University of Szeged

Faculty of Pharmacy

Institute of Pharmaceutical Technology and Regulatory Affairs

Summary of PhD Thesis

Optimization of a combined wet milling process to produce nanosuspension and its transformation into surfactant-free solid compositions to increase the product stability and drug bioavailability

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1. INTRODUCTION

Particle size reduction techniques are widely used to produce micro- or nanoparticles in order to increase the specific surface area thereby improve the dissolution rate of poorly soluble drugs as non-steroidal anti-inflammatory drugs (NSAIDs), (Shegokar et al., *Int. J. Pharm.,* 2010). Top-down processes comprise disintegration techniques, where the raw material is broken down via mechanical forces. Milling is a commonly applied technique to produce micro- or nanosized drug crystals. There are numerous types of milling techniques; dry and wet milling can be distinguished (Dahiya, 2017).

Wet milling is applicable for micronization (Pomázi et al., *Eur. Polym. J.*, 2013), but is usually used for nanonization (Bilgili et al., *Chem. Eng. Sci.,* 2006, Loh et al., *Asian J. Pharm. Sci.,* 2015). Media milling is a commonly used milling process in pigment-, photo- and magnetic industry. Commercially, in pharmaceutical industry the media milling is exemplified by the NanoCrystal® technology from Elan Pharma International Ltd. (Dublin, Ireland), (Junghanns et al., *Int. J. Nanomed.,* 2008). The process is time-consuming and requires lot of energy (Merisko-Liversidge et al., *Adv. Drug Deliver. Rev.,* 2011, Azad et al., *Particuology,* 2014). Planetary ball milling belongs to high energy milling methods. The working principles of the mill: during the operation the milling container rotates about its own and an external axel, so it carries out a planetary movement (Broseghini et al., *J. Eur. Ceram. Soc.,* 2016). Retsch method recommendation list, combination of the planetary ball mill and the grinding media of the pearl mill is preferred as a novel milling technique in order to prepare pre-dispersions with nano particle size of the API (Retsch GmbH, Haan, Germany).

This combinative milling technology results in nanosuspensions which can be defined as colloidal dispersions of nanosized drug particles (< 500 nm), (Müller et al., *Int. J. Pharm.,* 1998). Nanosuspensions can be applied as final liquid dosage forms using further different excipients (viscosity enhancer, flavoring, preservative agents, etc.), however, their stabilization is a major challenge. Despite the stabilization, nanosuspensions have a short expiration time, and there are patients who do not prefer this form or the presence of a surfactant. One way to overcome the instability and surfactant problem is to design dry nanosuspension (Fülöp et al. *Eur. J. Pharm. Sci.,* 2018) or other processes may be the transformation of the nanosuspension into solid-state dosage forms.

Present PhD thesis shows new results on this topic, which may help nanonization of active ingredients by combined wet milling, stabilization of nanoparticles without surfactant, and improved bioavailability of the final solid product.

2. AIMS

The aim of this work was to study the wet milling process, where the planetary ball mill was combined with pearl milling technology and optimize the process parameters (pearl amount, milling time and rotation speed) and predict the robustness of the process and design the amount of the additive (poly(vinyl alcohol), PVA) in order to produce Meloxicam (Mel) containing nanosuspension. PVA as a protective polymer had a dual function in this system, partly to increase the efficiency of milling (without any pre-milling procedure and surfactant) and to stabilize the nanosuspension as an intermediate product. Furthermore, the optimized Mel formulations were tested on the cell culture model of intestinal epithelium.

The purpose of the work was further to produce a surfactant-free product by solidifying of Mel containing nanosuspension. Critical product parameters were considered to be the particle size distribution of the drug ($d(0.9) < 500$ nm), stabilization of the degree of crystallinity altered during milling, and enhancement of the bioavailability of the solid product with fast absorption from the stomach for rapid analgesia. The transformation of the nanosuspension was done by fluidization and lyophilization.

The main steps in the experiments were as follows:

i. Optimization of the critical process parameters of the combined wet milling (ratio of pre-dispersion and pearls, milling time and rotation speed) in order to produce Mel containing nanosuspension as an intermediate product without any pre-treating procedure and surfactant (nanoMel).

ii. Investigation of the influence of the PVA amount on the milling effectiveness and the PSD and crystallinity of the Mel in the ground products which does not contain any additional excipients, thus surfactant.

iii. Perform *in vitro* dissolution test and cell culture studies (cell viability and permeability) to control the amount of PVA.

iv. Testing the robustness of combined wet milling process to determine the interval of the Mel amount and to predict the degree of crystallinity of the milled products.

v. Transformation of the surfactant-free nanoMel sample into solid-state products by fluidization and lyophilization and investigation of the product stability (particle size, crystallinity), and *in vitro* release of Mel

vi. Bioavailability of the products was studied by *in vivo* animal tests to justify applicability of the surfactant-free samples containing nanonized Mel.

3. MATERIALS AND METHODS

Mel was obtained from EGIS Ltd. (Budapest, Hungary). PVA-Mowiol^{R} 4-98 (*Mw* ~ 27,000) (Sigma Aldrich Co. LLC, St. Louis MO, USA) was used as a stabilizing agent. Zirconium oxide (ZrO_2) beds with a diameter of 0.30 mm were obtained from Netsch (Netsch GmbH, Selb, Germany). Microcrystalline cellulose (MCC) (Avicel^{R} PH 101, FMC Biopolymer, Philadelphia USA) was used as a carrier material for the fluidized product. D-(+)-trehalose dihydrate as a cake-forming agent was purchased from Karl Roth GmbH + Co. KG. (Karlsruhe, Germany).

3.1. Preparation of the products

The samples were milled with the steel jar with 50 ml volume of the Retsch PM 100 planetary ball mill (Retsch PM 100 MA, Retsch GmbH, Germany) combined with 0.3 mm ZrO_2 beads as the grinding media. The ratio of the amount of pre-dispersion and pearls (w/w) was 1:0.5, 1:1, 1:2 and 1:4; and the milling times were 10, 30 and 50 min. In these cases, the concentration of PVA solution was 2.5% (w/w), and the rotation speed was 400 rpm. In the second step, design and analysis of experiments with three levels were used to optimize the milling time (10, 30 and 50 min) and the rotation speed (200, 350 and 500 rpm) as independent variables.

3.2. Optimization of PVA concentration

After optimizing the process parameters, the determination of the adequate PVA concentration was executed. Various amounts of PVA (2.5-7.5%) were applied to prepare the concentrated pre-dispersions.

3.3. Robustness determination of the process

Eight samples were prepared from 0.5 to 4.0 g of Mel with 0.5 g increment per sample. As the stabilizing agent, 5% of PVA aqueous solution was added up to 20.0 g to each sample, which was selected on the basis of our previous experiments.

3.4. Transformation of nanosuspension into solid compositions: fluidization (fluidMel), lyophylization (lyoMel)

The Avicel PH 101 as the carrier material was used in a Strea-1 (Niro Aeromatic, Bubendorf, Switzerland) fluid bed chamber. Freeze-drying was performed in Scanvac CoolSafe 100-9 Pro type equipment (LaboGene ApS, Lynge, Denmark).

3.5 Particle size distribution measurements

The investigations on the particle size of raw Mel and nanoMel via laser diffraction were executed (Malvern Mastersizer S 2000, Malvern Instruments Ltd, Worcestershire, UK). In the case of the solid-state products (fluidMel and lyoMel), the particle size of Mel was determined by using Scanning Electron Microscopy (SEM) images (Hitachi S4700, Hitachi Scientific Ltd., Tokyo, Japan). For nanoMel and lyoMel samples, the Z-average particle size and the polydispersity index (PDI) of Mel were measured using a Malvern Zeta Nano ZS (Malvern Instruments Ltd).

3.6 Image analysis (scanning electron microscopy - SEM)

The shape and surface characteristics of the samples were visualized by SEM (Hitachi S4700, Hitachi Scientific Ltd., Tokyo, Japan).

3.7 X-ray powder diffraction analysis (XRPD)

XRPD patterns were produced by a Bruker D8 Advance diffractometer (Bruker AXS GmbH, Karlsruhe, Germany) system with Cu K λI radiation (λ = 1.5406 Å).

3.8 Stability test

The products (fluidMel and lyoMel) were stored in a well-closed container, at room temperature (23 ± 2 °C, 45 ± 5% RH) for 6 months. The crystallinity of Mel was investigated compared to freshly measured products.

3.9. Differential scanning calorimetry (DSC)

DSC measurements were carried out with a Mettler Toledo DSC 821e thermal analysis system with the STARe thermal analysis software V9.0 (Mettler Inc. Schwerzenbach, Switzerland

3.9. Raman spectroscopy

Raman spectra were acquired with a Thermo Fisher DXR Dispersive Raman (Thermo Fisher Sco. Inc., Waltham, MA, USA)

3.10. Zeta potential measurements

The zeta potential of the dispersions and the lyophilized product was measured via Malvern Zeta Nano ZS (Malvern Instruments Ltd, Worcestershire, UK).

3.11. Rheological investigations

Rheological measurements were carried out with Physica MCR101 rheometer (Anton Paar, Austria, Graz).

3.12. *In vitro* dissolution studies of Mel

To determine the dissolution extent of Mel from dispersions, the paddle method (USP dissolution apparatus, type II Pharma Test, Heinburg, Germany) was used. The Mel contents of the samples were determined by spectrophotometer (ATI-UNICAM UV/VIS Spectrophotometer, Cambridge, UK) at 362 nm (gastric juice) and 364 nm (enteric fluid). The number of parallels was three.

3.13. *In vitro* cell culture studies

3.13.1. Human Caco-2 intestinal epithelial cell line

Caco-2 intestinal epithelial cell line was purchased from ATCC (cat.no. HTB-37) and used until passage 60 for the experiments. The cells were grown in Dulbecco's Modified Eagle's Medium (Gibco, Life Technologies, Carlsbad, California, USA) supplemented with 10 % fetal bovine serum (Pan-Biotech GmbH, Aidenbach, Germany) and 50 μg/ml gentamycin in a humidified incubator with 5 % CO2 at 37°C.

3.13.2. Cell viability measurement by impedance

Impedance was measured at 10 kHz by RTCA SP instrument (RTCA-SP instrument, ACEA Biosciences, San Diego, CA, USA).

3.13.3. Permeability study on cell culture model

TEER was measured every 2 day to check the barrier integrity by an EVOM volt-ohmmeter (World Precision Instruments, Sarasota, FL, USA) combined with STX-2 electrodes, and was expressed relative to the surface area of the monolayers.

3.14. *In vivo* study of Mel

For per os delivery, the different formulations were individually diluted and were given at a single dose of 300 μg/kg of Mel to male Sprague–Dawley rats (8 weeks old, 240-260 g, n = 6) in a volume of 0.5 ml by gastric gavages.

3.15. *In vitro-in vivo* correlation calculation

In vitro–in vivo correlation (IVIVC) was calculated by Microsoft Excel (Microsoft Corporation, Redmond, Washington, U.S.) and Statistica for Windows (StatSoft GmbH, Hamburg, Germany).

4. THESES / RESULTS

i) Optimization of the critical process parameters of the combined wet milling

The PhD work reports a wet milling process, where the planetary ball mill was combined with pearl milling technology to produce nano-size meloxicam (Mel) (d(0.9) < 500 nm). Mel as a water-insoluble highly potent NSAID was milled in the presence of the PVA, in aqueous solution without any other stabilizer agent as surfactant. The critical process parameters as ratio of pre-dispersion and pearls and the milling time and rotation speed of the steel jar were optimized on the particle size distribution and crystallinity of the Mel was investigated. It was found that the ratio of pre-dispersion and pearls 1:1 (w/w) resulted in the most effective grinding system without any pretreating process (200-fold particle size reduction in one step) (Table 1) with 437 rpm and 43 min as optimized process parameters (Figure 1).

Table 1. Particle size of Mel (d(0.5)) in milled dispersion as a function of different pearl amount and milling time (d(0.5) of raw Mel was 34.260 ± 4.860 μm)

	Ratio of pre-dispersion and pearl amount (w/w)			
	1:0.5	1:1	1:2	1:4
	Particle size (μm)			
10 min	4.015±0.06	2.426±0.029	2.383±0.016	0.149±0.03
30 min	0.293±0.008	0.145±0.007	0.190±0.003	0.137±0.006
50 min	0.202±0.003	0.140±0.004	0.140±0.002	0.130±0.004

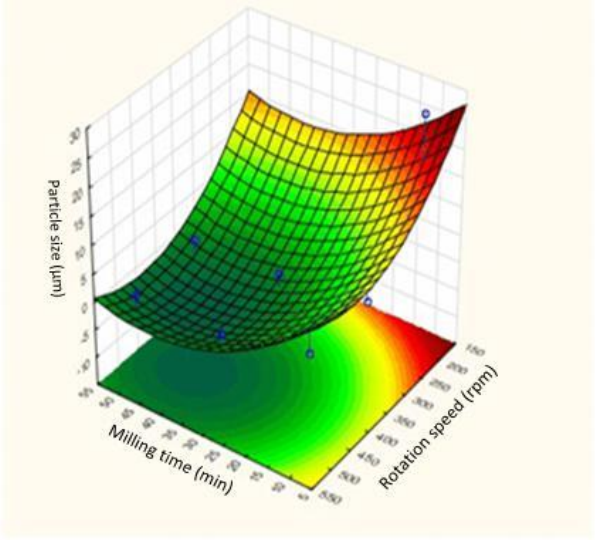

Figure 1. 3D illustration of the particle size changes during the second factorial experimental design

ii. Optimization of the PVA amount on the milling effectiveness, the particle size distribution and crystallinity of the Mel

The amount of PVA were also a critical parameter because it affected the investigated parameters. It can be concluded that the milling effectiveness of low concentration of PVA (< 4%) was not satisfactory, because the crushing / breaking effect of the pearls could less prevail. High concentration of PVA (> 5%) also resulted in unsatisfactory milling effectiveness, because of the formation of polymer layer on surface of particles which protects the particles from the fragmentation. Considering the effectiveness of milling, 5% PVA was proved to be an optimal quantity to meet the expected value (d(0.9) < 500 nm) (Figure 2). The different concentrations of PVA in the aqueous dispersion also influenced the

viscosity, consequently the particle size distribution and electrokinetic property of the particle (Table 2) and the stability of the dispersions.

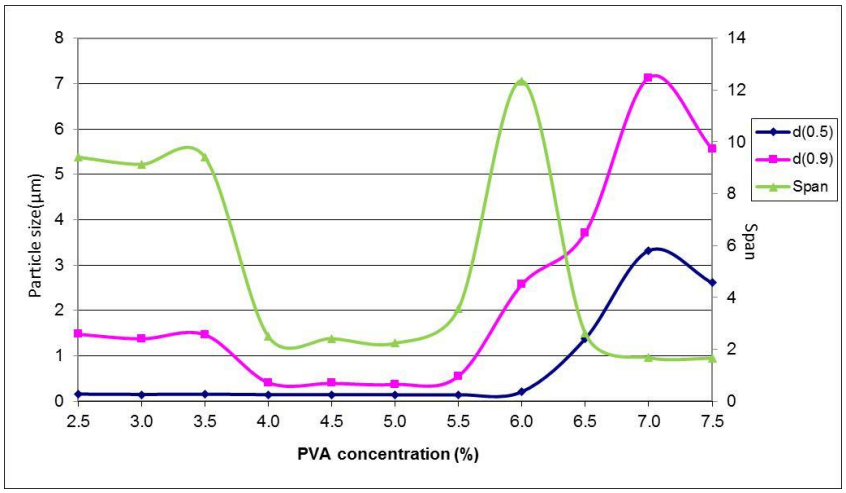

Figure 2. Particle size reduction effectiveness according to PVA concentrations and the Span values for the demonstration of particle size distribution (d(0.1) values are not shown)

Table 2. Zeta potential values of the diluted dispersions as a function of the PVA concentration and particle size distribution of Mel (±SD)

Samples	Particle size distribution			Zeta potential (mV)
	d(0.1)	d(0.5)	d(0.9)	
	Particle size (µm)			
Mel PVA 0%	2.508±1.1	5.762±2.7	135.640±12.9	-30.7
Mel PVA 0.25%	0.070±0.001	0.150±0.009	1.478±0.04	-20.9
Mel PVA 0.50%	0.067±0.001	0.130±0.005	0.371±0.01	-16.1
Mel PVA 0.75%	1.235±0.006	2.611±0.018	5.560±0.07	-15.7

The crystallinity degree of Mel measured by XRPD was also fundamentally influenced by the PVA content of the samples (Figure 3). It was established that low (2.5%) and high (7.5%) concentrations of PVA in the milled dispersion did not result in suitable milling efficiency. In this case, the crystallinity degree of Mel was 75.82% at low PVA content (2.5%), and it decreased to 51.44% at high concentration

of PVA (7.5%). These results are connected to the milling effectiveness. In this study, the 5.0% PVA-containing milled dispersions showed smaller crystallinity (13.43%) and the highest milling efficiency.

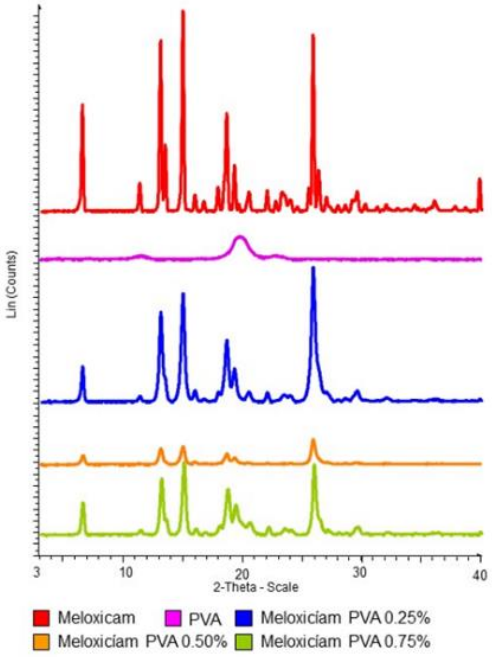

Figure 3. XRPD diffractograms of the Mel, PVA and the dried dispersion

Raman spectrograms and chemical maps of raw materials and products with different PVA content are presented in Figure 4 and Figure 5. The conclusion is as follows: the chemical map of dispersion profiled to Mel spectrogram shows homogenous distribution of Mel and there are no chemical degradation or interaction in dispersion which could detectable with Raman technique.

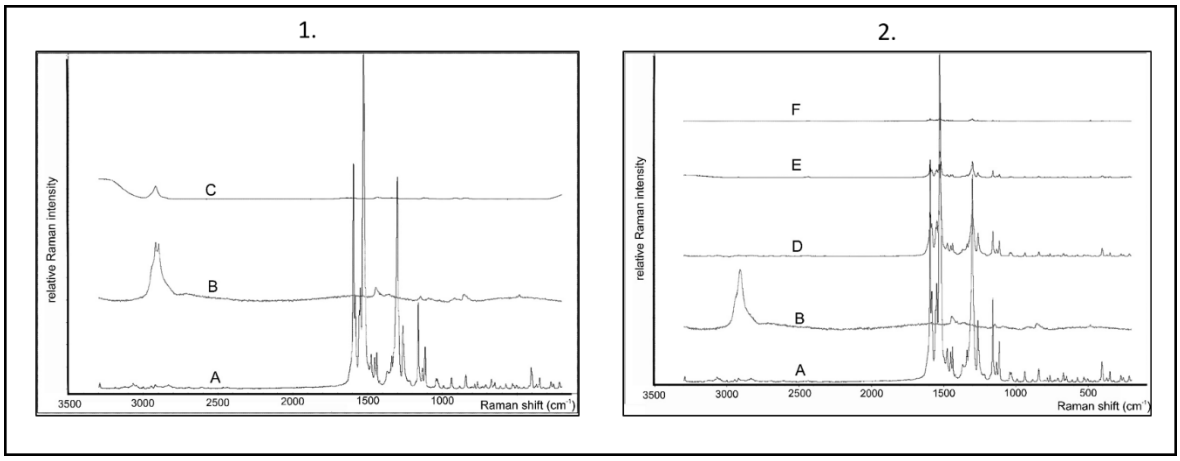

Figure 4. Investigation with Raman spectroscopy: 1. A: spectrum of raw Mel, B: spectrum of raw PVA, C: spectrum of raw PVA (0.50%) containing solution. 2. Comparison study of raw materials (Mel, PVA) and the dispersions, D: spectrum of dispersion containing 1% Mel and 0.25 % PVA, E: spectrum of dispersion containing 1% Mel and 0.50% PVA, F: spectrum of dispersion containing 1% Mel and 0.75% PVA

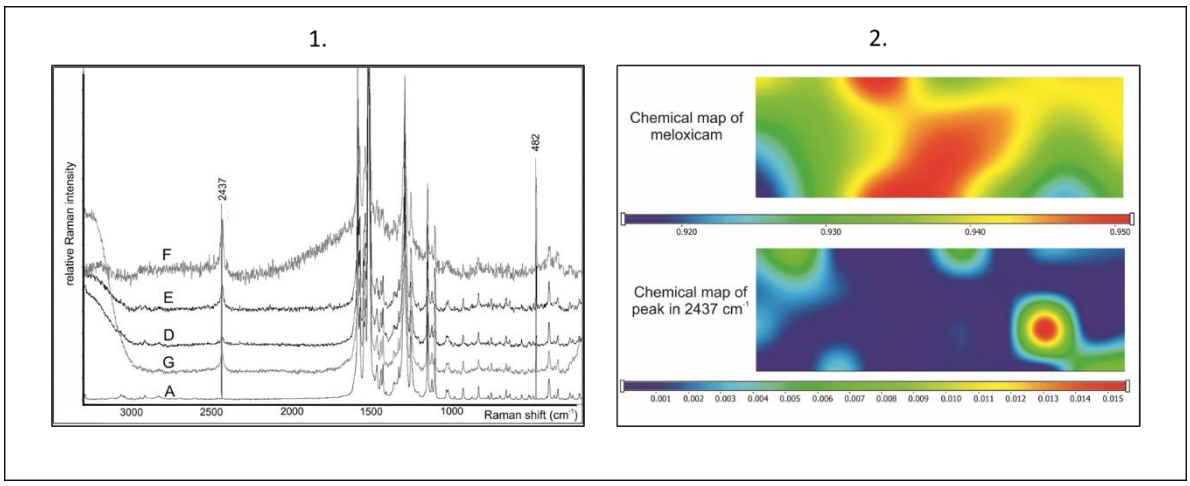

Figure 5. Investigation with Raman spectroscopy: 1. Comparing raw Mel and aqueous dispersion of Mel and PVA containing dispersions, A: spectrum of raw Mel, D: spectrum of dispersion containing 1% Mel and 0.25% PVA, E: spectrum of dispersion containing 1% Mel and 0.50% PVA, F: spectrum of dispersion containing 1% Mel and 0.75% PVA, G: spectrum of aqueous 1% Mel containing dispersion without PVA. 2. Chemical mapping of Mel containing dispersion (1% Mel and 0.50% PVA) and chemical mapping of its dried form profiled to peak in 2437 cm^{-1}

Finally, the sample (diluted dispersion) containing 1% Mel and 0.5% PVA produced by the optimized wet milling procedure fulfilled the requirements for the nanosuspension with respect to particle size

distribution (d(0.1) 0.067µm, d(0.5) 0.130µm, d(0.9) 0.371 µm). The intermediate product showed a stable system with 2 weeks of holding time.

iii. Perform in vitro dissolution test and cell culture studies to control the amount of PVA

In vitro dissolution tests have shown that the particle size of the Mel and its degree of crystallinity are interdependent critical parameters, which plays an important role in the fast drug release (Figure 6). The human Caco-2 cell culture studies justified that the penetration of Mel from different PVA-containing products was significantly increased as compared to Mel suspension without toxic effects (Figure 7). From all the tested samples, the permeability (P_{app}) value of Mel was the highest in the investigated sample containing 0.05% PVA, which belongs to the optimized nanosuspension (Figure 8).

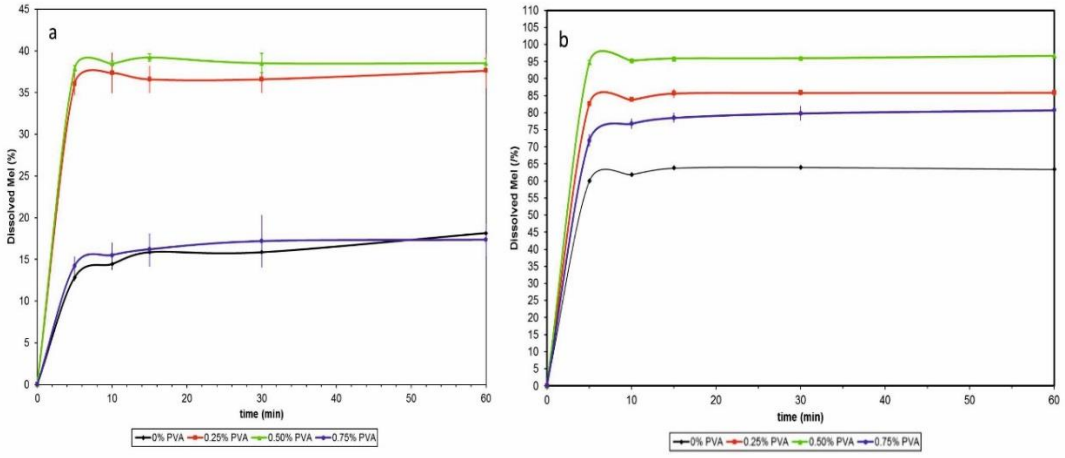

Figure 6. *In vitro* dissolution curves of Mel in artificial gastric juice (a) and intestinal juice (b)

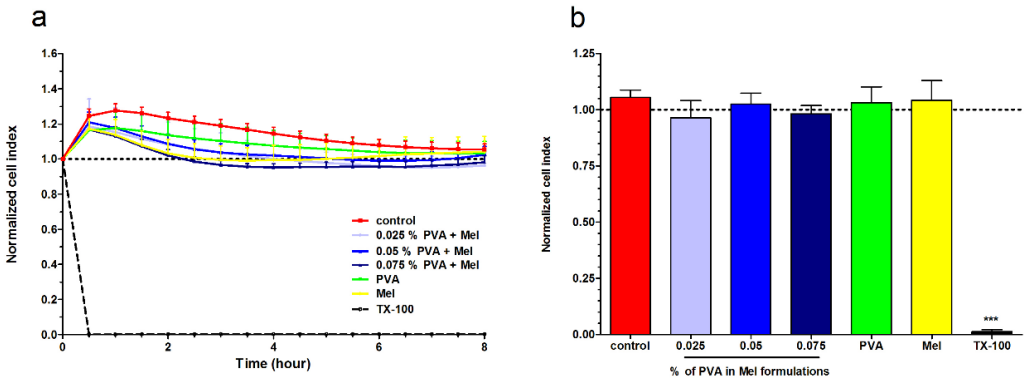

Figure 7. Cell viability kinetics (a) and results 8 hours after treatment (b) of Caco-2 intestinal epithelial cells with Mel, PVA and formulations measured by impedance.

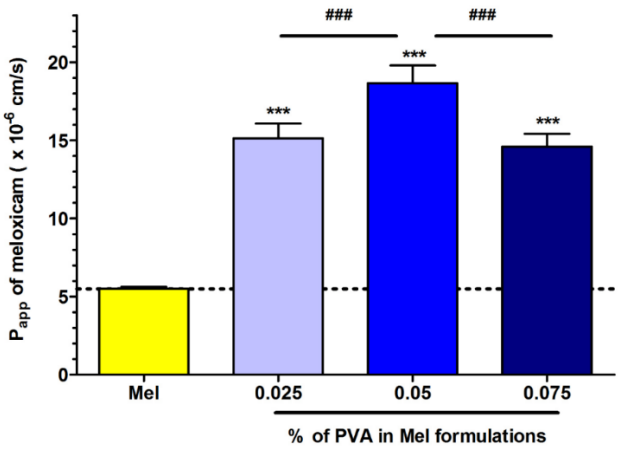

Figure 8. Evaluation of permeability of meloxicam across Caco-2 epithelial cell layers treated with Mel and optimized Mel-PVA formulations for 1 hour.

iv. Testing the robustness of combined wet milling process

The aim of this section was to discover the robustness of combined wet milling process to determine the interval of the Mel amount and to predict the degree of crystallinity of the milled samples as critical parameter using DSC and XRPD measurements. The samples had the PVA as the stabilizing agent. By increasing the amount of the Mel, its crystallinity increased and close correlation was found between the degree of crystallinity and the Mel amount. To achieve the desired particle size (< 500 nm), the Mel amount should be changed between 10.0 and 17.5% (w/w) and a PVA concentration should be used between 5.0 and 4.58% (w/w). In this specified range, the degree of crystallinity of Mel will be changed between 20 and 45%. The crystallinity of Mel investigated by DSC and XRPD did not show any significant difference at 95% significance level (Figure 9, 10).

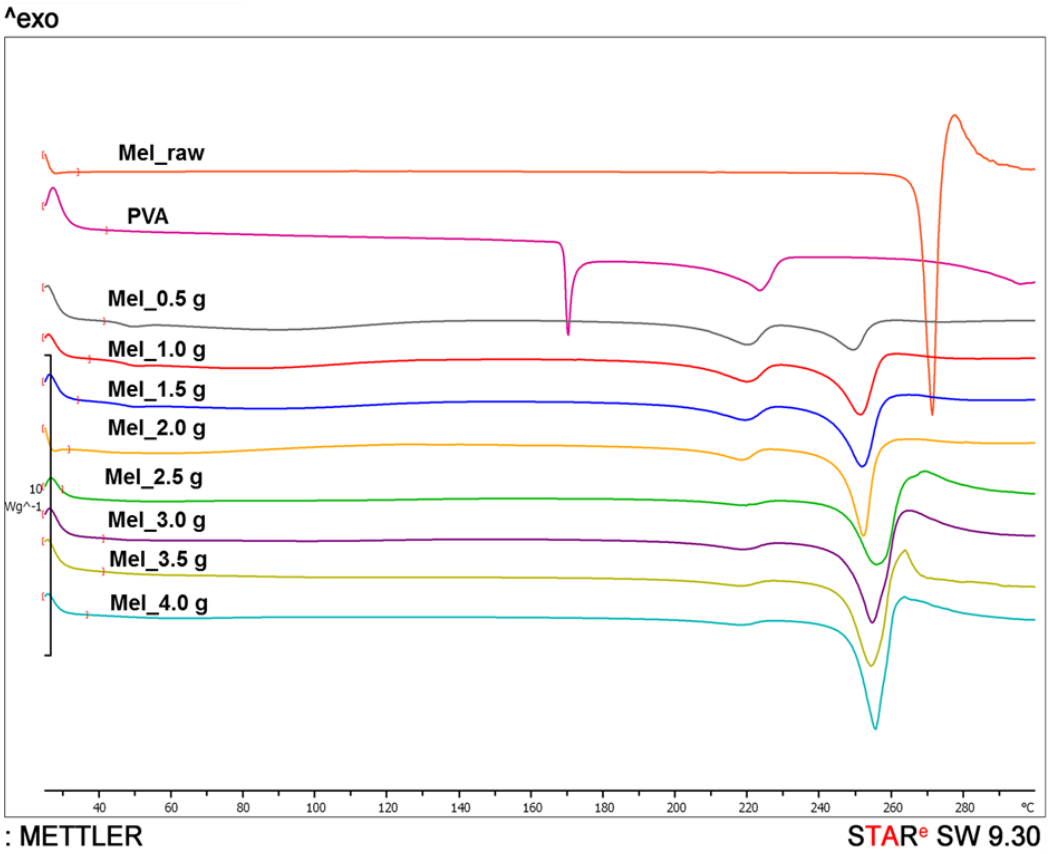

Figure 9. DSC curves of raw Mel, PVA and different Mel containing milled samples

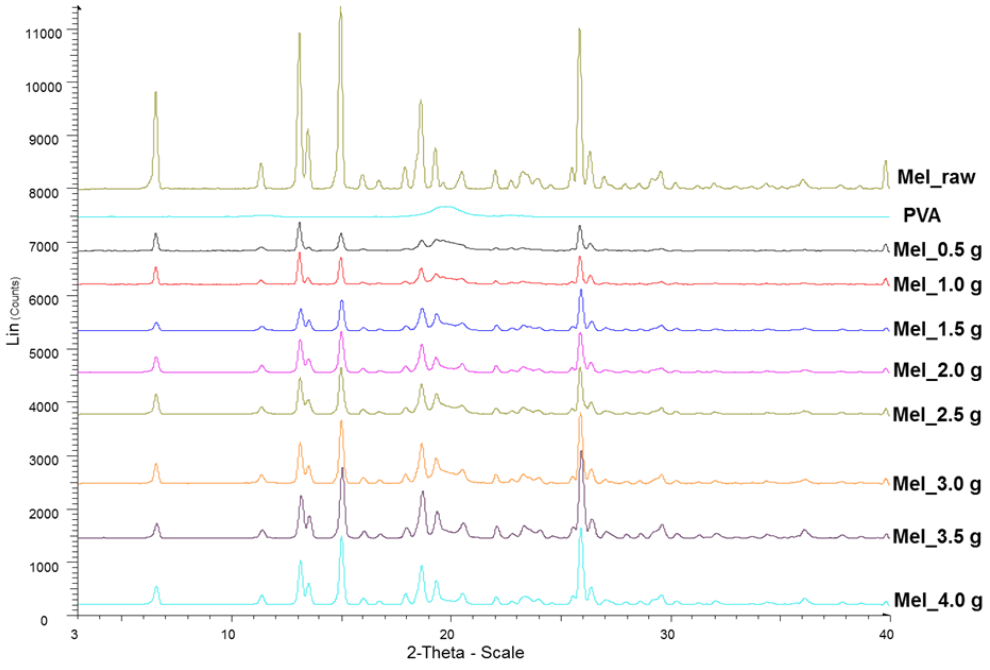

Figure 10. XRPD curves of raw Mel, PVA and different Mel containing milled samples

v. Transformation of the surfactant-free nanoMel sample into solid-state products by fluidization and lyophilization

Mel containing (1%) nanoMel sample with optimized amount of PVA (0.5%) resulted in 130.04 ±5 nm as mean particle size and a significant reduction in the degree of crystallinity (13.43%) of Mel. The fluidization technique using microcrystalline cellulose (MCC) as carrier resulted a quick conversion no significant change in the critical product parameters. Process of lyophilization required a longer operation time, which resulted in the amorphization of the crystalline carrier (trehalose) and the recrystallization of Mel increased its particle size and crystallinity. In accordance with this, the particle size (Z-average: from 284 nm to 374 nm) and the degree of crystallinity of Mel (to 36.54%) were also changed. The 6-month storage did not cause any further changes in the products (Figure 11, Table 3).

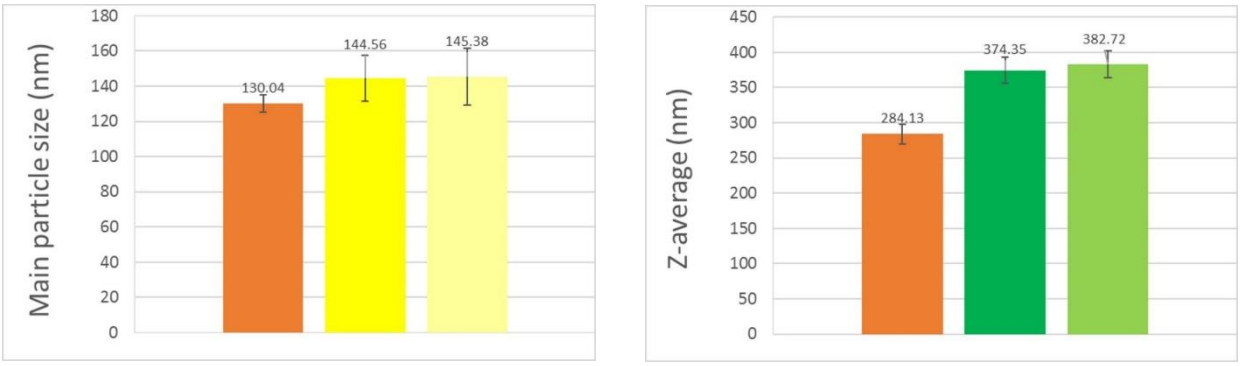

Figure 11. (from left to the right) Main particle size of nanoMel (measured by laser diffraction), fluidMel fresh and fluidMel stored (6 months) (measured by SEM images) and Z-average of nanoMel, lyoMel fresh, and lyoMel stored (6 months) measured by Zeta nano ZS)

Table 3. Enthalpy and calculated crystallinity values of the characteristic peak of Mel in the samples

Sample	Enthalpy (J/g)	Crystallinity of Mel (%)	Crystallinity of Mel after 6 months of storage (%)
nanoMel	12.24	13.43	-
fluidMel	11.83	12.98	13.02
lyoMel	36.54	40.11	40.16

vi. Bioavailability of the products

To justify applicability of surfactant-free samples containing nanonized Mel *in vivo* studies was performed. The nanonized Mel in solidified products (fluidMel, lyoMel) resulted in rapid absorption through the gastric membrane by passive transcellular transport. It was found that these products contained Mel in an adequate amount, and that the total amount thereof dissolved and absorbed. The fluidMel and lyoMel samples had nearly five-fold higher relative bioavailability than nanoMel application by oral administration. The correlation between in vitro and *in vivo* studies showed that the fixed Mel nanoparticles on the surface of solid carriers (MCC, trehalose) in both the artificial gastric juice and the stomach of the animals rapidly reached saturation concentration leading to faster dissolution and rapid absorption (Figure 12, Table 4, Figure 13).

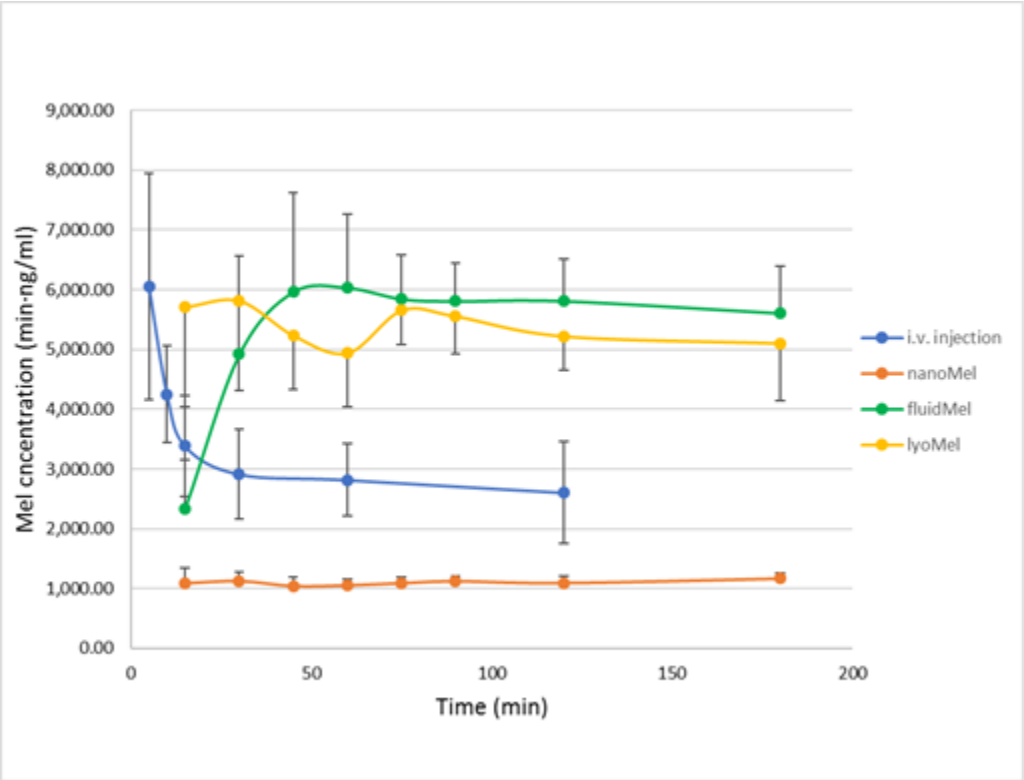

Figure 12. Plasma levels of MEL after the administration of different samples in rats. The preparations were administered orally (nanoMel, fluidMel and lyoMel) or intravenously (IV) as a single dose of 300 μg/kg.

Table 4. Plasma concentrations of Mel in time and its relative bioavailability in rats after IV and per os administration of Mel samples. Relative bioavailabilities were compared to nanoMel preparation

Sample	C_{15min} (nM)	C_{120min} (nM)	AUC_{blood} (min·ng/ml)	Relative bioavailability (%)
nanoMel	1,090.02	1,123.31	190,584.52	100.00
fluidMel	2,338.44	5,811.33	945,834.99	496.28
lyoMel	5,712.98	5,219.52	923,117.95	484.36
IV injection	C_{5min} 6,059.07	2,607.80	377,528.01	-

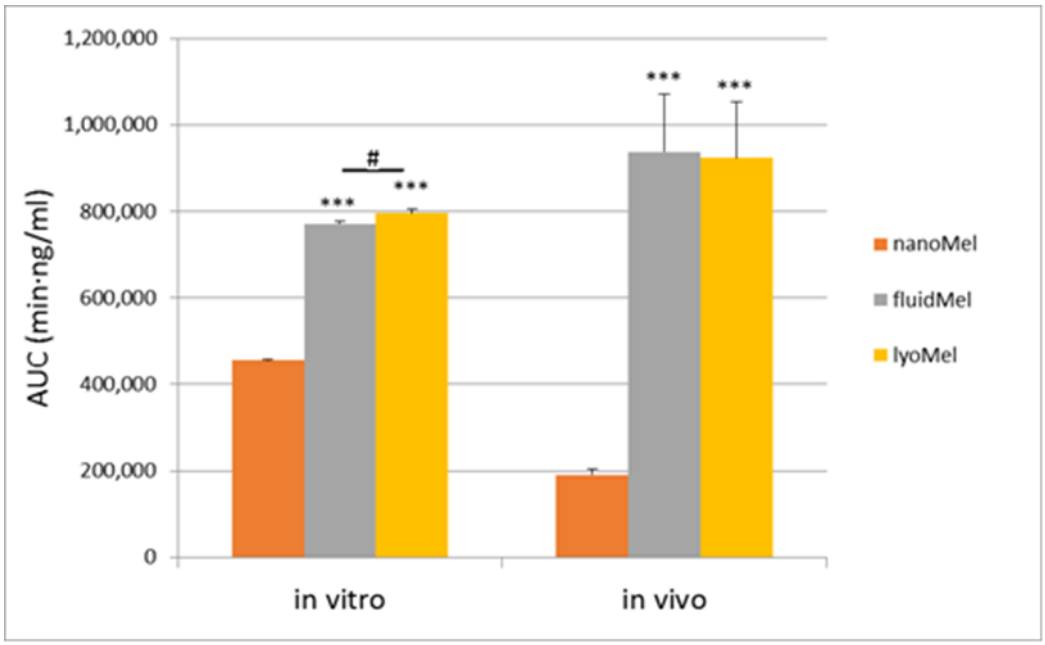

Figure 13. IVIV correlation of Mel containing samples. Notes: Values are presented as mean ± SD. Statistically significant differences are: ***p<0.001, compared to nanoMel separately in in vitro and *in vivo* groups; # p<0.05 compared to the indicated columns

New findings of this work:

Combined wet milling is considered to be a suitable process for nanonization of active ingredients, since it produces a desired particle size product in a single step without using pre-milling and surfactant (innovative technology). The novelty of the results is the determination and optimization of critical process and product parameters, which is well demonstrated by the production of Mel containing nanosuspension (nanoMel).

To discover the robustness of the milling process, it should also be considered that the amount of grinding media can be reduced by increasing the amount of the active ingredient and the crystallinity of the drug can be regulated. In this case, the DSC method can be suggested for the quantification of the degree of crystallinity because it can be used safely with high amorphous content.

The solidification of the nanosuspension not only increases the stability of the nanoparticles (particle size, crystallinity degree), but also allows the preparation of surfactant-free solid compositions (powder, tablet, capsule) with excellent bioavailability, which may be an important consideration for certain groups of patients (elderly and children) to achieve rapid effect. Further experiments are necessary to prove the therapeutic relevance of Mel containing formulations (innovative product).

PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

1. Bartos C, Szabó-Révész P, Bartos Cs, Katona G, Jójárt-Laczkovich O, Ambrus R. The effect of an optimized wet milling technology on the crystallinity, morphology and dissolution properties of micro- and nanonized meloxicam. Molecules 21 (2016) 507-518.

IF: 3.098 Q1

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