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Preparation of glyburide nanocrystals with improved dissolution properties by dry-ball- and wet-bead- milling: Systematic comparison by experimental design of the performance of the two methods

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ABSTRACT

The effectiveness of dry-ball and wet-bead nano-milling methods in producing nanocrystals of glyburide with increased dissolution rate has been compared. A full factorial design was applied to both methods to systematically evaluate the effect of the most critical factors (milling time, milling speed, ball/bead volume, drug amount) on the responses to be optimized (particle size and dissolution rate). Different experimental conditions were found to obtain the best results: the dry-method required to increase frequency and milling time and reduce ball volume and drug amount, while the wet-method to increase milling rate and drug amount and reduce bead volume and milling time. The results obtained under the respective optimal conditions evidenced a similar performance in nanocrystal production (120 and 180 nm for dry-ball and wet-bead milling, respectively) while a higher % dissolved at 10 min (28 vs 14 %) was found for the wet-method, principally ascribed to the presence, in this case, of the solubilizing polymer P188, added as stabilizer. Differently from the wet-method, the dry-method showed no direct relationship between particle size reduction and drug dissolution rate increase. Solid-state studies evidenced a role of the drug crystallinity loss, caused by the dry-milling, in affecting dissolution rate and proved the stability of the drug under both the milling processes.

1. Introduction

Irrespective of the administration route, dissolution is a compulsory requirement for a drug, so that it can be absorbed, and then it is a critical factor for its therapeutic efficacy. The poor drug aqueous solubility is in fact often associated to problems of erratic or low oral absorption, with consequent poor/variable bioavailability [1], thus representing an important obstacle to the development of therapeutically effective formulations [2]. Unfortunately, a large number of drugs are poorly water-soluble, being classified as class II or IV according to the Biopharmaceutics Classification System (BCS), and their number, in particular considering the new drug candidates coming through the industrial development pipeline, is constantly growing [3,4].

Therefore, significant efforts have been made in order to overcome this critical issue [5,6]. The main strategies explored with this aim include the use of: a) water-miscible co-solvents; b) complexation with cyclodextrins [7]; c) solid dispersions with hydrophilic carriers [8]; d) nanoemulsions or micro/nano self-emulsifying drug delivery systems [9, 10]; e) different kinds of colloidal nanovectors such as liposomes [11], polymeric micelles [12], lipid nanoparticles [13]. Another strategy is represented by chemical modifications of the drug molecule, either by synthesis of water-soluble prodrugs [14] or by salt formation [15,16]. This last approach is however applicable only to ionizable drugs; moreover, salt precipitation triggered by pH changes may occur during the gastrointestinal transit. A further type of strategy concerns modifications of the drug solid-state properties, obtaining amorphous systems [17], or metastable polymorphs [18,19], or micronized powders [20, 21].

Among these various techniques, particle size reduction by milling is considered a particularly attractive method for improving the drug

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dissolution properties. In fact, differently from the various formulation strategies above cited, it does not require (or in low concentrations) the use of particular excipients or carriers, thus avoiding the related problems of potential toxicity, limits in their useable amounts, etc., and it presents very little risks of unexpected and/or undesired changes of the drug chemical properties. Additionally, compared to the other techniques of solid-state modifications, it does not present the problems of poorly storage stability, typical of amorphous or metastable polymorphs. In fact, these "high-energy" systems spontaneously tend to transform into their corresponding thermodynamically more stable forms in a relatively short time [22,23], thus making it necessary to monitor possible changes during development, manufacturing and storage of the dosage form, in order to be able to guarantee the physicochemical stability of the drug during its full shelf life [24].

The favorable effect of micronization in improving dissolution rate and consequently bioavailability of different poorly soluble drugs has been proved [25–27]. However, in the case of very poorly soluble drugs, the increase in surface area obtained by micronization is not enough to give rise to an adequate improvement in dissolution rate. Thus, more effective techniques, able to reduce particle size to the sub-micron range, referred to as "nanonization", have been developed [28]. In the last two decades, nanosizing techniques have become a well-established approach for improving the performance of poorly-soluble drugs [29,30]. The reduction of particle size to nanometric dimensions more effectively facilitates drug solubilization, significantly increasing not only its dissolution rate, but also its saturation solubility, which is inversely related to the crystal size [31]. A loss of drug crystallinity, as a consequence of the nanonization process, has also to be considered: in fact, nanocrystals (as well as their physical properties) can be seen as intermediate between perfectly crystalline and completely amorphous solids [32].

It has been proved that the reduction of particle size to nanometric dimensions (below 1000 nm) increases the saturation solubility [33], and decreases the diffusion pathway, resulting in enhanced biopharmaceutical performance of hydrophobic drugs, and consequent improvement of their bioavailability and therapeutic efficacy [33,34]. Thus, nanocrystals emerged as an important tool, both at industrial and academic research levels, providing the possibility to overcome low drug solubility issues without requiring, unlike other nano-formulations such as polymeric or lipid nanoparticles, any special formulation changes or the use of special excipients (or only in low concentrations), thus allowing a faster and simpler development of many kinds of drug dosage forms for various administration routes and their ease authorization for commercialization [35].

Nano-reduction of drug particle size can be produced by bottom-up (controlled precipitation - solvent evaporation) or top-down (nanonization) approaches, where the second ones are the most widely used, due to their higher reproducibility and easier scalability; on the contrary, the first ones, despite their lower energy-requiring processes, are often limited by lower yield and poor reproducibility issues, as well as by the need to use organic solvents [36]. Among the different top-down nanonization techniques, media-milling is the most utilized one, being simple, rapid, eco-friendly, and easy to scale up [37].

Nanonization by media-milling involves breaking of drug macroparticles (mainly by pressure, impact, attrition and shearing forces) up to nanometer dimensions (<1 μm), giving rise to drug nanocrystals [38]. Milling can be performed in the dry state (dry-media-milling), or with the drug suspended in a suitable liquid medium (wet-media-milling), thus obtaining nanosuspensions. Despite the large use of both such kinds of nano-milling methods, at the best of our knowledge there are very few reports comparing these two techniques for their particle size reduction and drug solubility enhancement abilities [39], while they could be useful for a better understanding of their differences and helping in selecting the best methodology.

Based on these premises, in the present work, we considered it worthy of interest to compare the effectiveness of two commonly used dry- and wet-nano-milling methods (i.e. dry-ball milling and wet-bead milling) in reducing to nanometric dimensions and improving the dissolution properties of glyburide, a widely used oral hypoglycemic agent very poorly water-soluble. Since it is known that the effectiveness of every milling process is affected not only by the kind of apparatus used but also by the different process variables, in order to properly compare the performance of the two methods, it was first necessary to define their respective best experimental conditions. To find them, a statistical design of experiments (DoE) was initially applied to both milling methods aimed at investigating the effect of the most critical process parameters on drug particle size and dissolution rate, selected as the responses to be optimized. In fact, the use of DoE, allowing the simultaneous evaluation of multiple variables and the study of the interactions between them, proved to be a very suitable and valid approach for an efficient optimization of complex processes, including "nanomilling" [40]. A further objective of the work was to highlight eventual analogies and/or differences in factors determining the effectiveness of the two nanomilling methods and obtain more insight about the involved mechanisms of action.

2. Materials and methods

2.1. Materials

Glyburide (GLY) (also known as glibenclamide) was from Pura Quimica Laboratory (Cordoba, Argentina). Hydroxypropyl-methylcellulose (HPMC), poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (Kolliphor P188) (P188), sodium lauryl sulphate (SLS) were from Sigma Aldrich (St. Louis, MO, USA). Milli-Q® quality ultrapure water was used for the wet milling. All other chemicals and solvents were of analytical grade.

2.2. Experimental design

The software MODDE-GO® version 12.01 (Umetrics, Malmö, Sweden) was used for generation of the statistical experimental design and evaluation of the results obtained with the two grinding methods. A full factorial design with four factors at two-levels ($2^4 + 3$ central points) was employed to screen the most important process variables and their possible interactions. Analysis of variance (ANOVA) and multiple linear regression analysis (MLRA) were used, respectively, to test the model significance and validity and to evaluate the relations between dependent and independent variables.

2.3. Dry-ball milling method

GLY nanocrystals (NCs) were prepared by dry-ball milling in a high energy vibrational mill (Mixer Mill Type MM 200, Retsch, GmbH, Düsseldorf, Germany). Different amounts of GLY (100–500 mg) were ground for different times (30–90 min) and different vibration frequencies (12–24 Hz) within stainless steel grinding jars (volume 12 cm^3) containing different amounts of steel balls of 12 mm in diameter (25–75 % v/v of the jar volume).

2.4. Wet-bead milling method

GLY nanocrystals (NCs) were prepared as nanosuspensions in aqueous solution by wet-bead milling using a NanoDisp® laboratory-scale mill (NanoDisp®, Córdoba, Argentina) following a previously developed procedure slightly modified [34,41]. The apparatus consists in a grinding chamber and a shaft connected with a variable speed motor. Different amounts of GLY (as 3:1 w/w physical mixture with P188) were firstly ground in a mortar and ultrapure water was progressively added up to 100 mL to obtain suspensions with GLY contents of 3–6 % w/v. P188 was added as a stabilizer, since nanosuspensions tend to be physically unstable, due to thermodynamic and kinetic interactions, with consequent increase in particle size [34,41–43]. The suspension was put in a beaker, magnetically stirred for 10 min and then put in the milling chamber together with different amounts (25–50 % v/v) of zirconia beads (SiLi beads Typ ZS®, diameter 0.1–1.2 mm) and processed for different times (60–120 min) at different milling rates (800–1600 rpm).

2.5. Determination of nanocrystals (NCs) size

GLY NCs dimensions were determined by Dynamic Light Scattering using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). Determinations were made at 25 °C. NCs samples obtained by dry-ball milling, before analysis, were suspended in an ultrapure aqueous solution containing HPMC 1 % w/v and SLS 0.2 % w/v, added as dispersing agents, to obtain a homogeneous suspension [25,27,39,44].

The HPMC-SLS solution was pre-saturated with GLY, to avoid possible drug dissolution phenomena that could affect the particle size results. All measurements were performed in triplicate.

NCs suspensions obtained by wet-bead milling were properly diluted with ultrapure water before measurements to avoid multi-scattering phenomena. All measurements were performed in triplicate.

2.6. Dissolution studies

Dissolution experiments were carried out according to the dispersed amount method [45–48] that is widely used in pre-formulation studies. Samples containing 30 mg of drug were added to 75 mL of water in a 150 mL beaker at 25 °C. A vertical glass agitator shaft with three blades (19 mm diameter) was centrally immersed in the beaker at 20 mm from bottom and rotated at 100 rpm. Aliquots (3 mL) were withdrawn after 10 min with a syringe-filter (pore size 0.10 μm) and UV assayed for drug content at 302.0 nm. The % drug dissolved was determined with a syringe-filter (pore size 0.10 μm) and UV assayed for drug content at 302.0 nm. The % drug dissolved at 10 min was then determined and assumed as a parameter indicative of the drug dissolution rate to use as response in the experimental plan matrix. The test was performed in triplicate.

2.7. Production of solid NCs by spray-drying of nano-suspensions (NSs)

NSs obtained by wet-nanomilling were subjected to a spray-drying process in order to obtain solid NCs, according to a previously described procedure slightly modified [34]. Briefly, NSs were processed using a mini spray-dryer Büchi B-290 TM (Büchi Labortechnik AG, Flawil, Switzerland) equipped with a dehumidifier module, under the following experimental conditions: atomizing air flow: 50 L/h; aspiration: 75 m^3/h; inlet temperature: 45 °C; pump speed: 5 mL/min. The obtained powders were stored in dry conditions for solid-state characterization. The redispersion capacity of the GLY-NCs spray-dried powders was assayed in terms of particle size and redispersion time.

2.8. Solid-state characterization

The solid-state properties of GLY as such and of selected GLY NCs samples obtained by the two milling methods were characterized by the following techniques.

2.8.1. Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) analysis was performed with Discovery DSC 25P instrument (TA Instruments, New Castle, DE, USA) on 5–10 mg samples (Mettler M3 Microbalance, Mettler Toledo, Switzerland) scanned in pierced Al pans at 10 °C/min between 30 and 200 °C under N_2 flow (50 mL/min). The instrument was calibrated using Indium as a standard (99.98 % purity; melting point 156.61 °C; fusion enthalpy 28.71 J g^{-1}). All measurements were performed in triplicate (R.S.D. of crystallinity data about ±5 %). The residual crystallinity of GLY in the different samples, expressed as relative degree of crystallinity (RDC%), was determined using the following equation [46,53]:

$$RDC = \frac{\Delta H_{sample}}{\Delta H_{drug}} x\ 100 \qquad \text{(Eq. 1)}$$

where ΔH_{sample} and ΔH_{drug} are the fusion heat of treated and untreated GLY, respectively.

2.8.2. Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) spectra were recorded with a Cary 630 Transform Infrared spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Samples were scanned between 600 and 4000 cm^{-1}.

2.8.3. X-ray powder diffractometry

X-ray Powder Diffraction (XRPD) spectra were obtained with a Panalytical X-Pert ProVR apparatus (Malvern Panalytical B.V., Almelo, The Netherlands) under the following experimental conditions: Cu Kα radiation; scan range 5–50° 2θ; step size 0.04°/s.

2.8.4. Scanning electron microscopy and Energy Dispersive X-ray spectroscopy

Scanning electron microscopy (SEM) (FE-SEM Sigma, ZEISS Instrument, Oberkochen, Germany) with an acceleration voltage of 3–20 kV was used to examine the surface morphology of selected GLY NCs samples and of pure untreated GLY. The powdered samples were placed on aluminum stubs and subjected to an Au sputtering process before being examined. Magnification ranges between 100x and 20,000x were used to examine the morphology of the particles.

Energy Dispersive X-ray Spectroscopy (EDXS) was used in conjunction with SEM to determine the distribution of the chemical elements in the GLY NCs sample obtained by the wet-milling method, due to the presence of P-188 as stabilizer.

3. Results

3.1. Design of experiments

A statistical experimental design strategy was initially applied to both the dry- and wet-milling methods, in order to perform a systematic investigation of the effect of variations of the experimental conditions on the drug particle size and dissolution rate, and find the respective best conditions, thus allowing a correct comparison of their performance. Ball/bead volume, drug amount, milling speed and milling time were selected as the factors, since they have been identified as the potentially most critical process variables [49,50,51].

A four factors-two levels full factorial design was selected as a screening tool to evaluate the actual influence of the individuated variables on the responses, and to investigate if (and how) the effect of each variable is affected by the other ones. For each milling method, the low and high values of the considered independent variables (i.e. their experimental domain) were fixed in the light of experience previously acquired by the Authors from their respective applications [34,41,46–48]. The amphiphilic polymer P188 was added as stabilizer in the wet-milling process according to previous experiences of the authors [34,41].

The independent variables and their respective levels used for the full-factorial design are presented in Table 1.

Drug powder samples, obtained by both dry-ball- and wet-bead-milling methods according to the experimental conditions indicated by the respective experimental plan matrix, were then evaluated for mean particle size and % drug dissolved after 10 min (this last selected as an index of the drug dissolution rate) (Tables 2 and 3, respectively). For each series of studies, experiments were performed in a randomized order.

Table 1
Screening variables and their levels used for the full-factorial design.

a) Dry-ball milling method			
Variable n.	Independent variables	Levels	
		low	high
V_1	steel balls volume (% v/v)	25	75
V_2	drug amount (mg)	100	500
V_3	milling frequency (Hz)	12	24
V_4	milling time (min)	30	90
b) Wet-bead milling method			
Variable n.	Independent variables	Levels	
		low	high
V_1	beads volume (%v/v)	25	50
V_2	drug in suspension (%w/v)	3	6
V_3	milling rate (rpm)	800	1600
V_4	milling time (min)	60	120

Table 2
Experimental plan and obtained responses for the dry-ball- milling method.

Exp. code n.	V_1 balls volume (%v/v)	V_2 GLY (mg)	V_3 milling rate (Hz)	V_4 milling time (min)	nanoparticle size (nm)	% GLY diss. at 10 min
1	25	100	12	30	206	3.92
2	75	100	12	30	238	7.77
3	25	500	12	30	675	7.97
4	75	500	12	30	285	2.30
5	25	100	24	30	793	10.02
6	75	100	24	30	461	7.63
7	25	500	24	30	598	12.27
8	75	500	24	30	610	11.67
9	25	100	12	90	160	3.53
10	75	100	12	90	283	2.96
11	25	500	12	90	80	1.88
12	75	500	12	90	463	1.15
13	25	100	24	90	120	14.05
14	75	100	24	90	153	13.94
15	25	500	24	90	142	5.34
16	75	500	24	90	165	3.75
17	50	300	18	60	248	2.19
18	50	300	18	60	304	1.94
19	50	300	18	60	245	2.23

Table 3
Experimental plan and obtained responses for the wet-bead- milling method.

Exp. code n.	V_1 beads volume (%v/v)	V_2 GLY (%w/v)	V_3 milling rate (rpm)	V_4 milling time (min)	nanoparticle size (nm)	% GLY diss. at 10 min
1	25	3	800	60	500	2.51
2	50	3	800	60	250	12.08
3	25	6	800	60	432	1.00
4	50	6	800	60	290	6.50
5	25	3	1600	60	201	20.13
6	50	3	1600	60	166	7.04
7	25	6	1600	60	184	28.44
8	50	6	1600	60	220	18.12
9	25	3	800	120	299	8.05
10	50	3	800	120	211	21.14
11	25	6	800	120	373	3.02
12	50	6	800	120	210	19.63
13	25	3	1600	120	197	24.16
14	50	3	1600	120	167	7.04
15	25	6	1600	120	196	25.16
16	50	6	1600	120	163	6.54
17	37.5	4.5	1200	90	217	16.25
18	37.5	4.5	1200	90	217	17.61
19	37.5	4.5	1200	90	227	18.62

3.2. Evaluation of statistical significance and validity of the model

MLRA was applied to evaluate the relations between independent variables and responses. ANOVA analysis of the experimental results indicated that the assumed regression model was statistically significant ($p < 0.05$) and valid for both the responses for both milling methods.

The summary of fit plots, which is a graphical summary of the statistical key parameters of the experimental design, was firstly exploited to obtain insight regarding the strength and the robustness of the model. The key parameters considered were: R^2, representing the % of response variation actually explained by the model, whose ideal value is 1; Q^2, representing the % of response variation predicted by the model, whose value should be ideally higher than 0.5 for an optimal predictability; Model validity, whose value should be higher than 0.25 to exclude statistically significant problems of the model; Reproducibility, which should be higher than 0.5.

The results related to the dry-ball milling method are shown in Fig. 1A. Concerning the response “nanoparticle size”, the very high values obtained for both R^2 (0.99) and Q^2 (0.92) indicated a highly significant model fit, and a high prediction precision. Moreover, the applied model showed an optimal validity (0.98) and reproducibility (0.91). The applied model showed a high fit ($R^2 = 0.93$), good validity (0.82) and high reproducibility (0.86) also for the response “% dissolved drug”, thus confirming the validity of the model. A lower, even though still satisfactory precision for predictive ability was however obtained ($Q^2 = 0.49$).

The goodness of the fit of the selected statistical model was further supported by the plots of the observed-vs-predicted responses (Fig. 1B). As can be seen, for both the responses, a substantially good agreement was found between values experimentally obtained and those predicted by the model, confirming the adequacy of the model and its good predictive ability. Furthermore, the high R^2 value (0.987) obtained for the nanoparticle size response confirmed the very good fit of raw data to the regression model. A good R^2 value (0.935) was found also for the % dissolved drug.

The corresponding results obtained for the wet-bead milling method are shown in Fig. 2. As can be seen in Fig. 2A, the model resulted quite perfect for both responses in terms of fit and descriptive ability, as proved the very high R^2 values, very close to 1 (0.99 and 0.90 for “nanoparticle size” and “% dissolved drug” response, respectively), and showed an optimal reproducibility (0.98). In the case of the “nanoparticle size” response, high Q^2 value (0.84), indicative of a very good model in terms of predictive capacity, and very good validity (0.74) were also obtained. On the contrary, the “% dissolved drug” response showed less precision for predictability ($Q^2 = 0.32$) and model validity (0.31), although remaining within acceptable limits (>0.25) thus indicating the absence of statistically significant problems of the model.

The plots of the observed-vs-predicted responses (Fig. 2B) showed that in the case of nanoparticle size, almost all the experimental data were on a straight line, very close to the predicted data, indicating an ideal result; additionally, as observed for the other milling method, the optimum R^2 value (0.988) pointed out also in this case a really good fit of experimental data to the regression model. A greater dispersion of experimental data vs the predicted ones was instead observed in the case of the % dissolved drug response, as shown by the lower, even though still satisfactory, R^2 value (0.899).

3.3. Evaluation of the effects of the variables on the responses

Plots of coefficients were utilized to evaluate the actual contribution of each single variable to the considered responses. In such plots a factor is considered as significantly affecting the response if its related confidence interval does not cross the horizontal axis.

The plots of coefficients for “nanoparticle size” and “% dissolved drug” responses obtained for the dry-ball milling method are reported in Fig. 3.

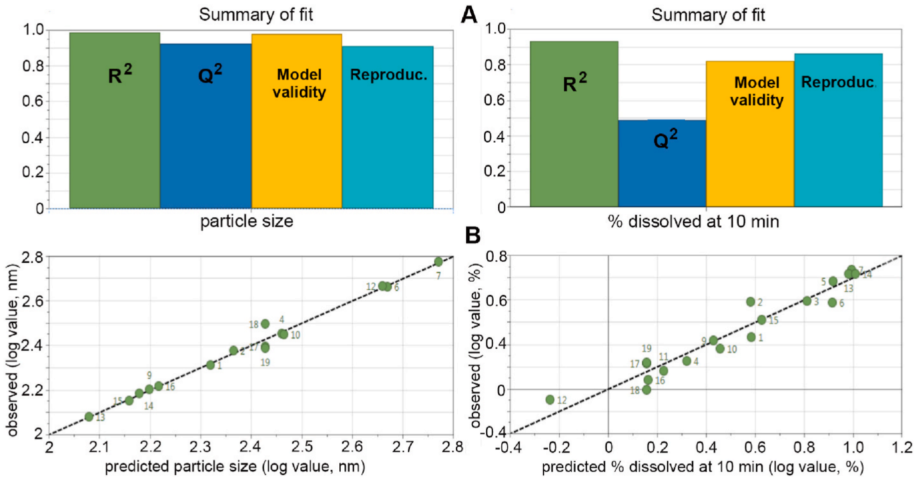

Fig. 1. Summary of fit plots (A) and observed vs predicted plots (B) for the responses nanoparticle size (left) and % dissolved drug (right) obtained with the dry-ball milling method.

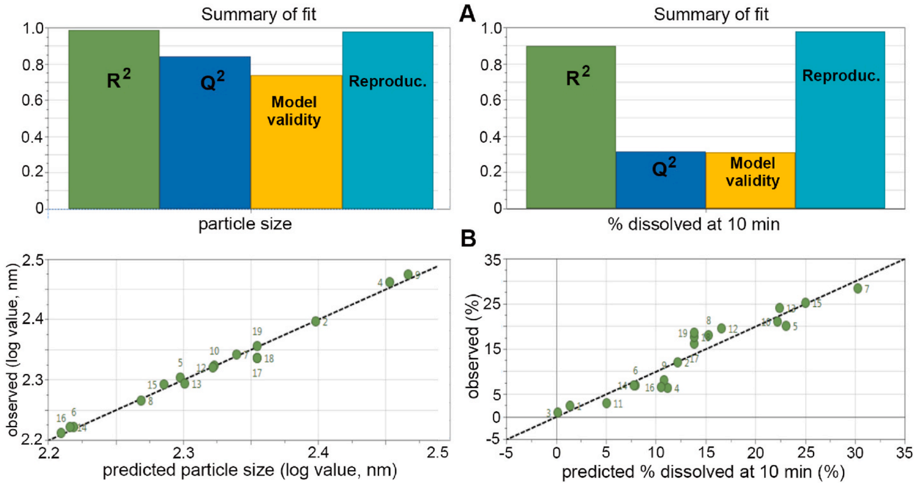

Fig. 2. Summary of fit plots (A) and observed vs predicted plots (B) for the responses nanoparticle size (left) and % dissolved drug (right) obtained with the wet-bead milling method.

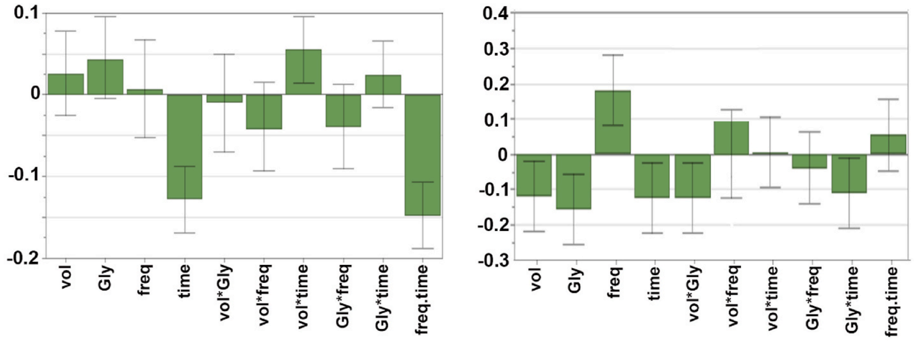

Fig. 3. Plots of coefficients for “nanoparticle size” (left) and “% dissolved drug” (right) responses for the dry-ball milling method (Key: Vol = V_1; GL_Y= V_2; freq = V_3; time = V_4).

As can be seen, in this case the most significant variable which favorably affected the nanoparticle size in terms of size reduction (Fig. 3, left) was the milling time (V_4). Beneficial effects were shown also by the combination of variables milling frequency*milling time (V_3*V_4). Probably the interaction between milling frequency and time variables was fruitful in promoting a greater mechanical energy transfer to the powder particles, which are trapped between colliding milling media or between mill walls and walls-impacting milling media, thus resulting in a more effective nano-comminution process [54].

On the other hand, the only parameter which had a significantly unfavorable effect on the response (in terms of increase in particle size) was the combination of variables V_1*V_4 (ball volume*milling time). It can be reasonably hypothesized that an excessive increase in balls volume decreased their impact effectiveness, as a consequence of the reduced empty volume within the milling chamber. An adverse effect, even though not statistically significant, was observed also for the V_2 variable (GLY amount).

The parameters which exhibited a significantly adverse effect on the "% dissolved drug" response (Fig. 3, right), were instead ball volume (V_1), GLY amount (V_2) and milling time (V_4), as well as the combinations of the variables ball volume*GLY amount (V_1*V_2) and GLY amount*milling time (V_2*V_4). The unfavorable effect of the milling time could be due to the formation of electrostatic charges on the particles surface, as a consequence of the prolonged milling process, responsible for a reduction of the powder wettability, and, consequently, of its dissolution rate. On the contrary, the only variable which had a significant positive impact in improving the drug dissolution rate was the V_3 variable (milling frequency). Probably an increase in milling frequency promoted drug dissolution facilitating the formation of a "mechanochemically activated" state [20,31].

The plots of coefficients for "nanoparticle size" and "% dissolved drug" responses in the case of the wet-bead milling method are reported in Fig. 4.

As can be observed, the most significant variable which favorably affected the nanoparticle size in terms of size reduction was V_3 (milling rate), followed by V_1 (beads volume) and V_4 (milling time). A beneficial effect was shown also by the combination of variables V_2*V_4 (GLY amount*milling time). On the contrary, the only parameter which had a significantly unfavorable effect on the response (in terms of increase in particle size) was the combination of variables V_3*V_4 (milling rate *milling time). Some unfavorable effect, even though not statistically significant, was found for the GLY amount (V_2) and the combination of variables V_1*V_3 (bead volume*frequency time).

Concerning the "% dissolved drug", the factor which had the most significant adverse effect on this response, causing its reduction, was the combination of variables beads volume*milling rate (V_1*V_3), followed by the combination V_3*V_4 (milling rate *milling time). On the other hand, the only parameter showing a significantly positive effect on this response was the milling rate (V_3). It could be assumed that, similarly to the dry-ball milling method, also in the case of the wet-bead milling method the increase in milling rate promotes a more effective transfer of mechanical energy to the system, and then the formation of an activated state of the drug, more prone to quickly dissolve. An advantageous effect, event though not statistically significant, was observed also for the drug amount* milling rate (V_2*V_3) combination.

3.4. Screening of the most significant variables for each milling method

3.4.1. Dry-ball milling method

The variables V_1 (volume of balls) and V_2 (GLY amount) did not had a statistically significant influence on the particle size response, while they had an adverse effect on the % dissolved drug. On the contrary, the variable V_3 (milling frequency) did not affect nanoparticle size, while favorably increased % dissolved drug, and the variable V_4 (milling time), which favorably decreased nanoparticle size, adversely affected the % dissolved drug.

As for the effects of variables combinations, V_3*V_4 was the only one that beneficially affected nanoparticle size, while it did not have effect on the drug dissolution rate. Instead the combinations V_1*V_2 and V_2*V_4 adversely influenced drug dissolution rate but did not affect nanoparticle size. On the contrary, the combination V_1*V_4 showed a negative effect on the particle size reduction, but did not influence the % drug dissolved.

In summary, the increase in milling frequency (variable V_3) favorably affected drug dissolution rate, without effects on particle size, while a compromise value should be found for milling time (variable V_4), whose increase was beneficial for size reduction but unfavorable for dissolution rate increase. Moreover, the lower level of variables V_1 (ball volume) and V_2 (GLY amount) should be selected to reduce their negative effect on the % dissolved drug.

Application of the desirability function substantially confirmed the above reported findings, indicating that in the case of the dry-ball milling method, the best results for the simultaneous optimization of both the responses should be obtained by setting the variables V_3 and V_4 at their highest levels, while keeping the variables V_1 and V_2 at their lowest levels. In such a way, the low levels of variables V_1 and V_2 should also reduce the negative effects of their interaction (V_1*V_2) and of the V_2*V_4 combination on the drug dissolution rate.

3.4.2. Wet-bead milling method

The variable V_3 (milling rate) was the only one that had a beneficial influence on both responses, while the variables V_1 (volume of beads) and V_4 (milling time) favorably influenced nanoparticle size, but did not have any statistically significant effect on drug dissolution rate, and V_2 (GLY amount) did not have any effect on both the responses.

Regarding the effects due to variables combinations, V_1*V_2, V_1*V_4 and V_2*V_3 did not influence both the responses, which, on the contrary, were both negatively affected by V_1*V_3 and V_3*V_4 combinations. Finally, V_2*V_4 was the only combination showing a favorable effect on nanoparticle size without affecting % dissolved drug. No important effects of GLY amount (variable V_2) were instead observed.

The desirability function in the case of wet-bead milling method suggested that the best conditions to optimize at the same time both the responses should be obtained by setting the variables V_1 and V_4 at their lowest level (25 % and 60 min, respectively), and the variables V_2 and V_3 at their highest level (6 % and 1600 rpm, respectively). In such a way it should be possible to better exploit the favorable effects of the milling rate (variable V_3) on both the responses, by minimizing the negative effects due to its interactions with the variables V_1 and V_4.

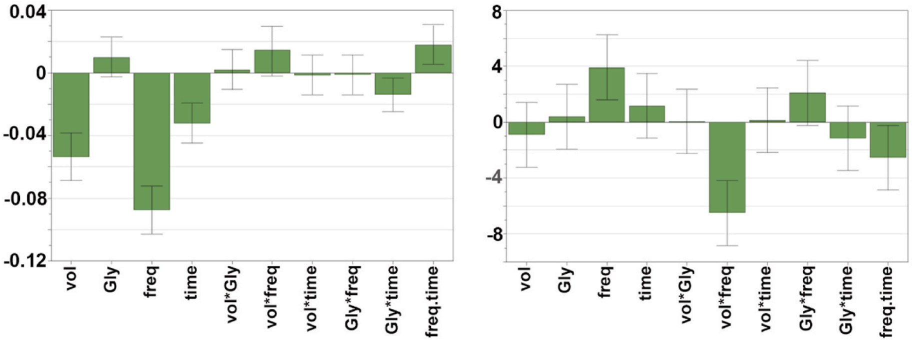

Fig. 4. Plots of coefficients for "nanoparticle size" (left) and "% drug dissolved" (right) responses for the wet-bead milling method (Key: Vol = V_1; GL_Y= V_2; freq = V_3; time = V_4).

3.5. Comparison of the performance of dry-ball and wet-bead nano-milling methods

Both methods were effective in reducing the starting micronized GLY powder (size 1.7–2.0 μm) to nanometric dimensions, giving rise, depending on the different experimental conditions used, to nanoparticles ranged from 800 up to 80 nm or from 500 up to 160 nm in the case of the dry-ball method or of the wet-bead method, respectively (see Tables 2 and 3). In particular, the mean powder dimensions obtained under the optimized conditions were around 120 and 180 nm for the dry and the wet method, respectively.

However, interestingly, as can be observed in Fig. 5, while in the case of the wet-milling method, an exponential increase of the drug dissolution rate (as % dissolved at 10 min) as a function of the particle size reduction was found (except for 3 and 1 outlier points at the lowest and highest particle size values, respectively), no relation was observed in the case of the dry-milling method.

In fact, as it is also evident by examining the data reported in Tables 2 and in the case of the dry-method the use of some experimental milling conditions, while always giving rise to a more or less intense particle size reduction, resulted sometimes in a worsening or at least in a minimal increase of the drug dissolution rate compared to that of the starting GLY powder (1.8 % dissolved after 10 min).

On the other hand, it is important to underline that the exponential relationship found for the wet-method was not followed by the lowest particle size values, lower than 170 nm, for which they would have observed the highest dissolution rate values.

These results suggested that, particularly in the case of the dry-method, the dissolution rate of the hydrophobic drug was also affected by other phenomena occurring during the process, including, for example, formation of electrostatic charges on the particles surface, changes in nanocrystal morphology (shape and surface characteristics) and loss of crystallinity. Evidently, the occurrence of these additional phenomena and their related relevance were influenced by the experimental conditions used for the milling process. Then, in order to obtain some more insight about the other possible factors concurring to affect the drug dissolution rate as a consequence of the dry-milling process, such as in particular the drug crystallinity variation, careful solid state-studies for the characterization of the obtained powders have been performed (see section 3.6).

Anyway, independently from their reasons, these findings highlighted the complexity of the process itself and proved the need of its proper optimization by the use of experimental design methodologies, in order to be able to exploit at the best its potential.

Moreover, the effects of the examined variables and of their combinations on the considered responses were different for the two methods, and, consequently, different experimental conditions have to be used to optimize the responses. This upshot further confirmed the complexity of the mechanisms operating during the milling process, as well as of their implications on the performance of the final product.

On the other hand, as for as the higher value of % drug dissolved at 10 min obtained under the optimized conditions with the wet-method (28.4 %) than with the dry-method (14.0 %), it has most likely to be attributed to the presence in the first case of P-188 (necessarily added as stabilizer in a 3:1 w/w drug:polymer ratio), considering its known solubilizing, dispersing and wetting properties. Then, to support this hypothesis, an analogous 3:1 w/w physical mixture of GLY with P-188 was subjected to the dry-ball milling under the optimal conditions found for this method. The obtained nanonized powder actually showed a clear improvement in GLY dissolution rate with respect to the sample ground under the same conditions in the absence of the polymer, reaching about 26 % of dissolved drug after 10 min. Therefore, based on the overall findings, it can be stated that the dry-method allowed to obtain nanocrystals of drug alone, and also made it possible, if considered opportune, the addition of a solubilizing polymer during the grinding process.

3.6. Solid-state characterization of the solid NCs obtained by the two methods

The absence of a direct relation between the drug particle size reduction and its dissolution rate increase, as emerged in particular in the case of the dry-milling method, required a thorough characterization of the solid-state properties of the obtained GLY nano-sized powders, for a better understanding of the results.

Moreover, for a more comprehensive comparison of the performance of the two nano-milling methods, it was considered interesting to investigate also the solid-state properties of the powders obtained by the wet-bead milling method. In the latter case, in order to be able to perform solid-state studies, the selected GLY samples obtained as nanosuspensions were dried by spray-drying. It must also be considered that this last step was in any case necessary to overcome the problems of physical instability of nanosuspensions [42,43] and to enable the formulation of solid dosage forms [55].

3.6.1. Differential Scanning Calorimetry (DSC) studies

The thermal curves of the starting GLY powder and of some representative GLY NCs samples obtained by dry-ball milling (samples n. 1, 13 and 14 of Table 2) or wet-bead milling (samples n. 1, 7 and 8 of Table 3) are presented in Fig. 6, while the corresponding thermal data are reported in Table 4.

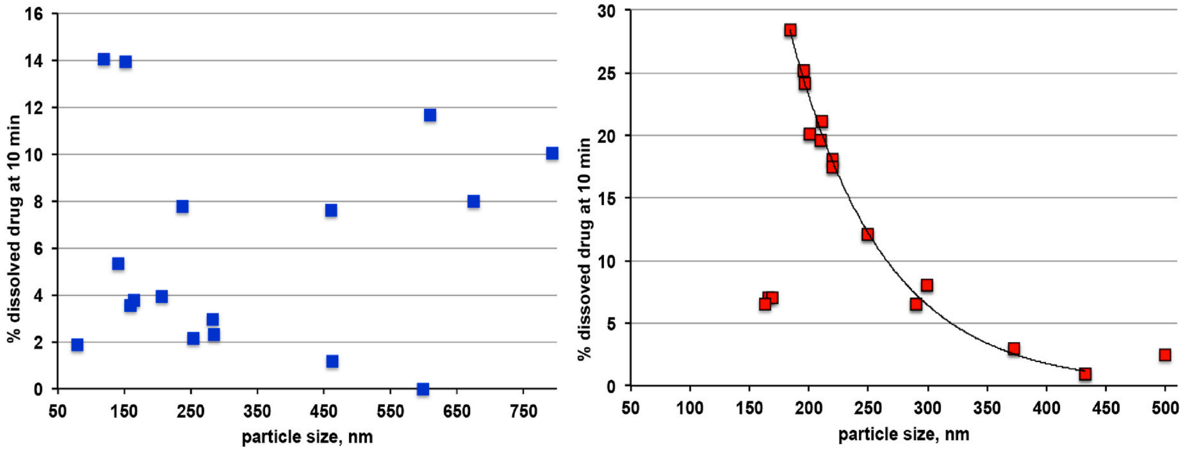

Fig. 5. Plots of particle size versus % drug dissolved at 10 min of nanocrystals obtained by the dry-milling (left, blue points) and wet-milling (right, red points) methods. Data were from Tables 2 and 3, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

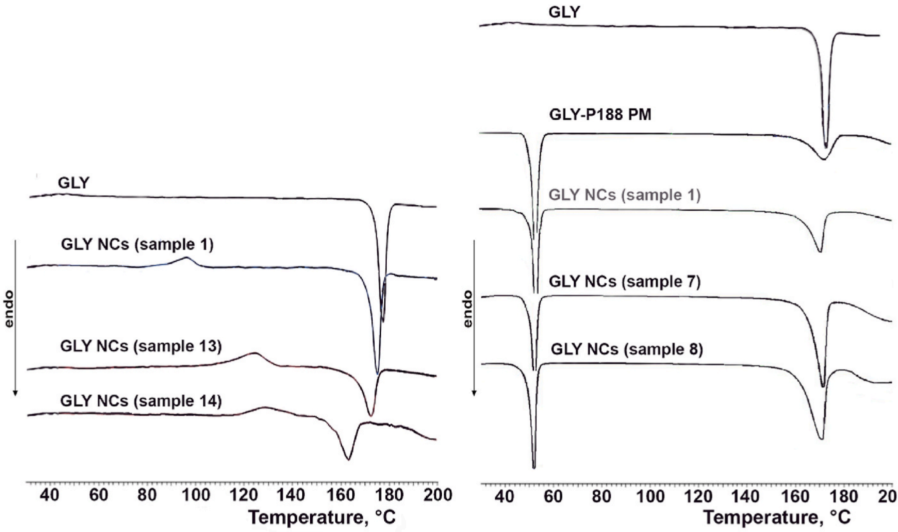

Fig. 6. DSC curves of the original GLY sample and of selected GLY NCs obtained by dry-ball milling (left) (samples n. 1, 13 and 14 of Table 2) and by wet-bead milling (right) (samples n. 1, 7 and 8 of Table 3). In the case of the wet-bead method, the DSC curve of GLY-P188 3:1 w/w physical mixture (PM) is also shown.

Table 4
DSC data and relative decrease in crystallinity (% RDC) for unprocessed GLY and representative GLY NCs samples obtained by dry-ball milling (DM) or wet-bead milling (WM).

sample	ΔH (J/g)	T_{peak} (°C)	T_{onset} (°C)	T_{endset} (°C)	% RDC
GLY	90.6	175.9	172.6	178.5	100
GLY NCs DM (1)[a]	77.7	173.9	171.7	177.0	85.7
GLY NCs DM (13)[a]	47.0	171.3	167.0	175.2	51.8
GLY NCs DM (14)[a]	41.5	163.1	157.4	167.4	45.8
GLY NCs WM (1)[a]	34.4	169.7	163.0	171.9	n.c.e.[b]
GLY NCs WM (7)[a]	54.7	170.8	164.7	172.6	n.c.e.[b]
GLY NCs WM (8)[a]	53.4	170.6	161.3	172.5	n.c.e.[b]

[a] Numbers in brackets referred to the codes in Tables 2 and 3 (column 1) to identify the different grinding conditions used to obtain the various GLY NCs samples by dry-milling (DM) or wet-milling (WM) methods, respectively.

[b] Not correctly evaluable.

The DSC curve of the original GLY powder, characterized by an initially flat profile followed by a sharp and intense endothermic event peaked at 175.9 °C, due to the drug melting, was typical of a pure, crystalline, anhydrous substance. As can be seen in Fig. 5 (left), the thermal behavior of GLY NCs obtained by dry-ball milling was different from that of the unprocessed sample, and it was affected by the grinding conditions. In fact, the presence of a small and broad exothermic band was observed in all ground samples, and it progressively shifted at higher temperatures with increasing grinding time and frequency, passing from 96 °C for sample 1 (30 min at 12 Hz) to 125–128 °C for samples 13 and 14 (90 min at 24 Hz). The appearance of an exothermic band as a consequence of the milling treatment was previously observed also in the case of glisentide (another sulfonylurea), and attributed to the partial recrystallization of the drug fraction converted into an amorphous state by the grinding process [52]. Then, the value of the crystallization enthalpy of each ground sample was subtracted to the corresponding fusion enthalpy value, to correctly determine the actual reduction of drug crystallinity (%RDC). Besides, a concomitant shift of GLY melting peak to lower temperatures, joined to a band broadening and reduction of its intensity and fusion enthalpy (see Table 4) was observed, due to a progressive loss of the powder crystallinity, as a consequence of the mechanical treatment. An analogous and even more evident lowering and broadening of GLY melting peak, with a concomitant reduction of the relative enthalpy, was observed after dry-milling in the presence of crystalline and amorphous cyclodextrins, and attributed to the drug partial amorphization [53]. On the other hand, the more evident lowering of the peak melting temperature observed for sample 14 compared to sample 13 could also indicate that a polymorphic transformation happened. However, this hypothesis was discarded, since the most common polymorphic form of GLY, i.e. form II, has a melting point of 151.0 °C [56] i.e. clearly lower than that of sample 14 (163.1 °C).

Finally, a DSC analysis of GLY NCs obtained by dry-ball milling, under the found optimal grinding conditions, of the drug:P188 3:1 w/w PM was also performed (Fig. 1 supplementary). The presence of the melting bands of both polymer and drug, which appeared almost unchanged, with respect to those of the single components, allowed to exclude any solid-state interaction between the components, as well as to rule out any possible amorphizing effect of the polymer towards the drug; in fact, compared to the DSC curve of the sample GLY NCs DM (13), obtained under the same grinding conditions in the absence of polymer (see also Fig. 6), only a very slight lowering of the GLY melting peak temperature was observed, while the fusion enthalpy (47.8 J/g) and the % RDC (52.7) were nearly the same (see Table 4). Such a finding further confirmed that the observed improvement in GLY dissolution rate in the presence of P188 has to be actually ascribed to the polymer solubilizing effect.

As for the samples obtained by the wet-bead milling technique, Fig. 6 (right) shows the DSC curves of the selected GLY NCs (obtained from samples 1, 7 and 8 of Table 3) compared to that of the 3:1 w/w GLY-P188 physical mixture. As can be seen, the thermal curves of all the samples showed a first endothermic peak at 50–52 °C, due to the P-188 melting, followed, at higher temperature, by the endothermic peak due to the GLY melting. This last gradually shifted from 175.9 °C (unprocessed pure drug) to 172.0 °C for the GLY-P188 physical mixture, up to 170.8–168.0 °C for the various NCs examined, together with a band broadening. The slight change observed in the case of the physical mixture can be considered simply due to the presence of the polymer, which acted as an impurity, and then it was possible to confirm the absence of possible incompatibility issues between drug and polymer. Instead, the further lowering of the GLY melting peak observed in the case of NCs thermal curves, as well as the observed reduction of melting fusion enthalpy can be considered the combined result of the mechanical treatment undergone by the powder samples, together with the effect of the spray-drying process and of a possible adsorption of the drug by the polymer. Therefore, in this case the variation of the GLY melting enthalpy could not be taken as a reliable measure of crystallinity reduction caused by milling.

3.6.2. Fourier transform infrared spectroscopy studies

Fourier transform infrared spectroscopy (FTIR) spectra of untreated GLY and of the selected GLY NCs obtained by dry-ball or wet-bead milling are shown in Fig. 7.

The FTIR spectrum of the original unprocessed GLY showed typical characteristic bands at 1715.5 cm^{-1} (carbonyl-stretch), 1155.9 and 1306.3 cm^{-1} (symmetric and asymmetric sulfonyl stretch) and 3315.7–3367.8 cm^{-1} (amide stretch) [57]. The characteristics absorption bands of the drug appeared almost unchanged in the spectrum of the NCs sample obtained by dry-ball (DB) milling, without any appreciable shift.

In the case of NCs obtained by the wet-bead milling method, the spectrum of the nanonized sample was compared also with that of the 3:1 w/w physical mixture of untreated GLY with P188, used as stabilizer. Also in this case, no significant changes or shifts of bands were observed between the spectra of the original GLY sample, the GLY-P188 physical mixture and the GLY NCs. Moreover, the presence of all the characteristic GLY peaks in the NCs and the almost superimposable spectra of GLY NCs and GLY-P188 physical mixture demonstrated the absence of drug-polymer solid-state interactions.

3.6.3. X-ray powder diffractometry studies

X-ray powder diffraction (XRPD) spectra of the original GLY powder sample and of the selected GLY NCs obtained by dry-ball-milling or wet-bead-milling are presented in Fig. 8. The diffraction pattern of the starting GLY powder showed several sharp and intense peaks at 11.9, 16.1, 18.8, 21.5, 22.9, 27.9, 30.1 and 32.5 2Θ, indicative of its crystalline nature.

A strong reduction of the number and intensity of drug crystallinity peaks was observed in the nanosized sample obtained by dry-ball milling (Fig. 7A), even if some residual peaks at identical angles (11.9, 18.8, 22.9 and 27.9 2Θ values) were still visible, thus confirming the absence of polymorphic changes. The observed GLY crystallinity loss could might be the result of the reduction of crystals size and of the shortening of the length-range order of the powder sample as a consequence of nanonization process [32].

Some reduction of intensity of the drug crystallinity peaks was observed in its physical mixture with the polymer P188, which was simply due to the effect of the mixing. On the contrary, while the two typical peaks of the polymer were still well evident in the nanonized sample obtained by the wet-ball milling method, a marked reduction or even the disappearance of most of the drug characteristic diffraction peaks loss was instead observed (Fig. 7 B). This result could be attributed to both the reduction of crystals size (as explained above), as well as to the spray-drying process. Nevertheless, some residual peaks typical of GLY, slightly broadened but at unchanged angles, were still detectable, allowing to exclude also in this case any polymorphic change, confirming the absence of solid-state interactions with P188, and indicating that the drug was still present in crystalline form.

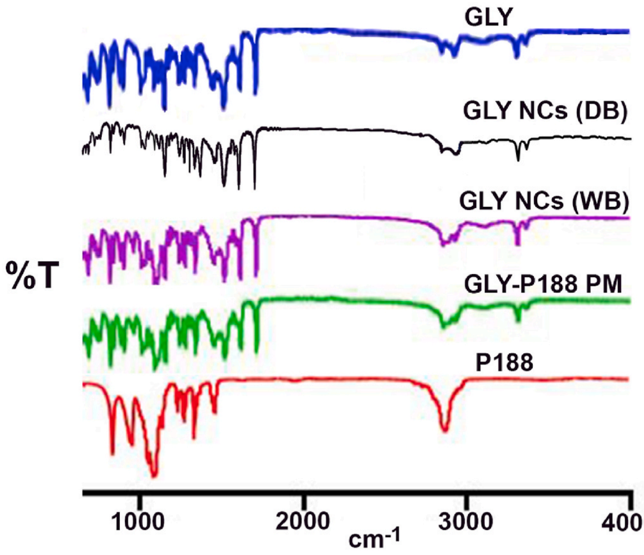

Fig. 7. FTIR spectra of unprocessed GLY and the selected NCs obtained by dry-ball-milling (DB) (sample n. 13 of Table 2) and by wet-bead milling (WB) (sample n. 7 of Table 3). In the case of wet-bead-milling method, spectra of P188 (used as stabilizer) and GLY-P188 1:3 w/w physical mixture (PM) are also shown.

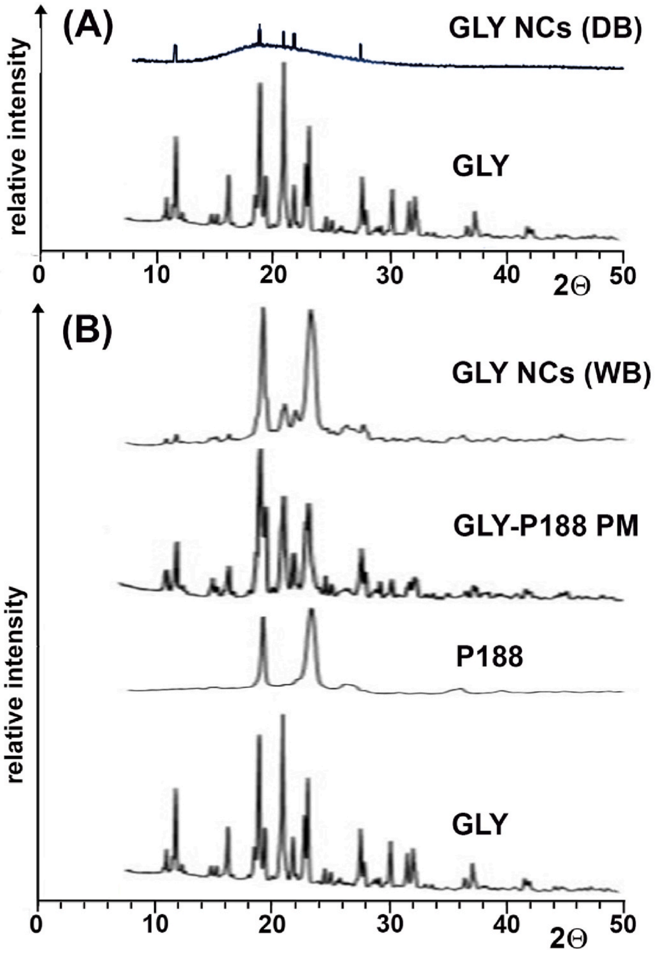

Fig. 8. X-ray powder diffraction patterns of unprocessed GLY and of selected NCs obtained by dry-ball milling (A) (sample 13 of Table 2) and wet-bead milling (B) (sample 7 of Table 3). In the case of wet-bead method (B), spectra of P-188 and of 3:1 GLY-P188 physical mixture (PM) are also shown.

3.6.4. Scanning electron microscopy (SEM) studies

SEM analyses were performed to examine in detail the morphology of the selected GLY NCs obtained by the two nano-milling methods. SEM micrographs of unprocessed GLY and of selected GLY NCs samples obtained by wet-bead milling (sample 7 of Table 3) and by dry-ball milling (sample 13 of Table 2) are shown in Fig. 9.

Untreated GLY powder appeared as micron-sized crystals of irregular and non-uniform shape (Fig. 9A–B). Both treated powder samples showed an evident reduction of the powder granulometry (Fig. 9, C-D and E-F, respectively), but their morphology was clearly different, as it is possible to better appreciate in more detail in Fig. 10.

In fact, GLY NCs obtained by spray-drying of the nano-suspension prepared by the wet-method were characterized by typical micro-sized agglomerates of small particles of ovoidal shape, easy to be redispersed (Fig. 10A and B-C). On the contrary, GLY NCs prepared by the

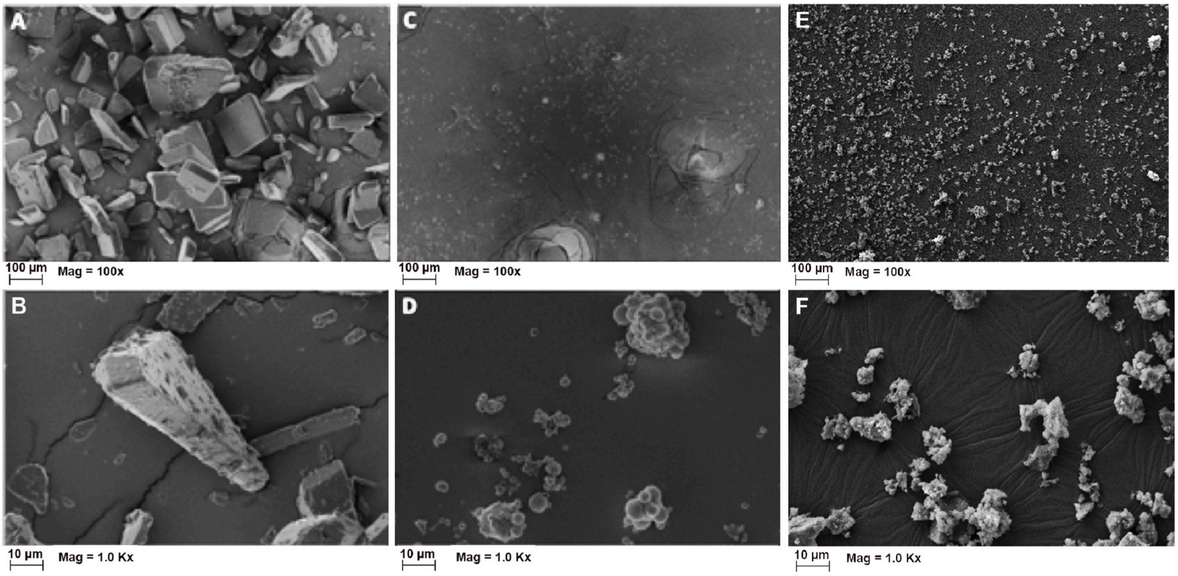

Fig. 9. SEM micrographs at 100x magnification (top) or 1.0Kx magnification (bottom) comparing pure GLY and the selected GLY NCs obtained by wet-bead milling (WM) or dry-ball milling (DM) methods. Key: A–B: pure GLY; C–D: GLY NCs obtained by WM (sample 7 of Table 3); E–F: GLY NCs obtained by DM (sample 13 of Table 2).

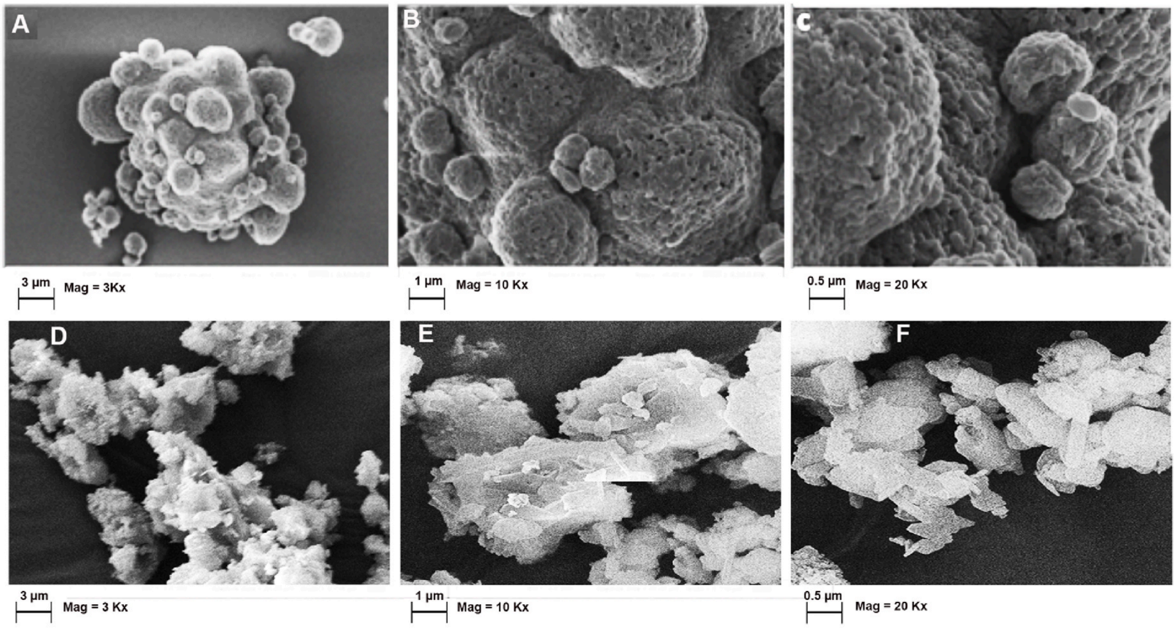

Fig. 10. SEM micrographs comparing the selected GLY NCs obtained by wet-bead milling (A, B, C) and dry-ball milling (D, E, F) methods at 3Kx, 10Kx and 20Kx magnifications.

dry-method appeared as micro-sized agglomerates of nano-sized crystals of flat and almost irregular shape, very smaller but rather similar in appearance to those of the original unprocessed sample (Fig. 10, D-E-F).

3.6.5. *Energy dispersive X-ray spectroscopy (EDXS) studies*

In the case of the sample obtained by the wet-bead milling method, since it consisted of a 3:1 w/w mixture of GLY with P-188, added as stabilizer, an additional elemental analysis using EDXS was performed, in order to assess the actual homogeneous distribution of the drug. This was possible by checking the distribution of sulfur and chlorine atoms, which are present only in the GLY molecules. Examination by EDXS of the color distribution of the elements (C, O, S and Cl) in the focused region of the sample (Fig. 2 Supplementary) allowed to clearly evidence the presence of the chlorine atoms (that resulted better detectable than the sulfur atoms), and then to confirm the homogeneous distribution of GLY in the mixture.

3.7. *Properties of the solid NCs obtained by the two methods*

Even though a direct comparison of the solid-state properties of the nanosized powders obtained by the dry- and wet-milling methods cannot be made, since this last method required a spray-drying process, a series of interesting and useful information was obtained.

In particular, as for the dry-ball milling method, the results of solid-state studies indicated that the milling process did not impair the drug stability, or caused the appearance of polymorphism phenomena, since the nanosized powder maintained unchanged its typical FTIR bands and XRPD diffractions peaks, only revealing a partial loss of drug crystallinity, as confirmed by DSC analysis, due to the mechanical treatment it has undergone. On the other hand, SEM micrographs showed that the nano-sized drug crystals maintained almost unchanged, even to in a nano-scale, the typical flat and rather irregular shape exhibited by the unprocessed GLY sample.

Instead, as for the wet-beads milling method, solid-state studies allowed to demonstrate not only the drug stability to the process and the absence of appearance of polymorphism phenomena, but also its full compatibility with P188, the polymer used as stabilizer. Even in this case, DSC, FTIR and XRPD analyses merely indicated a reduction of GLY crystallinity degree. On the other hand, SEM analysis showed in this case the formation of micro-sized agglomerates of nano-particles of roundish shape, typical of spray-dried products.

Moreover, the easy re-dispersibility of the spray-dried powder was assessed, obtaining the complete redispersion within only 60 s, with an average particle around 300 nm. The observed variation in particle size with respect to the original nanosuspension is in good agreement with previous results [34].

Finally, EDXS studies were useful to confirm the maintenance of the homogeneous dispersion of the drug in its mixture with the stabilizer also after the spray-drying process.

4. Conclusions

In the present work, the effectiveness of dry-ball and wet-bead milling methods in producing GLY NCs with improved dissolution properties has been thoroughly investigated and compared. DoE enabled a systematic evaluation, for each method, of the effect of the selected process variables (ball/bead volume, drug amount, milling speed and time) and of their combinations on the selected responses, i.e. particle size reduction and dissolution rate (as % drug dissolved at 10 min) of the model drug GLY. DoE indicated as the best conditions for the dry-method to set speed and milling time at the highest levels, while bead volume and drug amount at the lowest ones; on the contrary, setting to the highest levels of drug amount and milling rate and to the lowest ones of bead volume and milling time was suggested for the wet-method.

The results, obtained under the respective optimal conditions, evidenced a rather similar performance in drug particle size reduction, producing NCs with mean size around 120 and 180 nm for the dry- and wet-method, respectively. On the contrary, even if in both cases a clear increase in drug dissolution rate was achieved, a better result was obtained with the wet-than the dry-method, with a GLY dissolution rate increase, compared to the starting powder, of 15.8 times vs 7.8 times, respectively. This has been mainly ascribed to the solubilizing effect of P188, added in the wet-method as stabilizer.

Interestingly, and differently from the wet-method, in the case of the dry-method no direct relationship was observed between particle size reduction and drug dissolution rate. This was explained by the important role played in this regard by the loss of drug crystallinity during the dry-milling process, as evidenced by DSC and XRPD analyses.

Solid-state studies also proved the stability of the drug under both milling processes, the absence of polymorphism and, for the wet-method, the full compatibility of the drug with P188, as well as its homogeneous dispersion in its mixture with the drug.

Ultimately, both methods were effective to produce GLY NCs with good dissolution performance, provided that the respective most suitable experimental conditions are used.

CRediT authorship contribution statement

Paola A. Mura: Conceptualization, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. **Marzia Cirri:** Conceptualization, Data curation, Investigation, Methodology, Software, Supervision, Writing – original draft, Writing – review & editing. **Alan Rossetti:** Data curation, Formal analysis, Investigation, Methodology. **Daniel A. Allemandi:** Data curation, Formal analysis, Investigation, Methodology. **Alejandro J. Paredes:** Data curation, Formal analysis, Investigation, Methodology, Validation. **Santiago D. Palma:** Conceptualization, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jddst.2023.105222.

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