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Optimization by experimental design of grinding process variables for glibenclamide nanoparticles formation

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Resumo

A presente monografia visa a optimização, através de um desenho experimental, de variáveis implicadas no processo de produção de nanopartículas de glibenclamida pelo processo de moagem (com moinho de bolas).

A glibenclamida (GLB) é um antidiabético oral pertencente à classe II do sistema de classificação biofarmacêutica (BCS) sendo, por isso, um candidato ideal para optimizar as suas características físico-químicas. A absorção de GLB, em jejum, revela-se superior comparativamente a uma administração após ingestão de alimentos. Por outro lado, o tempo decorrido entre a administração e a absorção da GLB é inferior numa condição de jejum face a uma condição de presença de alimentos. Esta evidência sugere que o tempo ideal para administrar a GLB será 30 minutos antes das refeições. Contudo, a adesão a esta terapêutica pode estar comprometida porque, caso o doente não coma após a toma deste fármaco, desenvolver-se-á um quadro hipoglicémico grave. Desta forma, o desenvolvimento de estratégias que permitam melhorar as características físico-químicas da GLB poderá ter um forte impacto na melhoria da vida dos doentes diabéticos consumidores de GLB.

A utilização da nanotecnologia para a obtenção de fármacos com características mais favoráveis à sua biodisponibilidade, nos dias de hoje, tem surgido como uma ferramenta cada vez mais usual. Este facto verifica-se, especialmente, quando se tratam de fármacos pertencentes à classe II do BCS que apresentam elevada permeabilidade, mas baixa solubilidade. A formulação de nanopartículas pressupõe uma redução de tamanho das partículas até à escala nanométrica e, consequentemente, o aumento da área de superfície dessas mesmas partículas. Desta forma, promove-se o aumento da solubilidade do fármaco em questão o que, por sua vez, levará a um aumento da absorção e biodisponibilidade do mesmo.

O fundamento do processo de "ball-milling" baseia-se na redução do tamanho das partículas graças a forças mecânicas estabelecidas entre as paredes do frasco, as bolas contidas no mesmo e o fármaco.

Para o estudo que a presente monografia contempla pesaram-se 100 mg e 500 mg de GLB micronizada pura numa balança analítica. De seguida, para a obtenção das

nanopartículas, procedeu-se à moagem das amostras num moinho de alta energia vibracional (Mixer Mill Type MM 200) com diferentes frequências de vibração (12 e 24 Hz) e diferentes tempos de moagem (durante 30 e 90 minutos). Juntamente com a GLB pura adicionaram-se aos frascos 2 e 6 bolas de aço inoxidável, cujo diâmetro correspondia a 9 e 12mm. Desta forma, obtiveram-se 35 amostras de nanopartículas de GLB produzidas em diferentes condições.

Para a execução do teste de dissolução retiraram-se 30 mg de cada amostra e adicionaram-se a 75 mL de água purificada num copo de 150 mL a 25ºC. Uma pá de três lâminas foi colocada, submersa, no centro do copo, a 100 rpm. Alíquotas de 3 mL foram recolhidas, em tripilicado, após 10 minutos com uma seringa e analisadas espectofotometricamente a 302.0 nm.

O tamanho de partículas foi determinado através do método Dynamic Light Scattering (DLS), com recurso ao Zetasizer Nano-ZS90. As amostras foram previamente diluídas em água purificada para evitar o fenómeno de multidispersão. De seguida, foram suspensas numa solução constituída por 100 mg de hidroxipropilmetilcelulose a 1 % e 20 mg de lauril sulfato de sódio a 0,2% em 10 mL de água purificada. As suspensões foram colocadas directamente na cuvete e analisadas.

Para compreender quais as variáveis mais significativas na produção de nanopartículas de GLB pelo método "ball-milling" realizou-se um desenho experimental com 5 factores a dois níveis ($2^5 + 3$ pontos centrais), através do programa MODDE-GO®32 bit Trial. Um total de 35 experiências foi optimizado: foram selecionadas 5 variáveis independentes (factores) a dois níveis – quantidade de GLB (min: 100 mg; máx: 500 mg), número de bolas (min: 2; máx: 6), diâmetro das bolas (min: 9 mm; máx: 12 mm), frequência (min: 12 Hz; máx: 24 Hz) e duração (min: 30 min; máx: 90 min) - e 3 pontos centrais. O tamanho médio das partículas (medido através da técnica "Dynamic Light Scattering") e a percentagem de GLB dissolvida ao final de 10 min (realizado através do método da quantidade dispersa) foram as respostas seleccionadas. Todos os parâmetros estatísticos, resultantes do desenho experimental realizado, demonstraram que o modelo utilizado é estatisticamente significante. Para a obtenção dos resultados, estudou-se não só a influência dos 5 factores referidos acima (factores principais), como também, a influência das diversas interacções/combinações entre esses mesmos factores. Desta forma, obteve-se um total de 20 factores que afectaram quer o tamanho médio das partículas quer a percentagem dissolvida em 10

min. Desses 20 factores, verificou-se que 8 factores (principais e interacções) afectaram positivamente ambas as respostas. Considerando a resposta do tamanho médio das partículas, o factor com maior impacto positivo foi a interacção entre o número de bolas e a duração. As nanopartículas formam-se pelo impacto das bolas entre si, com o fármaco e com o frasco. Ao aumentar o número de bolas está a promover-se o aumento do número de colisões, e, consequentemente, o aumento da energia transferida das bolas para as partículas obtendo, assim, um maior número de partículas com as dimensões desejadas. Por outro lado, a interacção entre a frequência e o tempo apresentou-se como sendo o factor com um impacto mais negativo no tamanho médio das partículas. O processo de fraccionamento das partículas responsável pela diminuição do tamanho das mesmas atinge o seu máximo num curto espaço de tempo. Ao prolongar demasiado o tempo de moagem, bem como ao atingir frequências demasiado altas, alcançam-se condições demasiado drásticas que levam à agregação das nanopartículas, e consequentemente, ao aumento do tamanho das mesmas. Ao analisar os resultados, considerando um intervalo de confiança de 95%, os factores com significância estatística ($p<0,05$) obtidos foram os mesmos que referidos anteriormente, quer com impacto positivo como também negativo.

Para a resposta da percentagem de GLB dissolvida em 10 min, a frequência foi o factor mais importante com uma influência positiva. Aumentado a frequência há maior quantidade de energia transferida das bolas para as partículas, o que leva a que o fármaco atinja um "estado activado", e, por conseguinte, passe do estado cristalino ao estado amorfo. Assim, a percentagem de GLB dissolvida em 10 min é maior. Em contrapartida, a quantidade de GLB evidenciou ser o factor com maior impacto negativo. Ao aumentar a quantidade de GLB adicionada ao frasco, diminui-se o espaço livre de circulação das bolas. Consequentemente, a possibilidade de colisões entre as bolas e as partículas está diminuída. Dessa forma, a eficiência do processo decresce, visto que, a quantidade de partículas sujeita ao processo de fracionamento é menor, obtendo-se menor quantidade de partículas nanométricas. Ao analisar os resultados, considerando um intervalo de confiança de 95%, para além dos factores referidos acima, verificou-se também uma influência negativa por parte da interacção entre a quantidade de GLB e a duração. Isto demonstra que, a quantidade de GLB continua a ter um efeito negativo mesmo que se prolongue no tempo o tratamento mecânico-químico.

Em suma, existem diversos factores que influenciam o processo de produção de nanopartículas de GLB pelo método "ball milling" de alta energia. Contudo, nem todos têm um efeito estatisticamente significativo e podem ter uma influência quer positiva quer negativa. Regra geral, os resultados mais favoráveis, quer em relação à resposta do tamanho médio das partículas quer em relação à percentagem de fármaco dissolvida em 10 min, obtiveram-se quando se estabeleceram condições que promovem o aumento do número de colisões entre as bolas e as partículas, de forma a promover um aumento da energia transferida entre estas, sem nunca atingir condições drásticas responsáveis pela inversão dos resultados desejados.

Palavras-chave: Glibenclamida, Moinho de Bolas, Nanopartículas, Desenho Experimental

Abstract

The purpose of this study was to optimize the process parameters of glibenclamide by a top-down high-energy ball milling process. Glibenclamide is a poorly soluble drug, relatively permeable through Caco-2 cell monolayers, which enables it to be classified under the Biopharmaceutics Classification System as Class II drug. Improving the dissolution characteristics of glibenclamide may allow concomitant administration of the drug with food; it may also improve the bioavailability and, consequently, the compliance of patients taking this drug. Nanotechnology overcomes the problem of hydrophobic drugs, i.e., poor water solubility. Through the high-energy ball milling technique, it was possible to obtain glibenclamide nanoparticles. In this process drug particles are reduced due to the impact from the balls upon them, as well as the attritive forces that arise from the movement of such balls against each other. A full factorial design with 5 factors at two-levels (2^5 + 3 central points) was employed, by MODDE-GO®32 bit Trial program, to optimize five factors including amount of glibenclamide (mg), number of milling balls, diameter (mm) of such balls, frequency (Hz) and time of milling (min). Mean particle size (measured through Dynamic Light Scattering) and percent of dissolved drug after 10 min (carried out according to the dispersed amount method) were selected as response variables. Concerning particle size response, the factor that showed the most positive effect was the interaction between the number of ball and time. On the other hand, the most negative effect was the interaction between frequency and time (freq*time). Concerning the % dissolved in 10 min response, frequency is the main factor responsible for the most positive influence. Contrastively, the amount of glibenclamide, as main factor, and the interaction between the amount of glibenclamide and time were responsible for the most negative influence. In summary, there are several factors that influence the process of production of glibenclamide nanoparticles by the high-energy ball milling method. However, not all have a statistically significant effect and can have a positive or negative influence. In general, the most favorable results, both for the response of the mean particle size and for the percentage of drug dissolved in 10 min, were obtained in particular conditions promoting the increase in the number of collisions between the beads and the particles, thus consequently leading to an increase

in the energy transferred between them, without ever reaching drastic conditions responsible for the inversion of the desired results.

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Abbreviations

DM: Diabetes mellitus

GLP-1: Glucagon-like peptide-1

DPP4: Dipeptidyl peptidase-4

GLB: Glibenclamide

SUR1: sulfonylurea receptor 1

ATP: Adenosine triphosphate

K^+: Potassium

Caco-2: heterogeneous human epithelial colorectal adenocarcinoma

BCS: Biopharmaceutics Classification System

Cr-Ni: Chromium-Nickel

QbD: Quality by Design

DOE: Design of Experiments

USP: United States Pharmacopeia

HPMC: Hydroxypropyl Methylcellulose

SLS: Sodium Lauryl Sulfate

DLS: Dynamic Light Scattering

LDV: Laser Doppler Velocimetry

DTS: Dispersion Technology Software

PdI: Polydispersity Index

Ball size (min): balls with 9 mm of diameter

Ball size (máx): balls with 12 mm of diameter

num: number of balls

Gly: amount of glibenclamide, in mg

freq: frequency, in Hz

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1 Introduction

1.1 Diabetes

Diabetes is a chronic disease that can happen due to two main reasons: either the body cannot produce enough insulin, or rather, it cannot properly use the insulin produced. Diabetes is diagnosed by observing raised levels of glucose in the blood. Insulin is an endogenous hormone produced in the pancreas' β-cells, whose function is to transport glucose from the bloodstream into the body's cells where it is, in turn, used as energy. The lack, or ineffectiveness, of insulin in a person with diabetes means that glucose remains circulating in the blood. Hyperglycemia (the resulting high levels of glucose in the blood) causes damage to many tissues in the body, leading ultimately to the development of disabling and life-threatening health complications.(1)

Data provided by the Portuguese Society of Diabetology show that diabetes mellitus (DM) is the most frequent endocrine disorder, affecting roughly 415 million people worldwide in 2015; tendency expected to rise to 642 million by 2040. The tendency seems to be an increase of the number of people with Type 2 Diabetes in every country. In 2015, estimates showed the prevalence of Diabetes in the strand of the Portuguese population in the age gap of 20 to 79 years old (which corresponds to 7.7 million individuals) was 13.3%, i.e. more than 1 million diabetic Portuguese people in this age group.(2)

In fact, there are five distinct types of diabetes: Type 1 diabetes, Type 2 diabetes, gestational diabetes, monogenic diabetes and secondary diabetes.

As previously mentioned, the most prevailing type of diabetes is Type 2, which is most common in adults, but can also affect young children and adolescents. However still producing insulin, the human body in Type 2 diabetes grows resistance to it, thus insulin becomes ineffective. Indeed, insulin levels may become insufficient over time. Both, insulin resistance and deficiency, lead to high blood glucose levels. There are a great number of different medications that can be employed to type 2 diabetes, such as: metformin (biguanide class), gliclazide (2nd generation sulfonylurea), GLP-1 analogs (injectable treatments that are not insulin) and DPP4 inhibitors.(1)

1.2 Glibenclamide (ATC: A10BB01)

Glibenclamide (GLB) is a powerful second-generation sulfonylurea that has asserted its potential benefits like lower dose, rapid onset, lower insulin levels and less-pronounced glucagonotropic effects, and insulin-sensitizing and insulin mimetic effects. It is orally used as an hypoglycemic agent to treat non-insulindependent (type II) diabetes mellitus.(3) Blood glucose level in patients with Type 2 diabetes is lowered by GLB as it directly spurs the release of insulin from functioning beta cells of pancreatic Langerhans's islet tissues through binding to the SUR1 subunits and block the ATP-sensitive K^+ channel. Nevertheless, GLB is a poorly soluble drug (solubility < 8 μg/ml at pH 7.4 phosphate buffer), relatively permeable through Caco-2 cell monolayers, which enables it to be classified under the BCS (Biopharmaceutics Classification System) Class II classification.(4) GLB administration under conditions of fasting does sustainably increase the area under curve for 24 hours and, as well as, increasing the maximum concentration of GLB achieved in blood compared to its administration under feeding condition. Furthermore, there was also a significant decrease in the lag time in fasting condition, compared with the feeding situation. This suggests that GLB is effectively absorbed from the gastrointestinal tract. Nevertheless, it follows that its dissolution and, in turn, absorption, is indeed affected by the presence of food or other dietary supplements.(5) As for the time required for an optimal concentration in the plasma to be reached, GLB is thought to be more effective if given 30 minutes before meals. In contrast, patient compliance could be reduced if after taking the drug the patient is not able to have the meal; it would result in severe hypoglycemia. Alternatively, if taken with a meal, food sequentially would interfere with its absorption. Thus, by improving the dissolution characteristics of GLB may allow concomitant dosing of the drug with food, and may also see its bioavailability improved.(6)

1.3 Nanoparticles Technology

Nanotechnology in drug delivery is thought to have as one of its main characteristics the fact that it overcomes the problem of power water solubility of hydrophobic drugs. Roughly 40% of all developmental new chemical identities are difficult to formulate, as they are poorly water-soluble. Low solubility in drugs leads to low oral bioavailability and erratic absorption. This is particularly visible in drugs within class II of the BCS.(7)

Currently, a large percentage of drug compounds in drug development present poor aqueous solubility. Problems such as poor and highly variable bioavailability are frequent to the conventional formulations of poorly-water soluble drugs. Quite often is the dosage form affected by the fed-fasted state of the patient and its onset of action turns slower than previously anticipated. Sub-optimal dosing and poor performance are often results of the combination of the above-enumerated issues. Thus, it follows that one of the most challenging tasks of drug development is the improvement of drug solubility as to enhance the bioavailability of such drugs.(8)

Nanonization is a physical technique aimed at decreasing the particle size in order to improve solubility in water, thus, allowing better bioavailability. An increased surface area resulting from the size reduction leads to, according to the Noyes-Whitney equation (annex 1), an increased dissolution velocity. Thus, this method is used to increase drugs surface area and proportionally increase the rate of dissolution, as well as the rate of absorption. Drug nanocrystals are, as the name suggests, crystals with a size in the nanometer range; this means that they are nanoparticles with a crystalline character. In the pharmaceutical area, and having in consideration the size unit, nanoparticles should be defined as having a size between a few nanometers and 1000 nm (=1 μm).(9)

1.4 Ball milling Tecnhique

The unit operation where mechanical energy is applied to physically breakdown coarse particles to finer ones goes by the name of milling. It is, furthermore, regarded as a 'top-down' approach in the production of fine particles. Additionally, ball milling is another popular size reduction technique, especially used in research laboratories, for the production of nanoparticles. A ball mill is comprised by a vessel or vial filled with balls, or rods, that are constructed from a variety of materials, like: ceramic, agate, silicon nitride, sintered corundum, zirconia, chrone steel, Cr-Ni steel, tungsten carbide or plastic polyamide.(10) Inside said vessel, the material to be milled is placed and it is made to rotate at a certain speed or frequency. The balls are cascaded or made to move in a particular pattern by the movement of the vessel. They are also made to collide with one another and with the inner wall of the vessel. It follows, that the drug particles are reduced due to the impact from the balls upon them, as well as the attritive forces that arise from the movement of such balls against each other. In fact, during the grinding process mechanical energy is transferred by means of normal and shear stresses acting on solid material surfaces leading to crystal crushing and thus to the formation of new surface. The iteration of this phenomenon induces particle size reduction to some critical threshold. Further energy supply yields to the accumulation of defects into crystal volume or on its surface to finally lead to a complete amorphization.(11) The extent of the fill of the vessel and the intensity of the milling process is thus determined by the number of balls and their starting material.(12)

1.5 Experimental Design

Both the process optimization and validation have benefited from the usefulness of applying experimental design to formulation development. The traditional way of optimization of the manufacturing process is the 'trial and error' method. It evaluates one variable at said time, keeping others constant. However, this approach might lead to suboptimal results, as the interaction effects of process variables are ignored. This paves way for a better process to prevail in the studied conditions: the adaptation of

rational, systematic, efficient and cost-effective strategies using designing of experiments.(13) Quality by Design (QbD) has fundamentally integrated the pharmaceutical industry, as a result of recent quality initiatives and regulatory prospects. The result of a manufacturing process optimized using design of experiments (DOE) is likely to be a process marked by its robustness, amenable for seamless scale-up and validation.

A component part of QbD is DOE. This consists of a systematic and simultaneous evaluation of variables (process or formulation) to develop a product with the expected quality attributes. Though being a broad term, QbD encompasses pre-established target quality, physicochemical, physiological, pharmacological and clinical considerations that ultimately result in a product with the desired quality attributes, those being safeness and effectiveness. Variables associated with raw material characteristics, product design, process and scale-up issues ought to be carefully investigated, for the sake of thorough practical considerations. Thus, the understanding of factors and their interaction effects using a desired set of experiments becomes an extremely useful part of QbD.(14) Several statistical experimental designs have been acknowledged as useful techniques to the understanding of variables and their interactions amongst each other.

The influences of all experimental variables, factors and interaction effects on the response/s are investigated in a factorial design. Is important to pay attention and understand the meaning of terminology. In this sense, factor is a variable that potentially affects the response; treatment is a combination of one or more factors; levels are the values a factor can take on; and, effect is how much a main factor or interaction between factors influences the mean response.

A factorial design will consist of 2^k experiments when the combinations of k factors are investigated at two levels, these are given by – (minus) for a low level, and + (plus) for a high one. A centre, also known as a zero-level, is also included. In this all variables are set at their mid-value. Centre experiments, at least three or four, should always be included in factorial designs, as to a) minimizing the risk of missing non-linear relationships in the middle of intervals; and b) determining confidence intervals through repetition.

For each variable, - (minus) and + (plus) should correspond to is defined by what it is assumed to be a reasonable variation to investigate. In this way the size of the experimental domain is settled.(15)

2 Objective

Glibenclamide was used as a model drug for this study. The aim of this research work was therefore to investigate the feasibility of preparation of glibenclamide nanoparticles using the ball milling technique, in order to achieve fast dissolution, which would presumably yield quick onset of the peak plasma concentration. For this aim, the effects of different process variables (i.e. amount of glibenclamide, number of milling balls, diameter of such balls, frequency and time of milling) was evaluated by using experimental design, in order to find the optimal conditions to obtain drug nanoparticle formation and improve its dissolution rate.

3 Materials and Methods

3.1 Materials

Micronized (1.66 µm) glibenclamide USP (batch nº GLBA008MCR6) used in this study was manufactured by Laboratori Guidotti S.p.A., Pisa, Italy. Hydroxypropyl methylcellulose (HPMC) and sodium lauryl sulfate (SLS) were purchased from Sigma-Aldrich (currently is Merck). Purified water USP was used in this study.

3.2 Methods

3.2.1 Preparation of nanoparticles by top-down ball milling

Glibenclamide (GLB) nanoparticles were prepared using ball milling method. Samples of pure glibenclamide were weighed on an analytical balance (Mettler AE 166 DeltaRange, (Mettler Toledo, Switzerland) and then ground in a high energy vibrational mill (Mixer Mill Type MM 200, Retsch, GmbH, Düsseldorf, Germany) for different times (ranging from 30 to 90 min) and at different vibration frequencies (12-24 Hz). Grinding jars (volume 12 cm^3) and stainless steel balls (9 and 12 mm diameter, in a variable number from 2 to 6) were used. The sample weight ranged from 100 to 500 mg.

3.2.2 Dissolution Studies of Glibenclamide

Dissolution studies were carried out according to the dispersed amount method. Samples of 30 mg of drug were added to 75 mL of water in a 150 mL beaker at 25°C. A three-blade paddle (9.5 mm radius) was centrally put in the beaker and rotated at 100 rpm.

Aliquots (3 mL) were withdrawn after 10 min with a syringe-filter (nitrocellulose membrane, pore size 0.45 mm) and spectrometrically assayed for drug content at 302.0 nm. The test was performed in triplicate.

3.2.3 Particle size measurement

Particle size of GLB nanoparticles was determined by Dynamic Light Scattering (DLS) method using a Zetasizer Nano-ZS90 (Malvern Instruments, Malvern, UK).

DLS is a plain method to assess particle size, size distribution, and the zeta potential of nanomaterials in solution. DLS evaluates the velocity distribution of particle movement by calculating dynamic fluctuations of light scattering intensity caused by the Brownian motion of the particle. This approach produces a hydrodynamic radius, or diameter, to be calculated via the Stokes-Einstein equation from the aforementioned measurements. It yields a global measurement of the particle perpendicular to the light source at that instant. Laser Doppler Velocimetry (LDV) is the measurement technique used by the Zetasizer Nano-ZS to measure the zeta potential of particles in a solution. This technique uses a laser, which is being passed through the sample, to measure the electrophoretic mobility.(16)

Samples were suitably diluted with purified water before measurements to avoid multiscattering phenomena. Nanoparticles samples were suspended in a "solution A" (100mg of HPMC 1% plus 20mg of SLS 0,2% in 10ml of purified water, previously prepared). The obtained suspensions were directly placed into cuvette, and particle size was measured.

The Malvern Zetasizer Nano-ZS90 uses the Dispersion Technology Software (DTS) (V4.20) for data collection and analysis. The software collects and interprets data for the particle size, zeta potential, and molecular weight measurement functions of the device. For the particle sizing in solution (DLS), the software gives multiple aspects and interpretations of the data collected for the sample such as intensity, volume, and number distribution graphs as well as statistical analysis for each. The mean particle diameter is calculated by the software from the particle distributions measured, and the polydispersity index (PdI) given is a measure of the size ranges present in the solution (Malvern, Instruments Ltd., 2005).

3.2.4 Full factorial (2⁵) experimental design

A full factorial design with 5 factors at two-levels (2^5 + 3 central points) was employed to screen the significant process variables. A total of 35 experiments (N=35) were optimized by MODDE-GO®32 bit Trial.

Five independent variables (factors) at 2 levels were selected: the amount of GLB (min: 100 mg; max: 500 mg), number of balls (min: 2; max: 6), balls diameter (min: 9 mm; max: 12 mm), grinding frequency (min: 12 Hz; max: 24 Hz) and time (min: 30 min; max: 90min).

Mean particle size and % dissolved drug after 10 min were selected as response variables.

4 Results and Discussion

The results obtained from experimental design are show below. Figure 1 presents an overview of all the results obtained for each experiment. Experiment N14, N15, N28 (for % dissolved) and N30 (for particle size) were been excluded in order to achieve a better fit of the model. Five independent variables (factors) at 2 levels were selected: the amount of GLB (min: 100mg; max: 500mg), number of balls (min: 2; max: 6), balls diameter (min: 9mm; max: 12mm), grinding frequency (min: 12Hz; max: 24Hz) and time (min: 30min; max: 90min). N33, N34 and N35 represent the three central points (gly amount: 300mg; number of balls: 4; balls diameter: 9mm; grinding frequency: 18Hz; time: 60min). Mean particle size and % dissolved drug after 10 min were selected as dependent variables (response).

Exp No	Exp Name	Run Order	Incl/Excl	Ball size	Ball number	Gly amount	Frequency	Milling time	part size	% diss 10min
1	N1	20	Incl	min	2	100	12	30	206	2,91
2	N2	33	Incl	max	2	100	12	30	171	2,19
3	N3	35	Incl	min	6	100	12	30	38	6,07
4	N4	14	Incl	max	6	100	12	30	86	2,01
5	N5	13	Incl	min	2	500	12	30	675	6,23
6	N6	8	Incl	max	2	500	12	30	75	3,17
7	N7	21	Incl	min	6	500	12	30	285	1,8
8	N8	28	Incl	max	6	500	12	30	556	1,52
9	N9	3	Incl	min	2	100	24	30	843	9,39
10	N10	1	Incl	max	2	100	24	30	357	15,31
11	N11	18	Incl	min	6	100	24	30	461	5,96
12	N12	17	Incl	max	6	100	24	30	330	12,6
13	N13	2	Incl	min	2	500	24	30	598	11,93
14	N14	11	Excl	max	2	500	24	30		1,09
15	N15	24	Excl	min	6	500	24	30		19,68
16	N16	19	Incl	max	6	500	24	30	154	7,46
17	N17	27	Incl	min	2	100	12	90	160	2,76
18	N18	26	Incl	max	2	100	12	90	364	2,98
19	N19	15	Incl	min	6	100	12	90	283	2,31
20	N20	31	Incl	max	6	100	12	90	424	5,89
21	N21	5	Incl	min	2	500	12	90	80	1,47
22	N22	25	Incl	max	2	500	12	90	59	1,02
23	N23	32	Incl	min	6	500	12	90	463	0,8
24	N24	29	Incl	max	6	500	12	90	686	0,92
25	N25	6	Incl	min	2	100	24	90	120	10,88
26	N26	4	Incl	max	2	100	24	90	118	10,17
27	N27	23	Incl	min	6	100	24	90	153	10,89
28	N28	10	Excl	max	6	100	24	90		
29	N29	30	Incl	min	2	500	24	90	42	4,17
30	N30	22	Excl	max	2	500	24	90	100	2,74
31	N31	34	Incl	min	6	500	24	90	165	1,22
32	N32	7	Incl	max	6	500	24	90	106	10,56
33	N33	12	Incl	min	4	300	18	60	248	1,71
34	N34	9	Incl	min	4	300	18	60	313	0,98
35	N35	16	Incl	min	4	300	18	60	445	1,74

**Figure 1 : Worksheet – Overview of all experiments**

The "summary of fit" (figure 2) displays a graphical summary of the statistical key parameters.

$R2$ is the percent of the variation of the response explained by the model. $R2$ is a measure of fit, i.e. how well the model fits the data. A large $R2$ is a necessary condition for a good model, but it is not sufficient. In fact, $R2 < 0.5$ indicates a model with rather low significance and $R2 = 1$ indicates a perfect model. In the present study was obtained a large $R2$ ($R2 = 0.77$) for both response (particle size and % dissolved) which indicate that this a significant model that fits well the data.

$Q2$ is the percent of variation of the response predicted by the model according to cross validation. $Q2$ shows an estimate of the future prediction precision, i.e. how well the model predicts new data. $Q2$ should be greater than 0.1 for a significant model and greater than 0.5 for a good model. From figure 2 can be seen that $Q2$ value obtained was low, but significant, for particle size response ($Q2 = 0.195$) either % dissolved response ($Q2 = 0.332$). When exists a good $R2$, moderate model validity, and a design with many degrees of freedom of the residuals, then a poor $Q2$ is usually due to insignificant terms in the model. To increase $Q2$ value insignificant terms can be remove.

Model validity is a measure of the validity of the model. When the Model validity bar is larger than 0.25, there is no lack of fit of the model (the model error is in the same range as the pure error). A Model validity bar of 1 represents a perfect model. It can be seen from the data in figure 2 that was obtained a reasonable value of Model validity for both response indicating that there is no lack of fit of the model.

Reproducibility is the variation of the response under the same conditions (pure error), often at the center points, compared to the total variation of the response. The reproducibility value obtained for both response is above 0.5 which means that there is a low poor error, a high control of the experimental set up (the noise level is low) and it can assess the validity of the model.
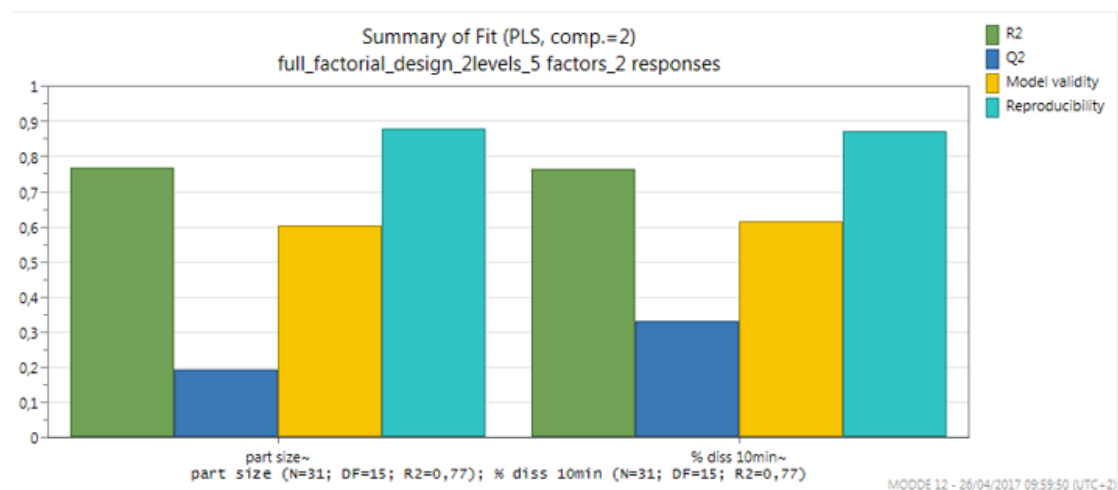
Figure 2: Summary of statistical key parameters concerning results fitting

Coefficient overview plot (figure 3) displays the coefficients for all the responses. To make the coefficients comparable when responses (Y's) have different ranges, the coefficients are normalized, that is, the coefficients are divided by the standard deviation of their respective response. This plot allows us to see how the factors affect all the responses.

Closer inspection of the figure 3 shows that exists 20 factors: 6 main factors and 14 interactions between factors affecting both responses. In total, there are 8 factors affecting positively both responses. An important interaction was detected between the number of balls and time (num*time) showing a positive effect on the particle size response. It can be probably due to an increase in the number of collisions between balls, powder and bowl with a consequent more energy transfer from ball to powder.(17) On the other side, the interaction between factors with most negative influence is the combination of frequency and time (freq*time). Increasing the frequency (freq) and the time, keeping other factors unalterable, drastic conditions are reached thus leading to the aggregation of the particles and, consequently, the most negative value. As a common rule, the maximum fracturing in the high energy milling takes place in a short time.(18) Moreover, also the interaction between the number of balls and the drug amount (num*gly) showed a positive effect on particle size response: such an effect could be explained since the concomitant increase of both the number of balls and the amount of the drug may increase the possibility of collisions between the balls and the powder. Otherwise, the interaction between drug amount and frequency

(gly*freq) seemed to have a negative effect, probably attributable to some aggregation phenomena induced by the drastic conditions.

Concerning the % dissolved in 10min response, figure 3 illustrates, two main factors as critical: the frequency (freq), with the most positive influence; and, the amount of glibenclamide (gly), with the most negative influence. The higher the milling frequency, the highest is energy transfer from ball to powder. Then the drug is brought in an 'activated state' and it can be hypothesized that the higher energy states produced by grinding converted the crystalline structure of the drug into an amorphous state, leading to an higher dissolution rate.(19) On the other hand the amount of glibenclamide (gly), seemed to be the factor with the most negative influence. Probably, higher is the amount of the drug, less is the efficiency of the grinding process to induce particle size reduction due to the reduced internal free space available for the movements of the balls within the jars, thus decreasing the possibility of collisions between the balls and the powder.
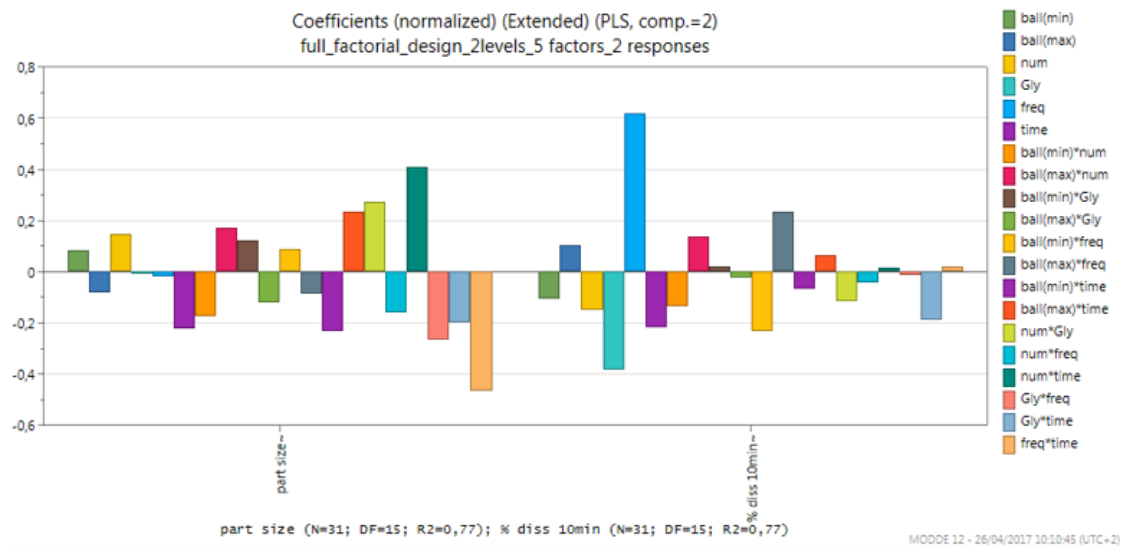

Figure 3 Coefficients overview plot

The figures 4 and 5 present the coefficient plots for particle size and % dissolved in 10min, respectively. The data of figure 5 were obtained through a full factorial design

(2^5) considering just one response (% dissolved in 10min) and 32 experiments (N14, N15 and N28 were excluded) which resulted in R2 and Q2 a bit higher. These plots display the regression coefficients with confidence intervals. The coefficient plot shows coefficients relating to scaled and centered variables, so, it can be used to evaluate the significance (p-value < 0.05) of the model terms. The size of the coefficient represents the change in the response when a factor varies from 0 to 1, in coded units, while the other factors are kept at their averages. The coefficient is significant (different from the noise), when the confidence interval does not cross zero. Thus, with a confidence interval of 95%, for particle size response only two factors are significant: num*time (positively) and freq*time (negatively), as observed in figure 4. Considering just % dissolved in 10min as single response, as can be seen in figure 5, there are two significant factors with negative influence: amount of glibenclamide (gly) and combination of amount of glibenclamide and time (gly*time). Probably, the efficacy of the grinding process to induce particle size reduction is lower when the amount of the drug is high even if in case of a prolonged mechano-chemical treatment. The frequency (freq), showed a positive influence, with a confidence interval of 95%, as explained above. The factors with statistical significance in Figure 4 are the same as the factors with most influence observed in Figure 3 for both response.
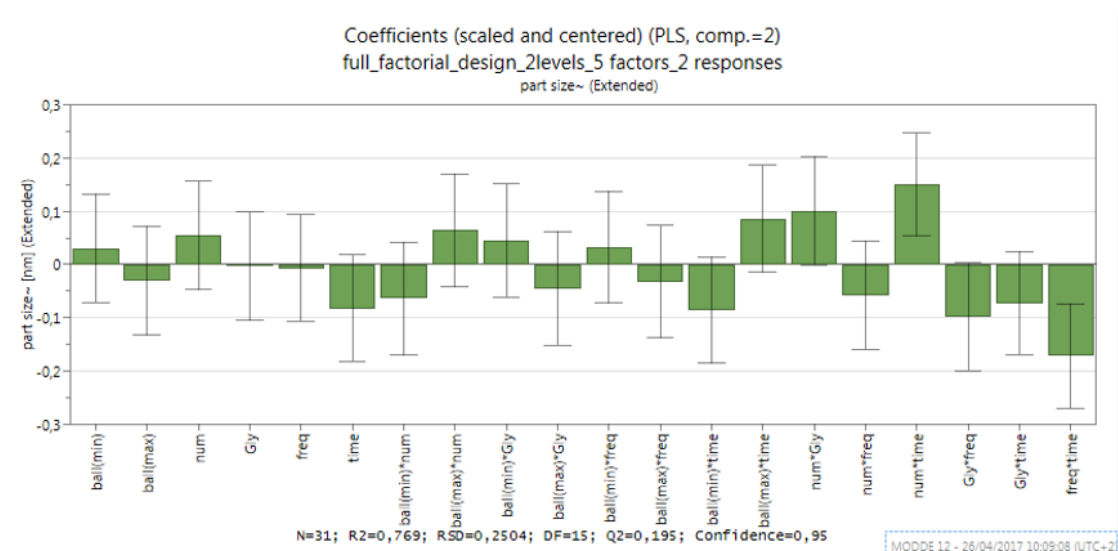

Figure 4 Coefficient plot: particle size

26
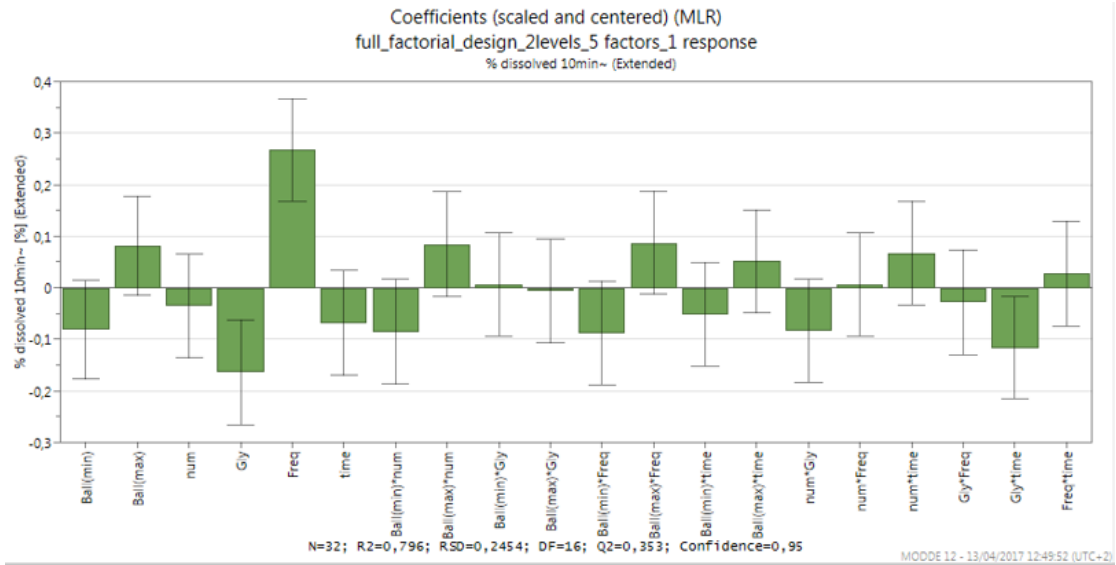
Figure 5 Coefficient plot: % dissolved in 10min

The normal probability of residuals, for both responses (particle size and % dissolved in 10 min), is shown in figure 6. This plot displays the residuals (standardized) on a double Log scale. It allows detect outliers and assess normality of the residuals. If the residuals are random and normally distributed, the normal probability plot of the residuals has all the points lying on a straight line between -4 and +4 standardized standard deviations. For both the particle size response and the % dissolved in 10min one all the points are on a straight line on the diagonal, indicating that the residuals are normally distributed noise.
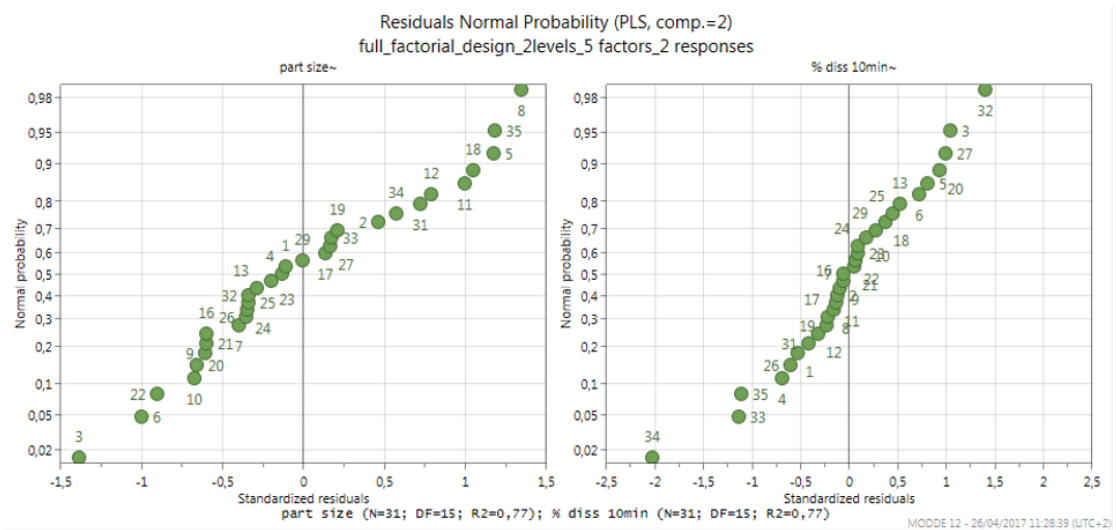
Figure 6 Normal probability plot of residuals

5 Conclusion

High-energy ball milling proves to be a useful method to produce glibenclamide nanoparticles. Low solubility drugs lead to low oral bioavailability and erratic absorption. This is particularly visible in drugs within class II of the BCS, as is the case with glibenclamide. Nanotechnology overcomes that problem since it promotes the decrease of the drug particles size, increasing the surface area leading to better dissolution, absorption and bioavailability. Some variables in the process of grinding of GLB in a high-energy ball mill positively influenced its size and dissolution properties. The different conditions utilized to appropriately produce GLB nanoparticles were analyzed and compared through a full factorial experimental design. Five independent variables (amount of GLB, number of balls, balls diameter, grinding frequency and time) at two-levels were studied to evaluate the influence of both each one separately and their interaction on the mean particle size and the percent of dissolved drug after 10 min (responses).

The study showed that the interaction between the number of balls and the time (num*time) was a crucial factor that positively influenced the mean size of particles. Increasing the number of balls, the number of collisions between the balls and the power also increase leading to a greater amount of nanoparticles produced. On the other hand, increasing the grinding frequency and the time (freq*time) drastic conditions are reached leading to particle aggregation process and, consequently, lower amount of nanoparticles.

A progressive increase of grinding frequency (freq) led to a better GLB dissolution performance, probably, due to drug amorphization process during grinding. On the contrary, an increase of the amount of glibenclamide (gly) decrease the efficiency of the dissolution process, even when extending the time of grinding (gly*time).

Through this study, it was possible to screen the significant process variables. These variables can be optimized in more detail in future studies with the propose of reaching the perfect conditions to produce glibenclamide nanoparticles.

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Annex

A1.   Noyes-Whitney equation

$$\mathbf{dc/dt = k \ (C_s - C_b)} \ (1)$$

dc/dt: dissolution rate of the drug

k: dissolution rate constant

C_s: concentration of drug in stagnant layer

C_b: concentration of drug in the bulk of the solution at time t