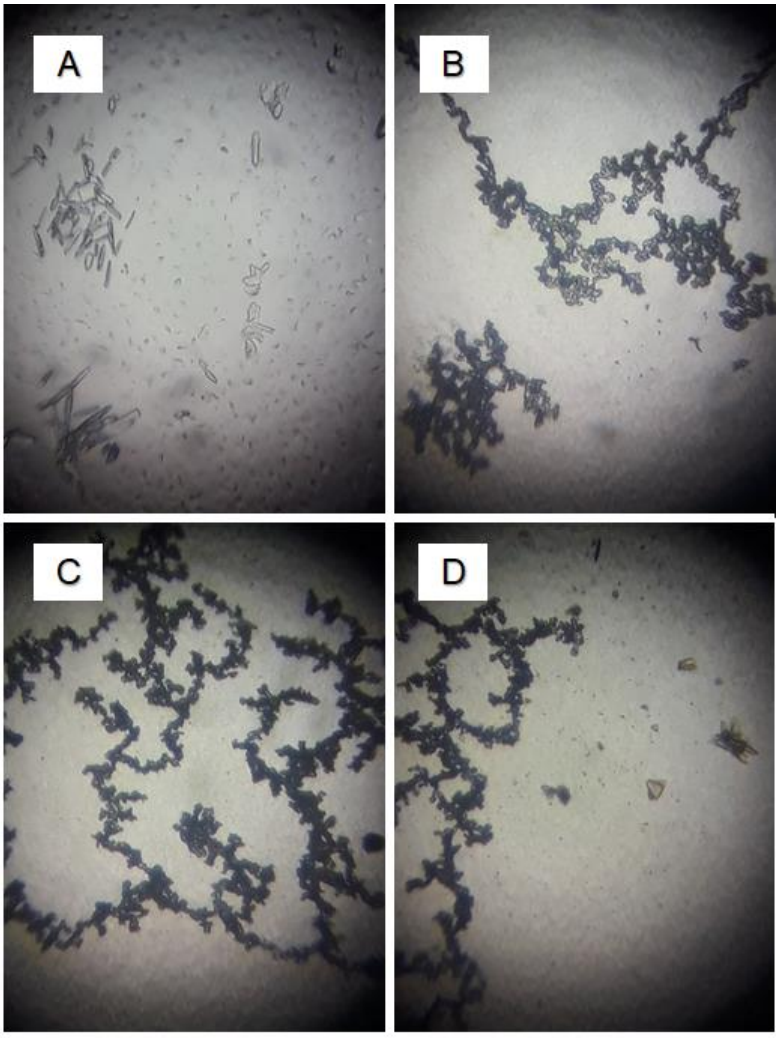


Figure 2. Optical micrographs (x10) of (A) NFOH; (B) Gelucire® 50/13; (C) Gelucire® 50/13 and NFOH (2.0mg/g) and (D) Gelucire® 50/13 and NFOH (10mg/g).

The melting enthalpy obtained from DSC data and the Cristalinity index (CI) of NFOH and solid lipids mixtures were used to select the best solid lipid (Table S1). The pure drug presents ΔH_{NFOH} 12.8 (kJ/mg); the greater the reduction in CI, the better the ability of the lipid to solubilize the drug [12]. Gelucire® 50/13 mixture (Figure 3) resulted in the lowest CI of NFOH, of 63.5 which is in consonance with the microscopic screening. Thus, it was the chosen SL for the preparation of nanostructure lipid carrier. Precirol® ATO 5, presenting the second-best CI (96.1%) was also chosen.

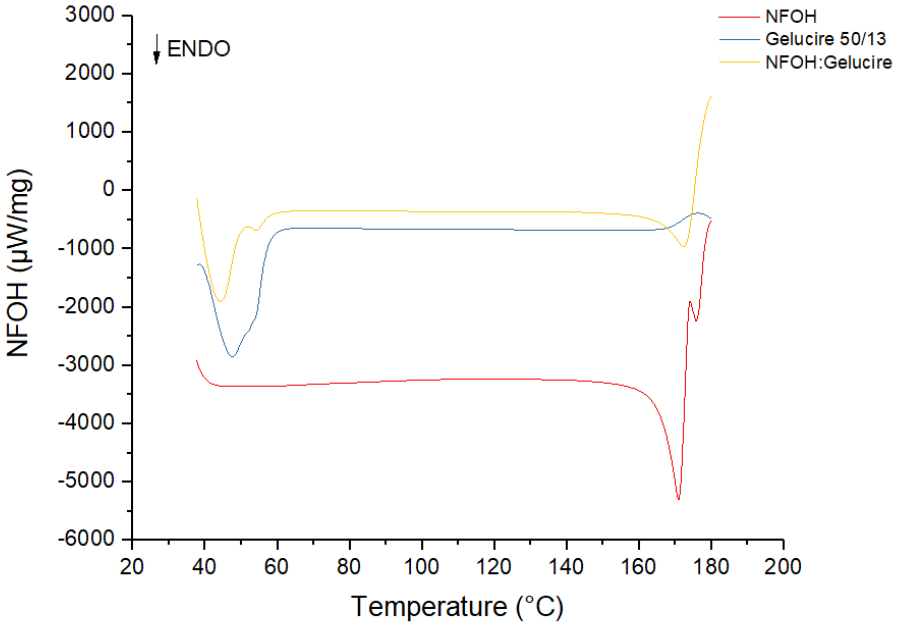


Figure 3. DSC curves of hydroxymethylnitrofurazone (NFOH), Gelucire® 50/13, and NFOH and Gelucire® 50/13 – physical mixture (PM) obtained at β = 10°C/min.

NLC development and optimization

The restrictions were determined in a preliminary study. Gelation was observed in a mixture composed of Precirol® ATO 5 and Miglyol® 840 at the ratio of 4:1. However, when Gelucire® 50/13 was added to this mixture, the gelation was not observed (data not shown). The addition of surfactants can also avoid the gelation [26]. Thus, Gelucire® 50/13 was added to all formulations of statistical design. The Z-average ranged from 100.9 nm to 294.0 nm and PdI from 0.12 to 0.66 (Table S2).

The R-square (R^2), adjusted-R^2 (adj-R^2) and predicted R^2 (pred-R^2) were 89.54%, 87.21% and 83.67%, respectively. The normal probability plot of the residuals was approximately linear revealing a normally distributed behavior (data not shown). The linear model was significant ($p<0.05$; $\alpha=0.05$) (Table S3). The mathematical model (Equation 4) showed the influence of independent variables for the z-average. The interaction of Gelucire® 50/13+Precirol® ATO 5, Gelucire® 50/13+Miglyol® 840, and Precirol® ATO 5 + Poloxamer® 188 resulted in a synergy that helped decrease the z-average. Interestingly, the mix of the three compounds, Gelucire® 50/13+Precirol® ATO 5+Poloxamer® 188, showed the opposite

response, significantly increasing the z-average. Table 2 shows the optimized formulations with a z-average less than 200 nm (monomodal distribution), which verified the validity of the mathematical model. The contour plots are presented in Figure 4. The EE% of F1 and F2 were 58.8% and 61.7%, respectively and DL% were 21,6% and 34,5%, respectively.

$$Z - average = +3210 * Gel + 4970 * Prec + 2216 * Mig + 1124 * Pol - 8.4 * Water - 98856 * Gel * Prec - 60703 * Gel * Mig - 98279 * Prec * Pol + 1351126 * Gel * Prec * Pol$$ **Equation (4)**

Table 2. Observed and predicted z-average, polydispersity index (PDI) and zeta potential (ZP) results from NLC-NFOH for F1 and F2.

	F1 (% w/w)	**F2** (% w/w)
Gelucire® 50/13	4.70	2.30
Precirol® ATO 5	2.45	3.70
Miglyol® 840	3.00	4.00
Poloxamer® 188	1.00	0.00
Water	88.85	90.00
Observed z-average (nm) ± SD	135.3 ± 2.1	198.6 ± 5.4
Predicted z-average (nm)	134.9	198.7
PDI ± SD	0.23 ± 0.02	0.11 ± 0.01
ZP (mV) ± SD	-13.7 ± 0.9	-13.7 ± 0.7
pH	4.92	4.98

PDI: polydispersity index; ZP: zeta potential; SD: Standard deviation.

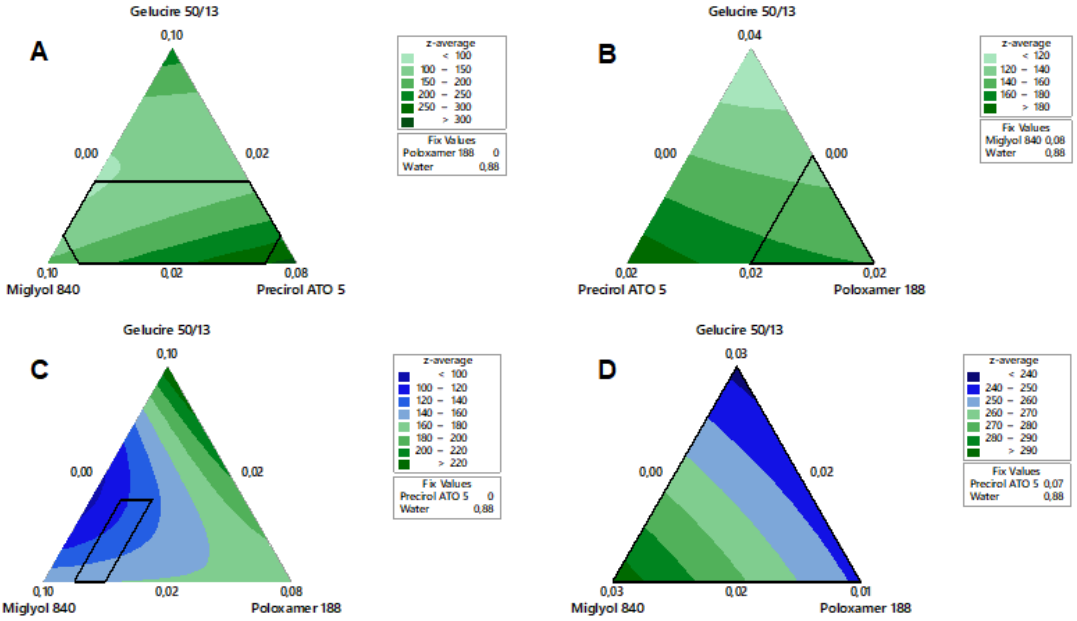


Figure 4. Contour plots of the NLC-NFOH mathematical model for z-average, containing the following variables: Gelucire® 50/13, Precirol® ATO 5, Miglyol® 840, Poloxamer® 188, and Water

F2 particle size was also determined using a laser-based particle size analyzer. The diameter 50%, d(50), was 170 nm and the media size was 220 nm (Figure S1).

Determination of NFOH in plasma

The NFOH was successfully detected in animal after oral administration of F2. The time of retention of NFOH was 2.6 minutes for NFOH solution and NFOH in plasma (Figure S2). The plasma concentration of NFOH after 5h of oral administration in rat was 0.22 µg/mL.

Discussion

The use of different tools for selecting lipids provided relevant scientific knowledge for the development of the NLC. Shake flask and spectrophotometry are the conventional approaches used to select liquid lipids [27]. However, the use of Crystal 16^{TM} helped to screen a large number of liquid lipids in a short time, low amount, without the use of organic solvent. Microscopy is also frequently used to select solid lipids [21,28,29], but it presents some limitations such as being time-consuming, presenting semi-quantitative results, and the experience of the analyst [12]. DSC used in this study could overcome these limitations. Thus, the association of these methods is substantial in saving time, material and labor.

The first selected liquid lipid was Miglyol® 840, a diester related to medium chain triglycerides (propylene glycol dicaprylate / dicaprate) [30]. It has been used for developing formulations in oral,[31–33] ophthalmic [34,35], subcutaneous [32], dermatological [36] and transdermal applications [37,38]. In order to increase the solubility of NFOH in Miglyol® 840, Lipoid® S100 was added. Lipoid® S100 is a lecithin derived from soybeans and contains 100% phosphatidylcholine, one of the main naturally occurring phospholipids. Lipoid® S100 offers a high transition phase temperature preventing leakage of drugs from the lipid matrix, and can therefore increase encapsulation efficiency [39]. In addition, when added to formulations, it can increase the oral bioavailability of drugs [40,41]. Despite these advantages, this component is highly susceptible to oxidation [42]. Therefore, it is desirable to use the smallest amount of Lipoid® S100 suitable to solubilized NFOH.

Gelucire® 50/13 was selected due to the superior ability to solubilize NFOH among the solid lipids tested, and its lower CI. This lipid is a mixture of propylene glycol mono, di and triglycerides and mono and diesters of palmitic (C 16) and stearic (C 18) acids, it has a melting point of around 50°C, and hydrophilic-lipophilic balance (HLB) of 13. It is an amphiphilic lipid compound that has been used to increase the oral bioavailability of hydrophobic drugs [43–45]. In addition, it has been used as solid lipid and surfactant in the preparation of lipid nanostructures [46–48]. Precirol® ATO 5 is composed of esters of palmitic (C16) and stearic (C18) acids, used to modify the release of drugs [49–51] and to increase oral bioavailability of drugs [52,53].

Lipid polymorphism can induce gelation and instability of formulation [54,55]. Precirol® ATO 5 and Gelucire® 50/13 present 3 and 4 polymorphic forms, respectively [46]. The interaction between these two components suppressed the polymorphism. Hazza et al [48]. demonstrated an increase in the z-average due to the interaction between Gelucire® 50/13 and Precirol® ATO 5. This confirms the findings of Jeon et al. [56], which revealed Precirol® ATO 5 is the main constituent of the particle walls. Gelucire® 50/13 molecules intercalate between Precirol® ATO 5 by Wan der Vaal forces between their lipophilic long hydrocarbon chain. When the concentration of Gelucire® 50/13 increases, the Gelucire® 50/13 molecules interact between themselves leading to a z-average increase. Thus, Gelucire® 50/13, as stabilizer, can provide suitable colloidal stability. Additionally, it can slow down the expulsion of the encapsulated drug [46].

In the present study, when Gelucire® 50/13 concentration increased from 2.0 to 5.0%, the NLC-NFOH z-average decreased, contrary to Jeon's study. This can be attributed to the addition of Miglyol® 840 to the formulation. This liquid lipid decreased the crystallinity of solid lipids reducing the particle size [57]. Furthermore, Precirol® ATO 5 (HLB = 2) may facilitate emulsification efficiency when combining with Miglyol® 840, leading to a lower particle size [35].

The use of a design space allowed for a systematic rational approach in the development of NLC-NFOH. The knowledge of the attributes that influence the z-average of the formulation provided an optimized NLC. Thus, the mathematical model demonstrated the

predicted z-average similar to the observed values (Table 2). Two preparations were selected to verify the model: F1 and F2. Gelucire® 50/13 (2.30 to 4.70% w/w) acted as solid lipid and also as a stabilizer for the formulation. The F2, without Poloxamer® 188, presented stability of three months (Table S4). Poloxamer was intended to act as gelation inhibitor in this system; however, the applied concentration of Gelucire® 50/13 seemed to be enough for this proposal. Therefore, the preparation F2 was chosen for the *in vivo* preliminary assay.

The analysis of plasma concentration of NFOH using Wistar rats (approx. 250g) was also performed and determined by Serafin et al. [58]; the authors found a plasma concentration of 0.25 mg/mL at 5h, which is similar to the concentration determined in our study (0.22 mg/mL) using NLC-NFOH at 2.8 mg/Kg. Thus, it was possible to achieve a similar plasma concentration using an almost 100-fold lower dose and despite the DL% below 40%. These preliminary results indicate that NLC might improve oral absorption of NFOH. Some studies have shown retard and/or sustained *in vitro* release from drugs in NLC [14,41,60]. Fathi et al. [60] and Kar et al. [14] showed an initial burst in the release study; this can be attributed to the entrapped drug on the surface of the nanoparticle. But both studies showed a sustained released, attributed, probably the lipophilic nature of the drug and the lipid formulation. Lipid-based formulations can enhance the solubility and bioavailability of poorly soluble drugs by keeping drugs solubilized in the gastrointestinal tract, enhance mucosal adhesion, and/or gradually releasing drug molecules from the lipid matrix into the blood. In addition, these formulations can improve therapeutic profiles compared to the free drug due to the protection from chemical and enzymatic degradation of the drug [61,62]. Therefore, our group is encouraged to continue researching the influence of NLC aiming at high bioavailability of NFOH.

Conclusion

A rational approach allowed to successfully develop the first stable NLC-NFOH. The D-Optimal mixture statistical design was effectively used to optimize the formulation. Besides, within the design space, it was possible to have a better understanding of the attributes that influence the z-average of the formulation. Gelucire® 50/13 decreased the z-average, while the addition of Precirol® ATO 5 increased the z-average. Additionally, the preliminary experiment of NLC-NFOH in rats demonstrated the presence of NFOH in plasma at a lower concentration of oral administration, compared to other studies. Under these promising results, we consider the NLC-NFOH as a potential candidate of oral medicine to treat leishmaniasis.

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