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Optimization of nanostructured lipid carriers loaded with methotrexate: A tool for inflammatory and cancer therapy

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ABSTRACT

The aim of this study was to optimize and assess the potential of nanostructured lipid carriers (NLC), prepared by the hot ultrasonication method, as carrier for methotrexate (MTX), highlighting the application of factorial design. Preliminary screening drug/lipid solubility, allowed us to select Witepsol® E85 as the solid lipid and Mygliol® 812 as liquid lipid for the NLC loaded with MTX. Then, a 3-level, 3-factor Box-Behnken design and validated by ANOVA analysis; the correspondence between the predicted values and those measured experimentally confirmed the robustness of the design. Properties of optimized MTX-loaded NLCs such as morphology, size, zeta potential, entrapment efficiency, storage stability, *in vitro* drug release and cytotoxicity were investigated. NLCs loaded with MTX exhibited spherical shape with 252-nm, a polydispersity of 0.06 ± 0.02, zeta potential of -14 mV and an entrapment efficiency of 87%. *In vitro* release studies revealed a fast initial release followed by a prolonged release of MTX from the NLC up to 24-h. The release kinetics of the optimized NLC best fitted the Peppas–Korsmeyer model for physiological and inflammatory environments and the Hixson–Crowell model skin simulation conditions. No toxicity was observed in fibroblasts. Thus, the optimized MTX-loaded NLC have the potential to be exploited as delivery system.

1. Introduction

Lipid based colloidal carriers have been widely used for drug delivery because they offer the possibility of modulating drug release, by facilitating its transport to the different targets, by increasing local penetration, prolonging residence time and by a controlled release mechanism to provide an effective dose to the target site (Marianecci et al., 2014). Among these lipid colloidal delivery systems (*e.g.* lipid nanoparticles, liposomes, and nano-emulsions) solid lipid nanoparticles (SLN) emerged in the early nineties as an alternative and current trials applying SLN consider them very promising in drug delivery (Müller et al., 2000). In fact, it is widely accepted that SLN combine the advantages and avoid the disadvantages of other colloidal carriers (Müller et al., 2000). Low drug loading and drug expulsion during storage period are taken as the disadvantage of SLN (Huang et al., 2008).

Nanostructure lipid carriers (NLC) have been developed to overcome the drug loading capacity and drug leaking phenomena that occurs after lipid polymorphic transition limitations of SLN. NLC are based on a mixture of solid and liquid lipids that results in an imperfect matrix thus, in an increase of the drug loading. As liquid lipids exhibit higher solubility for drugs, NLC have a higher loading capacity than SLN, as well as an improved drug controlled release (Uner, 2006). Many administration routes are being investigated for lipid nanoparticles (SLN and NLC), including topical, oral and parenteral ones. Also, these lipid colloidal carriers have been proposed for specific applications such as cancer treatment, gene therapy, diagnosis and medical devices production (Carbone et al., 2014).

Methotrexate (MTX) has been used in the clinics since the fifteens for the treatment of different solid tumours (*e.g.* osteosarcoma, lung and breast cancer) (Abolmaali et al., 2013) and in the therapy of autoimmune and inflammatory diseases as rheumatoid arthritis, Crohn's disease and psoriasis (Braun and Rau, 2009; Swierkot and Szechiński, 2006). MTX is a folate antagonist that competitively bind to dihydrofolatereductase (DFHR) hampering cell growth and arresting cell cycle in G1/S phases (Genestier et al., 2000). It has been reported that MTX induced apoptosis in several cancer cell lines (Padmanabhan et al., 2009) but its low tumor accumulation results in an ineffective exposure. However, MTX causes toxic side effects to normal cells as well as several adverse effects (hepatotoxicity,

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ulcerative colitis, nephrotoxicity) that hampers its therapeutic application (Visser and van der Heijde, 2009). To overcome these drawbacks colloidal delivery systems can be developed from biocompatible and biodegradable materials.

A formulation design requires full knowledge of the relationship between the process parameters and the quality attributes. To reach an optimized formulation using traditional screening approach (one factor at a time) is difficult, inefficient and time consuming. A few studies (Cun et al., 2011; Liu et al., 2010; Pradhan et al., 2015) have optimized lipid nanoparticles using factorial design and is widely accepted that the ingredients have great influence on the physico-chemical properties (Hao et al., 2011). The statistical formulation design is a validated and useful approach to develop a formulation with less experimentation and providing enough information on the relationship between independent and dependent variables (Gohel and Amin, 1998; Liu et al., 2010).

The present work reports the effect of the formulation composition in the optimization of MTX-loaded NLC by means of a Box–Behnken factorial design. Methotrexate was used as a model drug to be incorporated in the NLC due to its wide clinical application. The formulation was produced using the hot ultrasonication method and their physico-chemical properties (morphology, particle size, polydispersity, zeta potential, and entrapment efficiency), *in vitro* drug release studies and cytotoxicity were investigated.

2. Materials and methods

2.1. Materials

Methotrexate (MTX) was a kind gift from Excella (Feucht, Germany). The solid lipid, Witepsol® E85 and the liquid lipid, Miglyol® 812, were acquired from Cremer Oleo (Hamburg, Germany). The surfactant polyvinyl alcohol (PVA) was purchased from Sigma–Aldrich (St Louis, MO, USA). All other reagents and solvents were of analytical reagent grade.

2.2. Methods

2.2.1. Preparation of NLCs

MTX-loaded NLCs and NLCs were prepared by hot ultrasonication method. Briefly, the lipid phase composed by Witepsol® E85, Miglyol® 812 and MTX and the aqueous phase containing the surfactant (PVA) in 7 mL of double deionized water were heated to 60 °C in a water bath, separately. The aqueous phase was poured into the lipid phase and homogenized using a probe-sonicator (VCX130, Sonics & Materials, 115 Newtown, CT, USA) with amplitude frequency of 70% during 10 min, in order to obtain a nanoemulsion. Blank NLCs were prepared in a similar way, without the drug. Then, formulations cool down at room temperature.

2.2.2. Experimental design

The 3-level, 3-factor Box–Behnken design was applied to maximize the experimental efficiency, requiring a minimum of experiments to optimize NLCs produced by hot ultrasonication and study the effects of independent variables on dependent variables (Table 1). Independent variables were amount of liquid lipid (X_1), amount of surfactant (X_2) and amount of MTX (X_3). Other parameters, *i.e.*, amount of solid lipid, sonication time, sonication amplitude, final volume, were set at fixed levels. The established dependent variables were: Y_1 = mean particles size; Y_2 = polydispersity index and Y_3 = encapsulation efficiency. For each factor, the lower (−1), medium (0) and higher values (+1) were chosen on the basis of tested lower and upper values for each variable, according to pre-formulation studies and literature research. The data were analyzed using ANOVA by STATISTICA 10 (Statsoft®, Inc.) software.

Table 1
Variables with respective coded levels of the Box–Behnken design.

Factors	Coded levels		
Independent variables	Low Level (−1)	Medium Level (0)	High Level (+1)
X_1 = liquid lipid (mg)	40	50	60
X_2 = surfactant (mg)	40	50	60
X_3 = drug (mg)	2	10	20

Dependent variables	Constrains
Y_1 = particle size	Optimum (250 nm)
Y_2 = polydispersity index	Minimum
Y_3 = entrapment efficiency	Maximum

The polynomial equation generated from the experimental design is given below:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_{1^2} + b_{22}X_{2^2} + b_{33}X_{3^2}$$

where Y is the dependent variable, b_0 is the intercept, X_1, X_2, X_3 are the coded levels of independent variables, and b_1 to b_{33} are the regression coefficients computed from the observed experimental values of Y; the terms X_1X_2 and X_i^2 (i = 1, 2 or 3) represent the interaction and quadratic terms, respectively. The polynomial equation was statistically validated using ANOVA, by statistical significance of coefficients and r^2 values. Statistical analysis was considered significant when the p values were $\leq$0.05.

2.2.3. Optimization and validation

The graphical and numerical analyses were done by STATISTICA 10 to obtain optimum values of the variables based on the criteria of desirability (Table 1). The optimum variables were used to prepare a checkpoint NLC formulation and were compared with the predicted values to calculate the predicted error, in order to validate the chosen experimental domain and polynomial equations.

2.2.4. Entrapment efficiency

The entrapment efficiency of MTX within NLCs was determined as described previously (Pinto et al., 2014). Briefly, the non-entrapped drug was quantified at 303 nm, which is the wavelength of maximum absorption of MTX in aqueous solution (Lin et al., 2010). A standard curve of MTX was used to determine the concentration of MTX and the results are expressed as mean ± standard deviation (n = 3).

2.2.5. Particle size, polydispersity and zeta potential analysis

The particle size, polydispersity index (PDI) and zeta potential of all NLCs dispersions were analyzed using a ZetaPALS, ZetaPotential Analyzer (Holtsville, NY, USA). All samples were diluted with double distilled water to reach a suitable concentration before measurement. All analyses were carried out with a fixed light incidence angle of 90° at 25 °C.

2.2.6. Transmission electron microscopy (TEM) analysis

Optimized NLCs morphology was observed by TEM (TEM Jeol JEM-1400). Images were obtained after one drop of nanoparticles suspension was placed over a grid followed by negative staining with uranyl acetate and placed at the accelerating voltage of 60 kV.

2.2.7. Fourier transform infrared (FT-IR) spectroscopy

The freeze-dried optimized formulations of NLCs with and without MTX and pure MTX, as well as physical mixtures, were evaluated using an FT-IR spectrophotometer (Frontier™, PerkinElmer; Santa Clara, CA, USA) equipped with a horizontal