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Abbreviations: CMA, critical material attributes; CPP, critical process parameters; CQA, critical quality attributes; DE, dissolution efficiencies; DPIs, dry powders for inhalation; DSC, differential scanning calorimetry; ED, emitted dose; EF, emitted fraction; FPD, fine particle dose; FPF, fine particle fraction; FSI, fast screening impactor; GB, glyceryl dibehenate; HPLC, high-performance liquid chromatography; MMAD, mean mass aerodynamic diameter; NGI, next generation impactor; PCA, principal component analysis; QbD, quality by design; QTTP, quality target product profile; SA, stearyl alcohol; SEM, scanning electron microscope; SLMs, solid lipid microparticles; SS, salbutamol sulfate.

1. Introduction

Inhalation administration is attracting much attention in the past two decades as being the noninvasive route for not only local but also systemic drug delivery (Javadzadeh and Yaqoubi, 2017). Due to the recent appearance and rapid spread of a novel respiratory virus, pulmonary drug delivery, especially via dry powders for inhalation (DPIs), are attracting great interest (Sun, 2020). Currently marketed dry powders for inhalation (DPIs) are generally based on the so-called ordered mixtures of the active ingredient(s) and lactose as a carrier (Thalberg et al., 2004). It has been shown that addition of carrier may be associated with problems, such as poor uniformity of the blend and poor detachment of the drug from carrier surfaces (Carvalho et al., 2015), which can result in incomplete and uneven pulmonary drug delivery. Furthermore, there is no potential for modification of the drug release. There are many published examples of improved pulmonary drug delivery through formulation of polymer microparticles (Hitzman et al., 2006; Kim et al., 2012; Abdelazis et al., 2018 etc.). However, due to the potential issues with biocompatibility and/or biodegradability of polymers (Smith and Hunneyball, 1986; Armstrong et al., 1996; Mehnert and Mader, 2012), alternative materials such as lipids are being investigated for preparation of microparticles with the appropriate aerodynamic properties. Biodegradable lipids that do not present a toxicological risk for pulmonary delivery are available. One of the most significant properties of lipids is the possibility to develop low-density and low surface energy microparticles (Cipolla et al., 2014), which is an optimal feature from the perspective of aerodynamic properties of DPIs. Lipid materials can provide slow release for soluble drugs with a short elimination half-life, such as salbutamol sulfate (SS) (Daman et al., 2014), short-acting bronchodilator used for the treatment of bronchial asthma. Due to the high solubility and short half-life of SS, development of a DPI with modified drug release properties would be of significant therapeutical importance.

Solid lipid microparticles (SLMs) are predominantly prepared by the melt-emulsification method coupled with filtration, centrifugation and/or lyophilisation in order to obtain water-free SLMs (Scalia et al., 2015). This method excludes the usage of organic solvents, making it environmentally friendly (Scalia et al., 2015). However, spray-drying method may also be used for the production of inhalable SLMs, and usually it includes organic solvent(s) (Ben-Jebria et al., 1999; Vanbever et al., 1999; Cook et al., 2005; Jaspart et al., 2005; Sebti and Amighi, 2006; Daman et al., 2014). According to the published data, Mezzena et al. (2009) were the first and only group

who used melt-emulsification method for the preparation of microparticles and the spray-drying technique for removal of water (Mezzena et al., 2009). The main benefit of spray-drying in DPIs production is the potential to control various particle attributes such as size distribution, surface morphology and energy by regulating parameters of the spray-drying process such as feed rate, inlet/outlet temperature, and aspiration (Mehta, 2018).

Since there are many material attributes and process parameters that can impact the quality of DPIs, it is recommended to use a quality by design (QbD) approach in the formulation development and manufacturing process optimization (Buttini et al., 2018). In the presented study, a QbD approach was applied in the formulation and production of SS DPIs in the form of SLMs, by using glyceryl dibehenate or stearyl alcohol as the lipid matrix. The main objective of this study was to optimize the parameters of the complex melt-emulsification process coupled with spray-drying, in order to maintain the balance between powders aerodynamic performance and SS release rate. To the best of our knowledge this is the first study presenting a thorough QbD based approach in the development of SLMs to be used as DPI.

2. Materials and Methods

Materials

Salbutamol sulfate and stearyl alcohol were provided by Galenika (Belgrade, Serbia), glyceryl dibehenate (Compritol® ATO 888) was obtained from Gattefosse´ (Lyon, France) and poloxamer 188 (Kolliphor® P188) was supplied from BASF (Ludwigshafen, Germany) as gifts. Trehalose dihydrate was purchased from TCI Chemicals (Tokyo, Japan). Diammonium hydrogen phosphate was purchased from Merck (Rome, Italy), while Tween® 20 and Tween® 80 from VWR (Radnor, USA). Phosphoric acid (85%) was supplied from VWR (Radnor, USA). Hydroxypropylmethylcellulose (HPMC) size 3 capsules were obtained from Lonza Capsule Delivery Solutions (Capsugel® Vcaps® Plus DPI, Colmar, France) and RS01 Dry Powder Inhaler device (flow rate 60 L/min) was gifted by Plastiape® S.p.a. (Osnago (LC), Italy). Sodium chloride, potassium chloride and potassium dihydrogen phosphate were obtained from A.C.E.F. (Piacenza, Italy). Disodium hydrogen phosphate was obtained from Alfa Aesar (Haverhill, USA). All the solvents were of analytical grade and were purchased from commercial suppliers.

Methods

2.1. QbD-based development of SLMs

QbD strategy was initiated by definition of the Quality Target Product Profile (QTTP). QTTP was defined in order to balance the appropriate SLMs aerodynamic performance and modified *in vitro* dissolution of SS at the same time. The initial step of this QbD strategy was to create an Ishikawa diagram, in order to identify the parameters that could affect the quality of a SLMs powder intended to be suitable for inhalation as a DPI product. Ishikawa cause and effect diagram was constructed according to Buttini et al. (2018), and depicted in Figure 1, taking into account melt-emulsification coupled with the spray-drying process for production of the SLMs powder for inhalation.

The realization of the QTPP (Table 1) can be achieved by the formulation of the DPI with a quality profile described by the Critical Quality Attributes (CQAs). The fine particle fraction (FPF) is the most important characteristics for DPI performance. The emitted fraction (EF) is another important critical quality attribute to take into consideration, indicative of the percentage of the loaded dose that leaves the device upon inhalation and is available to the patient (Buttini et al., 2016). Targeted

CQAs can be accomplished through selection and optimization of the Critical Material Attributes (CMAs) and the Critical Process Parameters (CPPs). In this study, high-shear mixing time, high-shear mixing speed and washing of microparticles were identified as potential CPPs for the melt-emulsification, whereas airflow rate and temperature were chosen to be CPPs for the spray-drying process (Table 1, Figure 2). High-shear mixing speed and time were based on literature data (Sanna et al., 2004; Mezzena et al., 2009; Scalia et al., 2012; Scalia et al., 2013a) and preliminary experiments. Lipid type and content, surfactant (poloxamer 188) content and trehalose addition were chosen to be CMAs. The influence of trehalose addition was tested, since carbohydrates have been used successfully as (cryo)protectans in the freeze- and spray-drying of solid lipid nanoparticles. Trehalose was chosen since it was proven that it was the most efficacious protectant that could prevent particle aggregation during the spray-drying process of solid lipid nanoparticles with Compritol® (Freitas and Muller, 1998) or SLMs freeze-drying process (Zhang et al., 2008). Up to date, there are no available publication describing the effect of protectants addition on spray-drying of inhalable SLMs formulations. In addition, the effect of washing of microparticles on SLMs properties was evaluated.

QTPP, CQAs, potential CMAs and CPPs are listed in Table 1, according to Pallagi et al. (2016) and preliminary experimental findings, together with the desired targets and appropriate justifications.

2.2. Preparation of SLMs

The SLMs were prepared by the melt-emulsification method (Jaspart et al., 2007; Mezzena et al., 2009; Scalia et al., 2012; Scalia et al., 2013a; Scalia et al., 2013b), followed by the spray-drying of suspended microparticles. Lipids (glyceryl dibehenate, GB or stearyl alcohol, SA) and the active ingredient (SS) were heated to about 10-15 °C above lipids' melting points (90 °C in the case of GB and 70 °C in the case of SA), to allow the melting of the lipid phase. Poloxamer 188 and water were heated at the same temperature of the lipid phase. The hot aqueous phase was slowly added to the lipid phase (phase inversion process), in order to avoid the loss of SS and lipids during the manufacturing. The hot emulsion was maintained at 70 or 90 °C (depending on the used lipid), and subjected to the high shear mixing (at 13,400 or 17,400 rpm) with an Ultra-Turrax T-25 mixer (IKA-Werk, Staufen, Germany) for 2 or 8 minutes. The obtained emulsions were then cooled down to room temperature under magnetic stirring. During the cooling process, microparticles were

slowly formed. Some of the formed microparticles suspensions (as indicated in Table 2) were then centrifuged twice on 4000 rpms for 15 minutes (Universal 32, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany), in order to wash the microparticles. Microparticles washing was performed to remove the SS which was not encapsulated and remained on the microparticles' surface. Microparticles were washed with the poloxamer 188 solution (0.40-1.50% w/v, according to the poloxamer 188 concentration in different formulations) in order to avoid the agglomeration. In the case of four formulations (F8, F9, F10 and F14), trehalose solution (20%, w/v) was added before the spray-drying process in a ratio 60:40 (formulation:trehalose solution). Following washing and addition of trehalose, the suspensions were spray-dried (Büchi Mini Spray Dryer B-290, Büchi Laboratory-Technique, Flawil, Switzerland) in order to obtain the SLMs powders. The spray-drying conditions were as following: nozzle size 0.7 mm; inlet temperature 60-90 °C, (depending on the lipid); outlet temperature 43-62 °C (depending on the inlet temperature); aspirator 100%; feed rate (0.35-1.80 mL/min) and spraying air-flow rate 473-670 L/h. Prior to the spray-drying process, SLMs suspensions were weighed and their weights were approximately 150 ± 10 g. Following the spray-drying, SLMs powders were collected from powder collector and weighed. The yield of SLMs powders, calculated based on total weight of solid amount, ranged between 46% and 60%, depending on the applied process parameters. Scheme of SLMs preparation process is depicted on Figure 2, together with the characterization of the obtained SLMs powders.

2.3. Drug loading determination

The SLMs samples (20.00 ± 0.05 mg) were accurately weighed on an analytical balance (E 50 S, Gibertini, Novate Milanese (Mi), Milan, Italy) and placed in volumetric flasks with 1 mL of phosphate buffer saline, PBS (Scalia et al., 2013a; Scalia et al., 2015). The flasks were heated in a water bath at 90°C and sonicated for 15 minutes, to allow the melting of the lipid component of the lipid microparticles and then the solubilization of the active ingredient in the PBS. When the sonification was finished, the samples were diluted to 50 mL and filtered (0.45 μm cellulose acetate membrane filters, Sartorius, Göttingen, Germany). SS concentration in those samples was determined by the high-performance liquid chromatography (section 2.4). Drug loading was calculated based on Equation 1 (Scalia et al., 2015):

$$\text{Drug loading (\%)} = \frac{\text{Mass of drug assayed in microparticles}}{\text{Total weight of the sample}} \times 100 \quad (1)$$

2.4. High-performance liquid chromatography (HPLC)

The HPLC system (LC-10AT, Shimadzu Europe GmbH, Duisburg, Germany) consisted of a pump (LC-10AT VP, Shimatzu), a UV-VIS detector (SPD-10A VP, Shimatzu) set to 276 nm, a Waters 717plus Autosampler (Waters Corporation, Milford, USA) and a column (Supelcosil™ LC-SCX, 25 cm x 4.6 mm, 5 µm, Sigma-Aldrich, St. Louis, USA) was used for SS quantification. Mobile phase consisted of phosphate buffer (pH 7.0) and methanol in the ratio 40:60 (%, v/v) at a flow rate of 1.0 mL/min. The phosphate buffer (pH 7.0) was prepared by dissolving 6.00 g diammonium hydrogen phosphate in 1 L of MilliQ water, and phosphoric acid was used to adjust the pH value to 7.0. Temperature of the column was set to 30 °C and sample injection volume was 20 µL. The method's linearity (R^2 = 0.9999) was confirmed over the concentration range 5-200 µg/mL, using standard aqueous solutions of the SS. The sensitivity of the method was estimated in terms of limit of quantification (LOQ) and limit of detection (LOD). The determined LOQ and LOD were 2.09 µg/mL and 0.63 µg/mL, respectively. In addition, instrument repeatability precision was also confirmed (RSD = 0.75%).

2.5. SLMs morphology

Scanning electron microscope (SEM, Zeiss AURIGA, Oberkochen, Germany) was used for the morphological analysis of the formulations. Powders were deposited on a double-sided adhesive tape, pre-mounted on aluminum stubs, and inserted into the chamber for analysis without undergoing any metallization process. The SEM was operated under high vacuum conditions with an accelerating 1.0 kV voltage. Images were taken at random locations, at different magnifications.

2.6. Solid-state characterization studies

2.6.1. Differential scanning calorimetry (DSC)

Thermal behavior of the representative SLMs formulation (F7), SS raw material, GB and poloxamer 188 was analyzed by Mettler DSC 821e STARe system (Mettler Toledo, Greifensee, Switzerland). Samples (2–4 mg) in pierced aluminum crucibles, under a dynamic nitrogen atmosphere (100 mL/min), were heated from 25 to 250 °C at a scanning rate of 10 °C/min.

2.6.2. Powder X-ray diffraction

Powder X-ray diffraction patterns on SS, GB, poloxamer 188 and F7 powders were recorded on a Rigaku MiniFlex diffractometer (Tokyo, Japan) using a CuKα radiation (30 kV, 15 mA) at a step scan of 0.05/2 s in the 2θ° scanning range from 2 to 50.

2.7. SLMs size distribution

SLMs size distribution was determined by the laser light scattering (Mastersizer 2000, Malvern Instruments, Malvern, UK). The samples were dispersed in Scirocco dry dispersion unit by using a pressure of 4 bars and feed rate of 28-35%. Relative refraction index of 1.5 and absorption index of 0.01 were used. Analyses of all samples were performed in triplicate with an obscuration rate of 0.5-6%. The particle size was expressed as cumulative undersize volume diameter at 50% of particle population (d_{v50}), and the volume mean particle diameter ($D_{[4,3]}$), which is the average diameter, balanced by the total volume of particles contained in each histogram class (Jaspart et al., 2005; Depreter and Amighi, 2010). Particle size distribution was evaluated based on span values.

2.8. True density

The true density was assessed by helium pycnometer (AccuPyc II 1340, Micromeritics Instrument Corporation, Norcross, Georgia, USA). Each sample was analyzed in triplicate.

2.9. In vitro aerodynamic assessment of SLMs formulations

In vitro aerosol assessment of SLMs formulations was carried out using the Fast Screening Impactor (FSI, Copley Scientific, Nottingham, UK) and, based on the FSI analysis, six formulations were chosen for further testing by the Next Generation Impactor (NGI, Copley Scientific, Nottingham, UK).

2.9.1. Fast Screening Impactor study

The FSI divides the particles discharged from the inhaler into two parts, namely a coarse fraction and a fine fraction (lower than 5 μm as aerodynamic diameter), respectively. The Coarse Fraction Collector (CFC) is equipped with the insert that enables a cut-off of 5 μm at 60 L/min. The particles not captured in the CFC keep following the airstream and deposit in the fine fraction collector (FFC) where a filter captures all of them. The FSI was connected to the VP1000 vacuum pump

Erweka GmbH, Heusenstamm, DE) via TPK (Copley Scientific, Nottingham, UK). The flow rate of 60 L/min was set using the DFM 2000 Flow Meter (Copley Scientific, Nottingham, UK).

An amount of 20 ± 0.5 mg of powder, accurately weighed, was manually introduced into a size 3 hard HPMC capsule. The capsule was then inserted into the holder chamber of the DPI device (RS01, Plastiape S.p.A., Osnago (LC), Italy) and pierced. The device was connected to the FSI and passed by the air stream for 4 s at 60 L/min. The type A/E glass filter (76 mm, Pall Corporation, New York, USA) of FFC was weighed before and after the air actuation, in order to determine the amount of powder deposited, denoted as fine particle dose (FPD). Each powder was tested in triplicate.

2.9.2. Next Generation Impactor study

The aerodynamic parameters of representative SLMs formulations were obtained using the NGI with an USP induction port. Prior to use, the NGI cups were coated with a thin layer of ethanol containing 2% (w/v) solution of Tween® 20 to prevent particle bounce. The micro-orifice collector (MOC) was covered with a glass microfiber filter (82.60 mm, 934-AH, Whatman GE Healthcare, UK). The NGI was connected to the VP1000 vacuum pump via TPK and the flow rate of 60 L/min was set using the DFM 2000 Flow Meter.

An amount of 20 ± 0.5 mg of powder, accurately weighed, was manually introduced into a size 3 hard HPMC capsule. The capsule was then inserted into the holder chamber of the RS01 DPI device and pierced. The device was connected to the NGI through the induction port, and passed by the air stream for 4 s at 60 L/min. Each powder was tested in triplicate.

After actuations of three capsules for each formulation, the amount of SLMs powder, deposited in all components of the assembled NGI (induction port, cups and MOC), device and mouthpiece adapter, was recovered with a solution of 1% Tween® 80 (w/v) in MilliQ water (previously heated to 70°C) and sonicated to collect the deposited powders. After sonification, the content of all NGI stages and device was poured in separate volumetric flasks. Then, the flasks were placed in a water bath, heated at 90°C and sonicated to melt the lipid matrix of the microparticles and dissolve SS. The flasks were brought to the final volume with 1% Tween® 80 (w/v) MilliQ water solution. Each samples was filtered (0.45 μm cellulose acetate membrane filters) and analyzed by HPLC method, described in section 2.4.

The metered dose (MD) is the mass of drug quantified by HPLC, calculated by summing the drug recovered from the inhaler and the impactor (induction port, stages 1 to 7 and MOC). The emitted dose (ED) is the amount of drug leaving the device and entering the impactor (induction port, stages 1 to 7 and MOC). The MMAD was determined by plotting the cumulative percentage of mass less than the stated aerodynamic diameter for each NGI stage on a probability scale versus the aerodynamic diameter of the stage on a logarithmic scale. The FPD is the mass of drug < 5 μm, calculated from log-probability plot equation. The FPF is the ratio between FPD and ED in percent.

2.10. In vitro drug release studies

The *in vitro* drug release of the raw SS and from the selected SLMs formulations: F1, F7, F9, F10 and F11 was tested in Franz diffusion cells (Vertical Franz Type Diffusion Cells, diameter 4.6 mm, Area 18 mm^2) using the slightly modified method as described in a paper of Scalia et al. (2012). Formulations with the highest FPF values, based on FSI results were selected. A cellulose acetate hydrophilic filter (0.45 μm, Sartorius Stedim Biotech GmbH) was soaked in a crystallizer containing the dissolution medium (PBS pH 7.4, containing 0.1% Tween® 80) for conditioning, 16 hours before the experiment. Receiver compartment was filled with 18 mL of dissolution medium, heated to 37 ± 0.5 °C and kept under constant stirring during the test. Accurately weighted samples of raw SS (1.00 ± 0.05 mg) and the selected SLMs formulations (5.00 ± 0.05 mg) were put on the previously wetted cellulose acetate membrane and 1 mL of dissolution medium was put in the acceptor compartment, in order to facilitate uniform spreading of the powder over the whole membrane surface. At certain time intervals (2, 6, 10, 15, 20, 30, 45, 60, 75, 90 and 120 minutes), 1 mL of sample from the receptor compartment was withdrawn and immediately replaced with 1 mL of fresh medium, heated at 37 ± 0.5 °C. The samples were filtered (0.45 μm cellulose acetate membrane filters) and analyzed by the HPLC method, described in section 2.4.

When the experiment was concluded, the cellulose acetate membrane was sonicated in a crystallizer for 15 minutes with 5 mL of dissolution medium. The liquid was then poured in a 50 mL volumetric flask, to which 30 mL of dissolution medium was added. The flask was placed in a water bath and heated at 90°C, under magnetic stirring, up to 30 minutes to melt the lipid matrix of the microparticles and dissolve SS. The flask was then brought to the final volume with the dissolution medium. A sample was filtered (0.45 μm cellulose acetate membrane filters) and

analyzed by HPLC method, described in section 2.4. The percent of drug dissolved was calculated based on experimentally determined drug content.

The following dissolution parameters were determined: $\%DE_{15}$, $\%DE_{30}$, and $\%DE_{120}$, which are percentages of dissolution efficiencies (DE) after 15, 30 and 120 minutes, respectively, using DDSolver, an add-in program for Microsoft Excel, developed by Zhang et al. (Zhang et al., 2010).

In order to analyze SS release from the SLMs formulations, experimentally obtained dissolution data were fitted to the first-order, Higuchi, Peppas-Sahlin and Korsmeyer-Peppas models. First-order model was selected since this model is applicable for dosage forms containing water-soluble drugs in porous matrices (Costa and Lobo, 2001) and Higuchi model is usually applied for non-dissolving matrices (Karasulu et al, 2003). Peppas-Sahlin and Korsmeyer-Peppas models were used to determine the exact SS release mechanism from SLMs, as the drug can be released by diffusion and/or erosion of the lipid matrix.

The Higuchi model is described by equation 2 (Higuchi, 1961):

$$F = \frac{M_t}{M_\infty} = k_H \times t^{1/2} \quad (2)$$

where F is the fraction of the dissolved SS at time t, M_t is the amount of dissolved SS in any time t, M_∞ is the amount of dissolved SS at infinite time, k_H is the Higuchi release rate constant.

The first-order model is described by equation 3 (Polli et al., 1997):

$$F = \frac{M_t}{M_\infty} = (1-(e^{-k1st \times t})) \times 100 \quad (3)$$

where k_{1st} is the first-order release rate constant.

The Korsmeyer-Peppas model is described by equation 4 (Ritger and Peppas, 1987):

$$F = \frac{M_t}{M_\infty} = k_{kp} \times t^n \quad (4)$$

where k_{kp} is the Korsmeyer-Peppas release rate constant and *n* is the diffusional exponent.

The Peppas-Sahlin model is described by equation 5 (Peppas and Sahlin, 1989):

$$F = \frac{M_t}{M_\infty} = k_1 \times t^m + k_2 \times t^{2m} \quad (5)$$

where k_1 is the Fickian diffusion rate constant, *m* is the diffusional exponent, k_2 is the non-Fickian anomalous rate constant.

DDSolver was used for the calculation of coefficient of determination R^2, as well as *in vitro* release rate constants and diffusional exponent *n*.

2.11. Statistical analysis

As explained in the introductory section, QbD approach was used to better understand and establish a link between the CPPs and CMAs, and their influence on the CQAs of the final SLMs DPI product.

Statistical method based on Lenth t-ratios was used to determine CMAs and CPPs which significantly affected SLMs micromeritic properties, where *p* values < 0.05 were considered to be significant. Due to the fact that numerous parameters, both materials and process, potential affect CQAs, a multivariate data analysis method was also used to analyze the obtained results. Principal component analysis (PCA) is a classical data analysis technique that finds the linear transformations of data, by reducing the dimensionality and retaining the maximal amount of variance (Ilin and Raiko, 2010). This is achieved by the transformation of the whole dataset to a new set of uncorrelated variables, which are ordered in a manner that the first few retain most of the variation present in all of the original variables (Jolliffe and Cadima, 2016). PCA was performed in the present study in order to correlate the following parameters: addition of trehalose, spraying airflow rate, as well as SLMs micromeritic properties: particle geometric diameter, true density, and their aerosol performance - described by the two parameters, EF and FPF. Therefore, the aim of PCA was to determine the factors that could affect FPF and EF in order to optimize process parameters and obtain a formulation with highest possible FPF value with appropriate EF. PCA was carried out using PAST, version 4.03 (Hammer et al., 2001).

3. Results and discussion

3.1. Drug loading

SS loading in SLMs formulations was in the range from 0.87-14%, depending on the lipid type and the fact whether the SLMs were washed or not (Table 3). Formulations F13, F14 and F15 had the lowest drug loading. This was not expected since SA was chosen as a polar lipid (Scalia et al., 2015) with higher potential for encapsulating hydrophilic drugs in comparison to GB. As it was supposed, the higher drug loading was observed in non-washed SLMs formulations (F1, F6, F7 and F10), since part of SS was encapsulated in the SLMs matrix and part remained on the microparticle surface. The drug loading of F10 was not so high as in the case of other three non-washed formulations since this formulation included trehalose.

The addition of trehalose led to drug loading decrease (F9 vs F10 and F14 vs F15). For the non-washed formulations, it can be expected that the non-encapsulated SS will dissolve fast, whereas only the part of SS which is encapsulated in SLMs matrix will dissolve in a sustained manner.

Having in mind that a single dose of SS is 100-400 μg (Easyhaler® Salbutamol information leaflet, 2018), even SLMs formulation with the lowest drug loading will provide these doses with a relatively low and tolerable amount of powder.

3.2. SLMs morphology

SEM images of some SLMs formulations are reported in Figure 3. As can be observed from SEM images, GB formulation F7 (Figure 3a) showed small particles with a regular shape.

SEM image of the GB formulation with trehalose (F9) is illustrated in Figure 3b. It can be observed that those particles are also small and spherical, but more porous than those obtained from the formulation without trehalose (F7). High porosity is preferable for SLMs formulations since it can provide higher FPF. On the contrary, lipid SA provided most of the particles with irregular surface, although there were some perfectly spherical particles (F14) but with no porosity at all. In addition, two different particles generation were observed in SLMs F14 formulation with SA, indicating that components were not mixed homogenously (Figure 3c). In the case of F15 (formulation without trehalose), observed SLMs showed no sphericity, which indicated that this formulation is not suitable for inhalation administration (Figure 3d).

3.3. Solid-state characterization studies

Solid-state studies were performed in order to exclude the modification of the lipid solid state and/or drug degradation during the manufacturing of SLMs.

3.3.1. Differential scanning calorimetry (DSC)

DSC thermogram of the representative SLMs formulation, F7, displayed peaks of GB and poloxamer 188 without any changes in their thermal events, whereas a modification of SS peak was noticed. In the case of F7, SS endothermic peak was at 180 °C, compared to the raw SS with the endothermic peak at 204-205 °C (Figure 4). Both melting points for pure SS can be found in literature (Murphy et al., 2003; Scalia et al., 2013a). According to Murphy et al. (2003), a sharp discontinuity was seen in the region of 170-180 °C that corresponded to the melt/decomposition temperature of the spray-dried SS. It is interesting that they noticed a similar behavior in the case of both, amorphous and crystalline states of SS (Murphy et al., 2003). But, according to other authors (Shariare et al., 2011; Haghi et al., 2012; Scalia et al., 2013a; Ong et al., 2014) raw SS displayed an endothermic peak at around 205 °C. In the case of F7, it can be supposed that a change in SS peak can be attributed to the fact that SS is miscible and starts to dissolve in other components of the formulation prior to their melting (Medarević et al., 2019). Based on these studies, it is important to emphasize that there was no sign of SS degradation during the melt-emulsification and spray-drying process.

3.3.2. X-ray diffraction

The X-ray diffraction patterns of representative SLMs formulation F7, GB, SS and poloxamer 188 are shown in Figure 5. It can be observed that characteristic peaks of GB on 6.51°, 22.5° and 24.6° of 2θ/θ are present on F7 diffractogram. Also, the characteristic peak of poloxamer 188 on 20.6° of 2θ/θ can be observed in the F7 diffractogram, whereas the other characteristic peak on 22.5° cannot be claimed for sure since it is overlapping with GB peak and having in mind that poloxamer 188 concentration is lower than GB concentration in F7. All characteristic peaks for pure GB and poloxamer 188 are in agreement with the data available in literature (Jaspart et al., 2007; Scalia et al., 2013a). This is important since it can be observed that no lipid structure modification occurred during melt emulsification or spray-drying processes. The characteristic crystalline peaks of raw SS on 11.2°, 19.7°, 22.4° and 24.1° also comply well with the already published data (Raula et al.,

2008; Davies et al., 2013; Zellnitz et al., 2019), but they could not be detected in the diffraction pattern of the F7. ~~That is also a~~One of ~~the~~ possible reasons why SS peaks are not visible in the diffractogram of formulation F7 is that the drug~~. Actually, SS peak on 11.2° cannot be detected, and there is a possibility that other SS peaks are overlapping with other components peaks. In addition, SS~~ takes the smallest part of the sample in comparison to two other components GB and poloxamer 188. ~~That is also a possible reason why SS peaks are not visible in the diffractogram of formulation F7.~~ From the diffractogram of F7 can be observed that the baseline is not flat, which can indicate the presence of amorphous SS. However, it is difficult to determine the solid state of SS in this sample based on these results, so additional analysis should be performed.

3.4. Micromeritic properties

Results of the first set of experiments (formulations F1, F2, F3 and F4) indicated that parameters: 1.50% poloxamer, mixing time of 2 minutes and the mixing speed of 13,400 rpm used for obtaining formulation F4 (Table 2), gave the narrowest particles size distribution (represented by the span value of 1.14) and the smallest particle size (represented by the $D_{[4,3]}$ of 4.93 μm) although the d_{v50} value was slightly higher than d_{v50} value of F1 (Table 4). Those parameters (mixing time of 2 minutes with mixing speed of 13,400 rpm) were expected to be optimal since prolonged mixing time, coupled with the high mixing speed, could lead to larger particles with wider particle size distribution. This was explained by Sanna et al. (2004), who reported that prolonged time of emulsification leads to an increase in the particle size due to particle coalescence.

Jaspart et al. (2007) observed that the optimal results, in terms of SLMs size, could be achieved with the relatively lower mixing speed, which was set at 8,000 rpm. Melt-emulsification processing parameters were further kept at the optimal values (mixing time of 2 minutes and the mixing speed of 13,400 rpm), and formulations F5-F15 were prepared by varying the spray-drying parameters, and by evaluating the effects of SLMs washing and trehalose addition. Effect of the particle washing was evaluated and it appeared to have a significant effect on particle size ($p = 0.0087$). Namely, washed microparticles had smaller particle size than the unwashed (F4 vs F6 and F11 vs F7). Furthermore, increase in the spraying airflow rate to 670 L/h (F7) led to particle size decrease (F6 vs F7). This was also the case for F8 and F9, where higher airflow rate again gave smaller particles (5.90 vs 4.01 μm). The effect of trehalose addition can be observed by comparing F4 and F8, whereby the addition of trehalose solution to the SLMs suspension (F8) led

to the particle size increase upon spray-drying. This is confirmed in the case of F10 and F7, where the addition of trehalose (F10) has also led to increased particle size and has even increased the span value. Particle size decrease can be attributed to the higher spraying airflow rate, which significantly affected particle size ($p = 0.0054$). In addition, poloxamer 188 (%) was shown to have a negative effect on the particle size and span value ($p < 0.0001$ and $p = 0.0344$, respectively), meaning that smaller particle size with a narrower particle size distribution can be obtained with a higher content of surfactant poloxamer 188. This can be expected, having in mind that higher surfactant concentration could more efficiently prevent particle coalescence. All factors that significantly affect SLMs (GB formulations) micromeritic properties are represented in Table 5.

Powders true density was approximately 1.00 g/cm^3 for all formulations without trehalose, as shown in Table 4. However, with trehalose addition (formulations F8, F9, F10 and F14), powders true density increased, with the statistical significance of this effect ($p = 0.0009$). Similar results for the true density of SLMs formulations produced by the spray-drying method were reported by Daman et al. (2014). Namely, Daman et al. obtained powders with the true density of ≈ 1.1 g/cm^3, by using water-ethanol mixtures for the spray-drying process (Daman et al., 2014). It can be expected that lower true density can be achieved if ethanol is used instead of water, since ethanol will evaporate faster and more complete than water. In this study, we have managed to obtain the spray-dried powders with comparable true density without inclusion of organic solvents in the spray-drying process.

The effect of different type of lipid on the SLMs properties was further evaluated. Three formulations were prepared, in order to compare stearyl alcohol to glyceryl dibehenate as the lipid matrix for SLMs. Based on the results of SEM analysis and SS loading, as described previously, no further samples with SA were prepared. It can be observed that, however, contrary to GB formulations, in the case of SA being used as lipid, trehalose successfully stabilized SLMs (F14), which led to the smaller particle size (F14 vs F15).

3.5. In vitro aerodynamic assessment

FSI analysis were performed for all formulations except F6, F13 and F15, due to their large geometric particle size, $d_{v50} > 6$ µm. Particles of geometric size larger than 6 µm are not appropriate, unless their density is less than 0.40 g/cm^3, as in the case of large porous microparticles (Abdelazis et al., 2018). As it was already mentioned, F1-F15 powders true density

was approximately 1 g/cm^3, indicating that for particles with geometric diameter > 6 µm, theoretical aerodynamic diameter would be ≥ 6 µm, which is not the appropriate size for inhalation powders.

The results of FSI analysis are partly comparable to the results of geometric particle size analysis (Table 4), indicating that some formulations with smaller particle size (smaller d_{v50}) showed higher FPF as it was expected (Table 6). The exceptions from this assumption occurred in the case of formulations with trehalose such as e.g. F8, which had larger d_{v50} value (5.90 µm) than F5 (5.55 µm), whereas its FPF was higher (Table 6). In addition, it was noticed that even though F9, F11 and F12 had almost the same d_{v50}, F9 exhibited higher FPF compared to the F11 and F12 (38.04% vs 27.01% and 27.93%, respectively). This indicated that probably addition of trehalose in F8 and F9 improved these powders aerodynamic performance.

The EFs for most of the formulations were higher than 75%, indicating that the powders were well fluidized and emitted from the capsules and the device.

The results of PCA demonstrated that the first and the second component capture the most variability (56.72% and 30.17%, respectively), with EF and d_{v50} having the highest loading on Component 1 and Component 2, respectively (Figure 6a). According to correlation loadings (Figure 6b), trehalose addition is the factor that affects EF, showing negative correlation between trehalose addition and EF. This means that adding trehalose to formulations will result in powders with lower EF. It can be supposed that this is the consequence of higher powders true density comparing to powders without trehalose (Table 4) as it can be observed from a negative correlation between powders true density and EF. In addition, EF is in a negative correlation with FPF, indicating that optimization of formulation can be a challenge since increase in FPF could result in decrease in EF and vice versa. Since FPF is in positive correlation with trehalose addition, it can be expected that, trehalose addition can result in higher FPF values. Even though trehalose was added with an aim to protect the SLMs during spray-drying, it is possible that it also improved final powders aerosol performance since carbohydrates such as trehalose, lactose etc. are usually added to DPIs since it is well known that they can improve powder aerosol performance. Finally, it has to be mentioned that one process parameter, spraying airflow rate showed a positive correlation with powders FPF. This was expected having in mind that, as it was shown in the chapter 3.4, higher spraying airflow rate led to smaller particle size (smaller d_{v50}) which

consequently led to the higher FPF. Negative correlation between d_{v50} and FPF was also confirmed here by PCA (Figure 6). Another examples of applying PCA for DPI formulations can be found in the available literature (Guenette et al., 2009; Lakio et al., 2015; Buttini et al., 2016; Muddle et al., 2017; Sun et al., 2020 etc.).

Since MMAD cannot be calculated based on the FSI results, NGI analysis were performed for six GB formulations (F1, F7, F9, F10, F11, F12) with the highest FPF based on the results of FSI analysis.

NGI analysis of the selected formulations showed that all tested formulations had the mass median aerodynamic diameter (MMAD) smaller than 5 μm, which indicates that these formulations can be adequate for pulmonary administration (Table 7).

Based on the results from FSI and NGI analysis, it can be observed that all of the analyzed formulations showed the FPF > 20%, which is a respectable percentage for this type of formulations, where FPF values of 20-30% were usually observed (Mezzena et al., 2009; Scalia et al., 2012; Scalia et al., 2013b; Daman et al., 2014). Having in mind that, according to NGI results, EF is higher than 80% for all tested formulations, it can be assumed that lower FPF values are observed due to powder deposition in the induction port (30-40%). Formulation F9 showed the greatest FPF (35.77%) according to the NGI results as well as FSI, indicating that F9 can be chosen as an appropriate formulation to be further optimized.

3.6. In vitro drug release

The results of *in vitro* dissolution testing in Franz diffusion cells indicated that the release of SS from the five tested SLMs formulations was modified (sustained), in comparison to the raw SS release (Figures 7 and 8). This can be confirmed based on differences in %DE values between raw SS and five SLMs formulations, as illustrated on Figure 7. In addition, as it can be observed from Figure 8, almost complete amount of SS (96.29%) was dissolved after 30 minutes in comparison to SLMs, where 27.88% to 52.67% of SS was released depending on a SLMs formulation. As expected, F7 showed faster drug release in comparison to F11, due to the fact that F7 was unwashed formulation and the amount of SS on the particles surface (non-encapsulated) dissolved firstly. In addition, SS from the unwashed formulation F10 dissolved faster than from the washed formulation F11. On the other hand, SS release from washed formulation F9 was slightly faster

than from unwashed formulation F10. The fastest drug release was observed from F9, probably because of the combined effects of trehalose addition (which can enhance powders redispersibility; Freitas and Muller, 1998) and smaller particle size. Since the particle size of F10 (d_{v50} = 5.14) is larger than F9 (d_{v50} = 4.01), it can be assumed that the particle size had a predominant effect on SS release in this case. Formulation F1 showed the slowest SS release, probably due to the lowest content of poloxamer 188 (0.40%, in comparison to 1.50% in other four formulations). Therefore, it can be concluded that modified release rates are resulting from complex infuence and interactions of investigated factors.

The release from all five formulations was slower in comparison to the raw drug indicating the potential of the tested formulations to sustain the drug release. Scalia et al. (2013a) observed that the preparation technique influences SS dissolution rate from SLMs and this observation can be confirmed in this study. Namely, SLMs with GB, prepared by melt-emulsification/freeze-drying processes did not show drug release modification (Scalia et al., 2013a), whereas SS release was prolonged in this study where the SLMs formulations were prepared with the same excipients by melt-emulsification/spray-drying processes.

In vitro release kinetics parameters are presented in Table 8. The highest values for coefficient of determination (R^2) were obtained for Peppas-Sahlin model for F1, F9 and F10, as well as the first-order model in the case of F7 and F11, where $R^2 > 0.99$ was observed, although the other two models also showed high R^2 values (Table 8). Based on the results of the first-order model, it can be observed that the release mechanism involved dissolution controlled process, whereas the results of Korsmeyer-Peppas and Peppas-Sahlin models showed that the release mechanism involved combination of diffusion and erosion controlled processes. The Korsmeyer's-Peppas diffusional exponent *n* was in the range between 0.43 and 1 (the range for spherical samples) which indicates anomalous drug release i.e. a combination of diffusion and erosion mechanism (Ritger and Peppas, 1987). This was also confirmed by Peppas-Sahlin model, where constants k_1 and k_2 indicated that diffusion rate decreases (negative k_1 values) and erosion rate increases (positive k_2 values) with time. Apart from the diffusion, which is an expected mechanism of drug release from SLMs, erosion of the lipid matrix can also occur, especially due to the presence of surfactant (poloxamer 188) in SLMs formulations, as well as microparticles porosity.

In the studies by Bhoyar et al. (2011) and Rao et al. (2014) similar observations were reported for drugs' release mechanisms from lipid-based particles.

4. Conclusion

Melt-emulsification with spray-drying preparation method of SLMs is a complex process which requires optimization of a large number of various factors in order to achieve desirable final DPI product properties. Regarding the first target of QTTP-aerodynamic powders properties, it was observed that trehalose should be added in order to protect SLMs during spray-drying process since trehalose addition positively affected SLMs aerodynamic characteristics. Even though lower density is a desired property for SLMs DPIs, it has been shown that small increase in true density, due to trehalose addition, did not have a negative effect on SLMs aerosol performance. Beside trehalose addition, lipid type was proven to be an important CMA since changing the glyceryl dibehenate with stearyl alcohol led to inadequate SLMs morphology. Spraying airflow rate was shown to be the most important spray-drying process parameter for obtaining optimal particle size. Regarding the second target of QTTP-dissolution profile, it can be noted that beside particle size as one of the well-known factor, washing of SLMs formulations, as well as surfactant concentration could modify SS release rate. It can be concluded that numerous factors that affect CQAs of SLMs DPI were identified in this study, but further research is required to fully understand influence of all of them and to optimize them in order to obtain a formulation with maximized FPF values and the slowest SS release, as identified in QTTP. In addition, the optimized formulation should be characterized in terms of permeation potential, using Calu-3 cell monolayers. These experiments are planned within our follow-up studies.

Presented melt-emulsification/spray-drying method can be applied to develop dry powders for inhalation with other drugs and lipid excipients. This can provide basis for development of novel DPIs with improved aerodynamic properties that can be used for different indications, including treatment of respiratory viral infections that are currently the greatest threat to the worldwide population.

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