Development of oral lyophilisates containing meloxicam nanocrystals using QbD approach

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Abstract

The aim of this study was to develop oral lyophilisates with improved meloxicam (MEL) dissolution, optimizing each step of the preparation by design of experiments. First, meloxicam nanosuspensions were prepared by high-pressure homogenization (HPH), using PVP, Poloxamer or PEG as stabilizers and were subjected to freeze-drying using mannitol as cryoprotectant. The effects of the stabilizers and cryoprotectant were assessed and an optimal formulation was generated within the design space where the particle sizes and the PDIs are at their lowest values. The optimal formulation was used at the preparation of oral lyophilisates. Sodium alginate (SA) and croscarmellose sodium (CCS) were tested as matrix forming agents and three different freezing regimes were applied. The formulation was optimized, choosing the polymer that yielded

both high mechanical strength and fast MEL dissolution. Poloxamer led to particle size reduction down to 10.27% of the initial size, meaning 477.6 ± 7.5 nm, with a slight increase during freeze-drying process. PEG showed lower nanonizing capacity during HPH, but freeze-drying produced further diminution of the particle size. Since Poloxamer provided advanced size reduction while preserving MEL crystallinity, it was used for the optimized formulation containing 1% Poloxamer and 5% mannitol added before freeze-drying. SA showed good structural properties when compared to CCS and allowed fast MEL dissolution at low ratios. The optimal formulation contained 1.157% of SA was subjected to thermal treatment during freeze-drying. It disintegrated in 3.33 sec and released 77.14% of the MEL after 2 minutes. The quality by design (QbD) approach for the development of pharmaceutical products ensured high quality of the dosage form and good understanding of the preparation process.

Keywords

Nanocrystals, Meloxicam, Quality by Design, Oral lyophilisates, High-pressure homogenization

1. Introduction

Oral solid dosage forms are preferred by patients for the accurate dosing, their stability, easy of administration. Still, for the special groups of patients: pediatric, geriatric, patients with disphagia, their intake imposes serious limitations. The orally disintegrating dosage forms gained attention from the pharmaceutical industry and academia for their numerous advantages: easy swallowing without water, pleasant taste, enhancement of patent life cycle and increase of bioavailability of poorly water soluble drugs (Al Husban et al., 2010, 2011).

In the case of orodispersible tablets (ODTs), bioavailability increases due to the quick disintegration, followed by dissolution of the active pharmaceutical ingredient (API) in the saliva, the direct absorption through the oral mucosa to the systemic circulation, bypassing the liver first-pass metabolism (Al Husban et al., 2011). Fast disintegration occurs with highly porous products obtained either by compressing at low compression forces, molding or by freeze-drying (Chandraseckhar et al., 2009). Among these methods, freeze-drying provides light, porous structures that disintegrate in a matter of seconds, "officially" known as oral lyophilisates.

When fast disintegration of the dosage form is granted, bioavailability can be further limited by the API solubility and dissolution rate (Ghosh et al., 2012, Sarnes et al. 2014). To overcome this issue, researchers developed solid dispersions, drug-cyclodextrin inclusion complexes and nanosized particles (Samprasit et al., 2015, Blagden et al., 2007). Particle size reduction to the nano range with the effective surface area increase showed promising results in terms of dissolution rate and bioavailability improvement.

From the plethora of APIs being the subject of nanonization, meloxicam is a substance whose action consists in the selective inhibition of cyclooxigenase-2 isoenzyme and therefore it has effective anti-inflammatory and analgesic properties (Ochi et al., 2014) that recommend it for both human and veterinary use (Monteiro et al., 2016). Besides that, it is also emerging as a promising drug for the treatment of Alzheimer's disease and cancer. It was categorized into class II of the Biopharmaceutics Classification System (BCS), meaning it exhibits low water solubility (4.4 μg/ml) and good membrane permeability (Ambrus et al., 2009). Following the intake of classical

tablets, the peak plasma concentration is reached in 5-6 hours (Dellgado et al., 2014), far too long for a quick onset of the effect, which motivates the development of a fast dispersible dosage form with highly soluble meloxicam nanocrystals.

Nanocrystal technologies usually provide sub-micron colloidal dispersions of the drug crystals in a solvent, which has to be eliminated to obtain the dry powder for the further preparation of a solid dosage form (Kumar et al., 2015, Lai et al., 2015). One interesting approach is the combination of nanosuspensions with the production of freeze-dried orodispersible tablets (Lai et al.,2011, 2014). It involves the nanosuspension preparation and mixing with the matrix forming and cryoprotectant excipients, followed by freeze-drying, thus obtaining the freeze-dried ODTs. However, the main issues generated by the preparation of this new dosage form relate to the crystals' stability before, after freeze-drying, and to the balance between the disintegration and structure resistance of the freeze-dried products. The nanosuspensions are thermodynamically unstable systems, which can be stabilized for a pharmaceutically relevant time by adding surfactants or polymers that act as stabilizers. A high number of reports acknowledged the stability dependence on the type and amount of stabilizer, but when freeze-drying process is involved, data about crystal aggregation tendency is still controversial (Chung et al., 2012).

The design of a new formulation requires complete information about the process parameters and the way they control the quality attributes. Optimization via empirical screening approach is time consuming and does not reveal the collective effects of process and formulation factors. Design of Experiments (DoE) method has been used to overcome these issues by offering a broad understanding on the relationship between independent and dependent variables. Previous studies reporting the development of oral lyophilisates containing nanocrystals (Lai et al., 2011, 2014) used traditional screening approach and focused on API dissolution. They pointed out a complex preparation process with numerous variables, each one having a potential impact on product characteristics. Therefore, we believe that a research study conducted by a method that allows their simultaneous study could add to the knowledge base valuable data.

In this study, a DoE approach was applied to understand and optimize the two important steps in the preparation of oral lyophilisates (OLs) containing API nanocrystals: nanosuspension preparation and oral lyophilisates preparation. MEL was used as a model drug due to its emerging wide clinical applications and to the fact that such formulations of OLs containing MEL nanoparticles have never been used. In the first step, we established the optimal stabilizer and the mannitol ratios, after the evaluation of crystal behavior before and after freeze-drying. The second step focused on the matrix forming agents' functions and on the freezing regime. We studied their influence on the mechanical structure and the further on MEL dissolution.

The nanosuspensions were obtained by pre-sonication and high-pressure homogenization, followed by freeze-drying that led to oral lyophilisates. The particle size, polydispersity index (PDI) and Zeta potential were investigated as responses in the first step, while in the second preparation step we evaluated the disintegration time, the texture analysis and the *in vitro* drug release.

2. Materials and methods

2.1 Materials

The active pharmaceutical ingredient (API) – meloxicam (MEL) was purchased from Unichem Laboratoires Ltd., India. Mannitol (M) (Pearlitol 200M) and polyethylene glycol 4000 (PEG 4000)

were purchased from Merck, Germany. PVP K25 (Kollidon 25) and Poloxamer 188 (Polox) (Kolliphor P188) were kindly donated by BASF, Germany. Croscarmellose sodium (CCS) (Ac-Di-Sol) was obtained from FMC BioPolymer, Belgium and the alginic acid sodium salt (SA) from Sigma-Aldrich, United Kingdom.

2.2 Methods

2.2.1 Nanosuspension optimization

2.2.1.1 Design of experiment

Previous research in nanosuspension preparation revealed the importance of the stabilizer type and concentration for the API dissolution behavior. The stabilizers should assure wetting of the hydrophobic surfaces and increase of the activation energy of the agglomeration process, therefore be a barrier to agglomeration (Van Eerdenbrugh et al., 2008). The **type of stabilizer** was set as qualitative variable (**X_1**). We chose two polymers: PVP, PEG and one nonionic surfactant, Polox. Their **weight concentrations** in volume were varied on three levels: 0.25 – 1 – 1.75% (w/V) (**X_2**). During the freeze-drying, a cryoprotectant was added to avoid freeze damage due to ice formation and particle aggregation (Wang et al. 2013). We chose mannitol as **cryoprotectant, at concentrations** comprised between 0 and 5% (**X_3**). The effects of the aforementioned parameters on the crystal size, polydispersity (PDI) and Zeta potential were investigated using a three-factor, three-level DoE.

The critical quality attributes (CQAs) of the nanosuspensions were the average particle size and the MEL crystallinity.

As responses, we chose the **particle size** and **PDI after the size reduction** (**Y_1, Y_2**) and **after the freeze-drying** (**Y_3, Y_4**). In order to assess the size and PDI variations produced by freeze-drying process only and test if they have any statistical significance within the DoE, we calculated the size and PDI changes from the following equations:

$$\text{Size variation } (Y_5) = (\text{initial size} - \text{final size})*100/\text{initial size}$$

$$\text{PDI variation } (Y_6) = (\text{initial PDI} - \text{final PDI})*100/\text{initial PDI}.$$

The DoE modeling was performed using Modde 10.0 (Umetrics, Sweden) software and was used to provide a surface model for the six mentioned responses and an optimized formulation to take forward to the second step of the study.

2.2.1.2 Preparation of nanosuspensions

The micronized MEL (with 4.51 ± 0.57 µm average size and polydispersity index equal to 1) was suspended in the aqueous stabilizer (PVP, Polox or PEG) solution, using a magnetic stirrer to a concentration of 0.75% (w/V). The suspensions were stirred for 10 minutes at 1000 rpm. Each of them was then sonicated for 10 minutes at 70% amplitude using a high power ultrasound device (Hielscher UP 200S Ultrasonic processor, Germany) to wet the drug. Further size reduction to nanorange was achieved by applying high-pressure homogenization (HPH) with an Emulsiflex C5 apparatus (Avestin, Ottawa, Canada). 2 cycles at 500 bar were applied, followed by 20 cycles at 1000 bar.

2.2.1.3 Freeze-drying of nanosuspensions

MEL nanosuspensions were freeze-dried using a lab scale VirTis Advantage Plus freeze-drier (SP Scientific, Gardiner, USA). Briefly, four 0.5ml samples were taken from each of the nanosuspensions and poured into blister sockets. The blisters were placed on the freeze-dryer shelf and cooled to -50°C at a rate of 1°C/minute, thus we applied a fast freezing regime. The

temperature was kept constant for 6 hours for complete product solidification. The primary drying was performed at -20°C for 20 hours and vacuum of 0.2 mbar, followed by secondary drying at 5°C for 6 hours at 0.2 mbar.

2.2.1.4 Particle size analysis

Particle size measurements were performed by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS90 (Malvern Instruments, UK). Both the nanosuspensions before freeze-drying and the freeze-dried nanocrystals were subjected to this measurement. A mean particle diameter at an angle of 90° and a constant temperature of 25°C and the width of the size distribution (PDI) were determined by this method. 0.5ml of the suspension or corresponding freeze-dried product were diluted to 15ml with purified water. All samples were subjected to 60s of sonication prior to the size analysis in order to disperse the aggregates if present. Zeta potential was determined using the same equipment, by estimating the particle electrophoretic mobility in a thermostated cell. The results are presented as mean of three determinations and standard deviation.

2.2.2. *Oral lyophilisates optimization*

2.2.2.1. Experimental design

The optimal nanosuspension formulation revealed by the first DoE (containing 7.5 mg MEL/ml, 1% (w/V) Polox and 5% (w/V) mannitol) was taken forward to the next step, the formulation and optimization of the oral lyophilisates. Once the nanosuspension characteristics were established, we identified other factors that could influence the quality profile of the OLs: the type and content of matrix forming agent and the freezing rate. The chosen matrix forming agents (**X_1**) were sodium croscarmellose (**CCS**) at a ratio of **1%, 3% or 5%** and sodium alginate (**SA**) at a ratio of **1%, 2% or 3%.** The matrix forming agent **percentages** (**X_2**) were chosen from viscosity studies (results not shown); the viscosity had to be high enough to maintain suspension stability and still the suspensions had to be fluid enough to be accurately poured into blister sockets. The CCS percentages, of 1%, 3% and 5% were chosen from previous viscosity measurements that ranged between 40 and 250 mPa s, while for the sodium alginate, the viscosities of the 1%, 2% and 3% dispersions varied from 200 to 2000 mPa s. These dispersions were considered to be consistent enough to maintain meloxicam stability before freezing, but still fluid enough to be accurately poured into the blister sockets. The third independent variable was the **freezing type: fast, slow or annealing** (**X_3**).

The CQAs of OLs were the disintegration time, the mechanical strength and the MEL dissolution profile.

Several evaluation methods were selected in order to monitor the CQAs and their results were set as responses within the DoE. Therefore, we measured the **disintegration time** (**Y_1**), the **hardness** (**Y_2**), the **fracturability** (**Y_3**), the **% of dissolved MEL** after 2 minutes (**Y_4**), 4 minutes (**Y_5**), 6 minutes (**Y_6**), 12 minutes (**Y_7**), 18 minutes (**Y_8**) and 30 minutes (**Y_9**).

The same software was used to test the model and obtain the optimized formulation.

2.2.2.2. Preparation of oral lyophilisates

The previously optimized nanosuspension (containing 7.5 mg MEL/ml, 1% (w/V) Polox and 5% (w/V) mannitol) was prepared according to the described methods (*2.2.1.2.*). For each of the OL formulations, the corresponding matrix forming agent was added in the indicated ratio and the

formed viscous suspension was kept under gentle stirring until the complete polymer dispersion and homogenization.

0.5 ml of the obtained suspension that contained 3.75 mg MEL was poured into 30 blister sockets (blister material: PVC-Aclar®, PCTFE, poly-chloro-tri-fluoro-ethylene, cavity sizes: 12.70 mm diameter x 5.50 mm depth) and freeze-dried (VirTis Advantage Plus, SP Scientific, Gardiner, USA). The freeze-drying cycle started with one of the three proposed freezing profile (fast, slow or annealing), followed by primary drying at -20°C for 20 hours and vacuum of 0.2 mbar and by secondary drying at 5°C for 6 hours at 0.2 mbar (Fig. 1).

Figure 1

Fig. 2 illustrates the graphical procedure comprising the complete OL preparation process.

Figure 2

2.2.2.3. Characterization of oral lyophilisates

The disintegration time was measured according to Eur. Pharm. 8.0 method, by placing an oral lyophilisate in 200ml distilled water kept at 20 ± 0.5°C. The time necessary for complete disintegration, until no solid residue was perceived, was recorded using a digital stopwatch. The average disintegration time and the standard deviation of six tested tablets were calculated.

The texture analysis was performed using Brookfield TexturePro CT V1.5 (Brookfield Engineering, USA). Tablets were extracted from the alveolae, placed on a horizontal rigid surface and subjected to constant pressure. Pressure was applied by an acrylic probe (TA10), to a constant deformation of 80%, at a test speed of 0.1mm/s and a load of 10 g. Load vs. distance curves were recorded using Texture Pro Software. For each of the formulations, three measurements were carried out for the average hardness at 1.6 mm, fracturability and their corresponding standard deviations were calculated. The fracturability, as well as the hardness, derives from the texture calculations. The analyzer measured the resistance of the sample to the advance of the acrylic probe with a constant speed. The fracturability is calculated as the load value at the first fracture. A fracture is a sudden load drop that indicates an abrupt resistance decrease in the sample. Low fracturability values are correlated to brittle products. If no fracture occurs during the compression cycle, the fracturability value equals the hardness value. The fracturability was determined for three samples from each formulation; the mean value and the standard deviation were calculated.

The in vitro dissolution test was performed according to the Eur.Pharm. 8.0, using the paddle method. 900 ml of phosphate buffer with pH 7.4 at 37°C were used as dissolution media, at a rotating speed of 50 rpm. At certain time intervals, 5 ml samples were withdrawn, filtered through 0.2 μm cellulose filters (Phenomenex Syringe filters) and analyzed spectrophotometrically at 360 nm (Jasco V-560 UV-VIS, Easton, USA) against phosphate buffer pH 7.4 as a blank. Every sample was replaced with the same volume of the fresh media. The experiment was done in triplicate and the average meloxicam release at each sampling time and their standard deviations were calculated.

2.2.3. Solid state characterization of MEL nanocrystals

2.2.3.1. X-ray powder diffraction (XRPD)

The physical state of MEL in the different stages of the preparation process (for raw MEL and the freeze-dried nanosuspensions) was evaluated by XRPD. XRPD spectra were recorded with a BRUKER D8 Advance X-ray diffractometer (Bruker AXS GmbH, Karlsruhe, Germany) system with Cu Ká1 radiation (ë = 1.5406 Ĺ) over the interval 5-30°/2. The measurement conditions were as follows: target, Cu; filter, Ni; voltage, 40 kV; current, 40 mA; time constant, 0.1 s; angular step 0.010. In the determination of the degree of crystallinity, the total area of the characteristic three peaks with largest intensity was examined, after smoothing and background removal.

2.2.3.2. Scanning electron microscopy (SEM)

The morphology of the raw MEL, nanocrystals dried at room temperature without cryoprotectant and freeze-dried products was examined by SEM (Hitachi S4700, Hitachi Scientific Ltd., Tokyo, Japan). A sputter coating apparatus (Bio-Rad SC 502, VG Microtech, Uckfield, UK) was applied to induce electric conductivity on the surface of the samples. The air pressure was 1.3-13.0 mPa.

3. Results and discussion

3.1. Optimization of nanosuspension

3.1.1. Experimental design

In order to achieve an optimal pharmaceutical product, all the possible variables that may influence the characteristics of the product should be studied in detail. Two types of variables are involved in the development procedure: formulation and process variables. In this study, we chose to focus on the formulation parameters, while maintaining the process and its parameters at a constant level. The preparation of nanosuspensions via high-pressure homogenization (HPH) offers a set of advantages over the other size reduction methods, including low processing time, narrow size distribution and few contamination sources (Wang et al., 2013). Moreover, the organic solvents are not necessary, thus it is also ecologically friendly. Furthermore, HPH does not induce crystal form transformation, which could relate to higher drug and dosage form stability (Wang et al., 2013).

In this study, US preceded HPH, in order to disperse the eventual drug agglomerates and increase the initial contact between the drug and the stabilizer. A constant number of HPH cycles was chosen for each of the suspensions, at low pressure for the beginning and then at high pressure.

As stabilizers, we chose two polymers (PVP and PEG) and a nonionic surfactant (Polox). PVP was previously used as an agglomeration inhibitor at the preparation of MEL microparticles and nanocrystals (Pomazi et al., 2013, Bartos et al., 2015). In a grinding procedure to obtain MEL nanocrystals, both PVP and PEG were used as stabilizers and were reported to significantly improve the API dissolution rate (Kurti et al., 2011). Polox was confirmed as one of the most efficient stabilizers with respect to the achieved size reduction, size distribution but also concerning the morphology and aggregation capacity (Wang et al., 2013). Its high solubilizing capacity was another reason for selecting it as excipient (Mata et al, 2005).

For the freeze-drying process, mannitol (M) was chosen as a cryoprotectant for its ability to dispose between the nanocrystals in the cryoconcentrated product, to prevent aggregation and increase the structure strength.

Based on preliminary experiments and literature data, the stabilizers (X_1), their ratios (X_2) and the cryoprotectant (M) ratio (X_3) were set as critical factors. Thus, the study was conducted after a 3^3

factorial design for MEL nanosuspension formulation. To evaluate the nanosuspensions' behavior through the preparation process and through freeze-drying, we chose to measure the size and PDI after HPH (Y_1, Y_2), then after freeze-drying and reconstitution (Y_3, Y_4) and the size and PDI variation caused by freeze-drying (Y_5, Y_6) (see Supplementary material, Table 1).

~~**Table 1**~~

Multiple linear regression analysis and ANOVA were used to develop a mathematical model for each response. Equation 1 represents the general form of each model:

$$Y = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_4X_1^2 + B_5X_2^2 + B_6X_3^2 + B_7X_1X_2 + B_8X_1X_3 + B_9X_3X_2 + B_{10}X_1X_2X_3 \quad \text{(eq.1)},$$

where Y is the dependent variable (response), B_0 is the mean response (intercept) and B_i are regression coefficients derived from the obtained experimental values. X_1, X_2, X_3 are individual effects; they represent the result of the variation of one factor, while keeping the other factors at a constant value. X_1^2, X_2^2 and X_3^2 are quadratic effects that indicate non-linear correlations with the response. X_1X_2, X_3X_2, X_1X_3 and $X_1X_2X_3$ are interaction effects which reveal the variations of the responses when 2 or more factors change simultaneously.

The revised values obtained for eq. 1 regression coefficients are listed in Table 1 for each of the responses, together with their statistical significance, expressed as *p*-value. The coefficients indicate the magnitude of the effect of each of the independent variables and the way in which the factors influence the responses is given by + sign for positive influence, or – sign for negative influence. It was observed that all chosen factors produced significant changes with the responses. The regression coefficient R^2 was above 0.90 for responses Y_1, Y_2, Y_3 and Y_5, 0.89 for Y_4 and 0.74 for Y_6, which demonstrates the good fit of the data to the created model. When the size and PDI were measured after HPH, the critical factors were the stabilizer type and ratio ($p < 0.05$). After FD, the M ratio became the third critical factor. Two-way interactions between the type of stabilizer /its concentration and after FD between the stabilizer concentration/ M ratio had a significant influence on the nanocrystal size and PDI.

Table 1

3.1.2. The average size and PDI after HPH (Y_1, Y_2)

The average sizes after HPH ranged between 477.6 nm and 1232.0 nm, with PDIs between 0.29 and 0.88. The lowest particle size was met with the PVP and Polox, while the sizes reached after the same procedure using PEG as a stabilizer led to high values up to 1.2 μm. The wide data variations indicate the strong influence of the selected factors on the dependent variables. The regression coefficients listed in Table 1 pointed out the way stabilizers influence the nanocrystal formation. It seems that PVP caused the average size decrease, but the interactive effect between the type of stabilizer and its ratio indicates the opposite, a positive effect on the size. The response surface helps further understanding on this subject showing that up to 1.3 %, PVP generates MEL size reduction, while upper ratios hold back the nanonization process. Similarly, the PDI decreased up to 1% PVP and at higher concentrations it increased again. PEG produced the lowest size decrease, down to a minimum of 854 nm. The high viscosity polymers create in the suspension could be the reason for the impaired size reduction at high levels (Patel et al., 2014), due to the homogenization process hindering. Polox caused the most abrupt size and PDI decrease and kept

its behavior even at high ratios, as indicated by the response surfaces in Fig.3. 1% Polox led to the lowest crystal size of 477.6 nm and the corresponding PDI of 0.34.

3.1.3. The average size and PDI after FD (Y_3, Y_4)

The size and the PDI after FD were measured to assess the influence of independent variables on the global process. After HPH, M was dissolved in the obtained nanosuspensions, therefore its effect became visible in the statistical data. The average particle sizes ranged between 489.5 nm and 836.1 nm, so the upper limit decreased during FD. The results (Table 1) showed that PVP ratio did not have a significant influence on the size, but its presence determined a high PDI. Polox yielded low crystal sizes with no change of polydispersity degree (Fig. 3), while PEG determined the lower size reduction and wide size distribution.

M displayed a significant negative effect on both average crystal size and PDI, because it prevented aggregation in the cryoconcentrated system.

Figure 3

3.1.4. The size and PDI variations caused by freeze-drying

For a statistical evaluation of the crystal size and PDI variations induced selectively by the freeze-drying process, we calculated the percentages of size and PDI increase/decrease and tested them as responses in the experimental design (see Supplementary material, Table 1).

Apparently, for most of the freeze-dried nanosuspensions, the crystal size decreased (14 out of 19). The regression coefficients indicate which of the formulation factors influence the variation and how (Table 1). An individual positive effect for PVP as stabilizer was obtained, meaning that at low concentrations, PVP determined the size increase during FD. If the PVP ratio is too low, it is possible that it does not cover the entire particle surface by adsorption (Wang et al., 2013). Therefore, the uncovered sides of MEL particles could exhibit aggregation capacity and lead to average size increase. An interactive negative effect between PVP as stabilizer and the stabilizer concentration showed that high PVP content led to size reduction during FD, which confirms the previous hypothesis. According to the statistical data, Polox had a positive effect on crystal sizes, along the entire concentration range. On the opposite, freeze-drying nanosuspensions with PEG as stabilizer determined a significant particle size decrease. ~~As PEG is highly hydrophilic, it could adsorb water by hydrogen bonding and the freezing water molecules could break the stabilized conglomerates into smaller particles.~~

M was added to the system when size reduction process was finished, therefore we expected it to prevent aggregation and not to decrease the average size below the initial values. Surprisingly, the sizes decreased even further. The explanation could be that M solution penetrated into the particle fissures and when M crystallized during FD, the volume increase generated cracks into the crystal aggregates with consequent size reduction.

3.1.5. The Zeta potential

The Zeta potential is a parameter that indicates the stability of nanosuspensions with values between -100 mV and 100 mV. Products with Zeta potentials in the range [-25mV; 25mV] are considered less stable, with high agglomeration probability. The values we obtained after HPH ranged between -35.6 mV and -20.1 mV and suggest good stability for most of the nanosuspensions (results not shown).

3.1.6. Solid state characterization of MEL nanocrystals

SEM analysis

The SEM images with reduced size MEL containing the same amount of stabilizer (1%), showed the changes in the appearance and morphology of the obtained nanoparticles (Fig. 4). The large raw MEL crystals (A) presented regular prismatic shape with smooth surfaces. The particle size reduced, regardless of the type of stabilizer (B-D). PVP softened and covered the MEL particles (Martha et al., 2013) that kept their shapes within an amorphous conglomerate (B). The aggregation tendency of the MEL-PVP particles is visible in the SEM captions.

Polox as stabilizer led to oval shaped individual crystals (C) with soft edges that could be attributed to erosion phenomena during the homogenization process. Partial dissolution of Mel crystals mediated by Polox could also be incriminated, but since Polox critical micelle concentration is higher than the ones used in our study (between 24 and 32 mg/ml according to Moghimi et al., 2004), mechanical softening of crystal edges is more probable. The analyzed sample showed good size uniformity, also confirmed by PCS.

PEG particles preserved their sharp edges, but they had a wide size distribution and the size reduction did not reach the level of the other two stabilizers.

Figure 4

XRPD analysis

X-ray powder diffraction was performed for raw MEL and the freeze-dried nanosuspensions prepared according to the experimental design. It was meant to assess the crystalline changes that MEL might have suffered during the HPH and freeze-drying processes. The diffractogram of raw MEL exhibits numerous distinct peaks, at diffraction angles 2Θ of 13.22, 15.06 and 26.46 (Kurti et al., 2011), which confirm its initial crystallinity. Fig. 5 shows the diffractograms of the freeze-dried nanosuspensions and the changes that appear with different types and ratios of stabilizers. For the sample with low PVP content (N8), the MEL characteristic peaks can be observed, but at high PVP ratios the areas under the peaks considerably diminished (N11, N13), revealing the amorphous structure of the nanoparticles. The products samples containing Polox as stabilizer displayed all the MEL characteristic peaks at all concentration levels of the stabilizer, which indicates that the crystalline state was preserved (N9, N12, N13). The PEG freeze-dried nanosuspensions exhibited partial loss of crystallinity at low PEG concentrations (N15) (Martha et al., 2013).

Figure 5

3.1.7. Optimization of nanosuspension

Based on the revised equations and surface response plots, the software was used to generate the set of conditions/ optimum formulation variables in order to obtain the nanosuspensions with desired CQA profile. The selected criterions were to minimize the average sizes and PDI after HPH and after freeze-drying and to have a crystalline product. The software generated the following conditions: Polox as stabilizer at 1% ratio and 5% M as cryoprotectant during freeze-drying. Through statistical analysis, from the initial experimental area, a Design Space was identified (Figure 6), where all the conditions imposed to the nanosuspensions formulations would be fulfilled at a specified risk level. Each point from the Design Space represents a nanosuspension formulation obtained with a risk level expressed as Defect per one Million Opportunities (DPMO). The green areas could deliver a series of formulations that would comply to the conditions of low

average size and PDI, with a probability of 99.95%.The optimal nanosuspension was obtained using the indicated parameters and the same procedure: the US, HPH and freeze-drying regimes applied during the initial experiments. It exhibited a Zeta potential of -28.31 ± 0.48 mV, therefore good stability. The characterization of the optimal formulation compared to the predicted values and the calculated residuals are presented in Table 2. The closeness between the experimental and the predicted results confirmed the validity of the statistical model and its predictive power. The optimal suspension evaluated in Table 2 contains 7.5 mg MEL/ml, 1% (w/V) Polox 188 and 5% (w/V) M.

Figure 6

Table 2

3.2. Optimization of oral lyophilisates

3.2.1. Design of experiment

The optimal nanosuspension was taken forward to the next step, an experimental design for the optimization of oral lyophilisates. At this stage, the CQAs are disintegration time, mechanical strength and MEL dissolution which is granted by the size reduction achieved in the first step (Mauludin et al., 2009). A thorough study of the formulation factors is necessary in order to balance the quick disintegration time and the high mechanical strength. Both disintegration time and mechanical strength are conditioned by the type and ratio of matrix forming agents, while the structure depends on the freezing rate (Harnkarnsujarit et al., 2012).

As matrix forming agents we chose CCS, superdisintegrant used for its high hydrating and swelling capacity that grants suspension stability up to the freezing step. Moreover, it was mentioned for the ability to increase API dissolution (Lai et al., 2014). SA was selected for its capacity to yield highly viscous dispersions at low polymer concentrations (Vicini et al., 2015). The concentration levels were chosen from previous viscosity studies (results not shown) so that the dispersions would be fluid enough to be poured into blister sockets and viscous enough to prevent settling phenomena: 1-3-5% for CCS and 1-2-3% for SA.

During the lyophilization process, the freezing step is of high importance due to the crystallization processes that further impact the texture of the frozen matrix and the morphological characteristics of the dried cake. For orally disintegrating dosage forms, slow freezing is more appropriate due to the formation of structures with large pores, easily disintegrating upon hydration. Large pores promote disintegration, but when it comes to systems containing suspended nanocrystals, a dense cryoconcentrated phase during freezing could induce aggregation phenomena. Therefore, we planned to study the influence of two different freezing rates: 0.5°/minute decrease (slow freezing), 1°/minute decrease (fast freezing) (Fig. 1). An annealing step was also considered for its reported benefits on size distribution of ice crystals, on accelerating primary drying and reducing heterogeneity between samples (Abdelwahed et al., 2006). Moreover, mannitol as bulking agent is known to yield a mixture of amorphous and crystalline forms (Kim et al. 1998, Torrado et al., 2002) especially at low mannitol ratios, therefore an annealing step integrated into the freezing phase could maximize its crystallization (Mehta et al., 2013). The aforementioned independent variables were included in a quadratic D-Optimal design: the type of matrix forming agent (X_1), the matrix forming agent ratio (X_2) and the freezing rate (X_3). As responses, we chose the disintegration time (Y_1), the hardness (Y_2), the fracturability (Y_3) and the percentage of dissolved

MEL at 2 minutes (Y_4), 4 minutes (Y_5), 6 minutes (Y_6), 12 minutes (Y_7), 18 minutes (Y_8) and 30 minutes (Y_9). For the evaluation of the results (listed in Supplementary material, Table 2), multiple linear regression and ANOVA test were applied. They showed high variability, which indicates a strong dependence on the selected independent factors. A model was developed for each response, expressed as an equation of the response as a function of the independent variables and their interactions (eq. 1, *3.1.1*.).

The statistical analysis showed that all the selected independent variables had a significant influence on the responses. R^2 was above 0.9 for responses Y_1, Y_3, Y_4, Y_5 and Y_6 and between 0.8 and 0.9 for Y_2, Y_7, Y_8 and Y_9, meaning that more that 80% of the responses variability was explained by the model.

~~**Table 4**~~

3.2.2. The disintegration time (Y_1)

The OLs disintegrated between 0.83 and 58 s~~, as shown in Table 4~~. The results proved that the type of matrix forming agent (X_1), its ratio (X_2) and the freezing rate (X_3) significantly influenced the disintegration time (Fig. 7). As expected, high CCS content determined a fast disintegration, while high SA percentages led to slowly disintegrating OLs. The type of freezing also influenced the disintegration: annealing decreased the disintegration time, while progressive freezing delayed it. An interactive effect was noticed between the matrix forming agent (MFA) content and the type of freezing: when annealing, the higher the MFA content, lower the disintegration time. On the contrary, if progressive freezing was applied on highly concentrated MFA dispersions, disintegration was delayed. Disintegration depends on the structure of the 3D freeze-dried matrix. Annealing procedure allows the rearrangement of crystals with the structure relaxation; therefore, it usually leads to highly porous products, easily permeated by the dissolution media.

Figure 7

3.2.3. The texture analysis

The OLs were completely dry freeze-dried matrices, with the diameter of 12.75 ± 0.15 mm and the height of 5.14 ± 0.18 mm. The texture analysis revealed the OL's behavior when being subjected to constant pressure. It yielded two parameters: the hardness (Y_2) and the fracturability (Y_3), which describe the mechanical properties of the structures. The two MFA yielded quite different products. The OLs with CCS were extremely soft and fragile, while the OLs containing SA gave firm and stiff structures, easy to extract from the blister sockets. Therefore, the only significant influence was assessed with the MFA variation: CCS determined hardness decrease, while SA determined the hardness increase, with no significant influence from their ratios (Fig.8 A).

Fracturability is an indirect indicator of the brittleness of a product and is calculated as the load value at the first fracture, more precisely, it shows the resistance of a product to fractures. The influence of the MFAs was, as expected, the most important: SA gave high fracturability, while CCS gave low fracturability and their ratio increase determined higher fracture resistance in both cases (Fig. 8B).

Figure 8

The mechanical profile of the oral lyophilisates was represented as load (N) vs. distance (mm) curves (Fig. 9) and clearly shows the differences between CCS and SA behavior as structural excipients. The weak CCS matrix appears like a very heterogeneous porous structure with thin, disrupted pore walls. As for the suspensions containing SA, the dehydration led to a highly porous structure, with big cavities produced by water crystals sublimation, but with slightly thicker pore walls, linked to each other.

Figure 9

Interestingly, the type of freezing had no significant effect on the product's hardness or fracturability parameters (Fig. 8) when analyzed within the experimental design, but a closer texture curve analysis (results not shown) shows that progressive freezing yields weak mechanical profiles as compared to the other freezing treatments.

3.2.4. In vitro dissolution test

The drug release from the lyophilized matrix was assessed by *in vitro* dissolution studies. Dissolution is a limiting factor for the oral absorption and thus for the pharmacological effect. In this study, the dissolution profiles were studied on a 30 minutes range, in PBS pH 7.4.

After 2 minutes, the ratio of dissolved MEL ranged between 21.57% and 100%. The wide range shows the strong influence of independent variables on the responses. The influences of independent variables on the dissolution profile were constant at all the tested times. MEL dissolution was favored by the presence of CCS and delayed by SA. The higher the CCS ratio, more MEL was dissolved. On the contrary, at high SA ratios, the dissolution percentages decreased. The freezing rate influenced the dissolution profile during the first 6 minutes of the test; after 6 minutes, more than 90% of MEL was dissolved for 15 formulations out of 21.

The sudden freezing delayed the dissolution, while the progressive freezing and the annealing seemed to have a less significant enhancing effect on dissolution. The faster dissolution caused by slow freezing could be a consequence of the weak mechanical strength it delivers and the higher porosity produced by ice crystal growth in the freezing phase (Iurian et al., 2016).

Figure 10

3.2.5. Optimization of oral lyophilisates

The statistical calculations and experimental observations led to an accurate knowledge of the variables that influence OL's characteristics. MEL dissolution was granted by its size reduction, while the OL's disintegration and mechanical properties were controlled by the MFA type and ratio and by the freezing regime. The statistics software was used to generate the optimal OL formulation, by applying a set of constraints. We chose to minimize the disintegration time and maximize the mechanical strength and MEL dissolution after 2, 4 and 6 minutes. The software indicated the optimal formulation with SA as MFA, at -0.843 concentration level, meaning 1.157% SA and being subjected to annealing as a thermal treatment before freeze-drying.

The optimal OLs were prepared and tested following the same techniques as the previous formulations using the independent variables that resulted from the experimental design analysis. The optimal formulation characterization is listed in Table 3. All the responses were in the predicted range, therefore the experimental design was considered valid and could be further used for the development of oral lyophilisates with desired characteristics.

Table 3

4. Conclusion

The study reveals a two steps QbD strategy for the development of oral lyophilisates with high drug bioavailability. The first stage handles the development of an optimal nanosuspension with respect to the crystal size and PDI, with focus on the changes brought by freeze-drying process and how do different factors influence those changes. The optimal formulation that achieved the lowest average size and PDI both before and after freeze-drying contained 1% Polox as stabilizer and was submitted to lyophilization having 5% M as cryoprotectant. It was included in the second step of the study for oral lyophilisates optimization, when three more variables were added: the matrix forming agent type, ratio and the freezing regime. Sodium alginate granted high structural stability but also fast disintegration and drug dissolution, therefore it was selected as matrix forming agent in the optimal formulation.

The experimental design approach was a valuable tool for the thorough study of variables influencing the nanosuspension and oral lyophilisate preparation. Creating such models offers high versatility; the two validated experimental designs could be further used together for the preparation of oral lyophilisates or separately as basis for other research studies. The optimal oral lyophilisates according to Table 3 contain: 3.75 mg MEL/OL, 5 mg Polox 188/OL, 25 mg mannitol/OL and 5.78 mg Sodium Alginate/OL, obtained from the freeze-drying a suspension that contained 7.5 mg MEL/ml, 1% (w/V) Polox 188, 5% (w/V) mannitol and 1,157% (w/V) sodium alginate.

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Table 1
The revised quantitative factor effects and associated *p* values for the responses

Term	Size after HPH (Y_1)	PDI after HPH (Y_2)	Size after FD (Y_3)	PDI after FD (Y_4)	Size variation after FD (Y_5)	PDI variation after FD (Y_6)
Constant	681.437 (0.000)	-0.429 (0.000)	2.770 (0.000)	-0.472 (0.000)	-11.168 (0.000)	-41.753 (0.000)
X_1 PVP	-76.058 (0.000)	-0.071 (0.000)	-0.007 (0.471)	0.031 (0.087)	5.329 (0.002)	7.205 (0.000)
X_1 Poloxamer	-138.297 (0.000)	-0.059 (0.000)	-0.037 (0.002)	-0.072 (0.000)	8.854 (0.000)	-0.344 (0.000)
X_1 PEG	214.355 (0.000)	0.130 (0.000)	0.044 (0.001)	0.040 (0.037)	-14.184 (0.000)	-6.860 (0.000)
X_2	106.637 (0.000)	0.058 (0.000)	0.005 (0.593)	-0.062 (0.006)	-9.267 (0.000)	-6.906 (0.000)
X_3	3.470 (0.804)	-0.000 (0.916)	-0.025 (0.008)	-0.008 (0.102)	-4.603 (0.002)	-2.035 (0.002)
X_2*X_2	90.858 (0.017)	0.129 (0.000)	0.037 (0.068)	-	-	7.695 (0.000)
X_1 PVP*X_2	54.710 (0.007)	-0.008 (0.375)	0.019 (0.072)	-	-7.576 (0.000)	-
X_1 Poloxamer *X_2	-114.159 (0.000)	-0.027 (0.012)	-0.048 (0.000)	-	4.927 (0.005)	-
X_1 PEG *X_2	59.449 (0.004)	0.035 (0.008)	0.029 (0.010)	-	2.649 (0.088)	-
X_2*X_3	-	-	0.026 (0.007)	0.057 (0.003)	5.574 (0.000)	7.658 (0.002)
X_3*X_3	-	-	-	-	-	-4.595 (0.000)

Table 2
Optimal nanosuspension formulation results

	Experimental values	Predicted values	Residual
Size after HPH (nm)	463.5 ± 9.71	453	10.5
PDI after HPH	0.312 ± 0.014	0.302	0.01
Size after Freeze-drying (nm)	501.7 ± 9.18	491	10.7
PDI after Freeze-drying	0.301 ± 0.037	0.291	0.01

Table 3
Optimal oral lyophilisate formulation results

	Experimental values	Predicted values	Residual
Disintegration time (s)	3.33 ± 0.76	1.89	1.44
Hardness (N)	16.34 ± 0.48	16.10	0.24
Fracturability (N)	22.58 ± 4.36	21.19	1.38
% of dissolved Mel after 2 minutes	77.14 ± 6.55	71.79	5.34
% of dissolved Mel after 4 minutes	86.82 ± 8.64	93.77	-6.95
% of dissolved Mel after 6 minutes	96.71 ± 4.11	100.41	-3.70