Bioavailability Enhancement of Nanostructured Microparticles

of Carvedilol

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Abstract: A high throughput encapsulation technique termed as electrospraying assisted by pressurized gas technology (EAPG) was used to produce nano-within-micro structures of a Biopharmaceutics Classification System (BCS) Class II model drug. Carvedilol is a lipid soluble compound, poorly absorbed in the gastrointestinal tract. The produced formulations were characterized in terms of morphology, crystallinity, in vitro dissolution test, in vitro Caco-2 cells permeability and in vivo pharmacokinetics in rats. Spherical microparticles with a carvedilol loading of 80% were produced with sizes around 4 µm. DLS and TEM suggested that carvedilol is released in the form of nanoparticles of controlled size when the microparticles are put in solution, and WAXS and DSC confirmed that carvedilol was in an amorphous state. In vitro dissolution tests showed that the produced microparticles dissolved 4-fold faster than the commercial carvedilol in the first 30 min. The apparent permeability in Caco-2 cells of the produced formulations was approximately 2.5-fold higher than the apparent permeability of the commercial carvedilol. The preliminary pharmacokinetic assay suggested a reduction in 2 h of the C\text{max} for the prepared formulations, but due to the high variability observed, the results need to be confirmed in further studies. This work showed the potential of nanostructured microparticles of an API via EAPG to increase dissolution rate and hence the bioavailability of a BCS Class II drug.

Keywords: Carvedilol, encapsulation, enhanced bioavailability, electrospraying, nanostructured microparticles.
Highlights

- Nanostructured microparticles of carvedilol were produced via EAPG technology
- 4 µm spherical microparticles with a carvedilol loading of 80% were formulated
- Carvedilol nanoparticles of controlled size were dispersed within the microparticles
- The microparticles dissolved 4-fold faster than the commercial carvedilol in 30 min
- Apparent permeability in Caco-2 cells was 2.5-fold higher than that of the control
**Graphical abstract**

Carvedilol nanostructured microparticles

![Graphical abstract image]

- **SD1** and **SD2** comparison graphs showing cumulative dissolution percentage and cumulative transport (pg/ml) over time.
1. Introduction

According to the Biopharmaceutics Classification System (BCS), drugs are divided into four classes taking into account its aqueous solubility and intestinal permeability. In this fashion, Class I drugs have high bioavailability. In contrast, Class IV drugs present low solubility and low permeability. Class II and Class III drugs have a poor bioavailability because of their low solubility or their low permeability, respectively [1]. More than 40% of newly developed drug candidates are categorized as Classes II and IV according to BCS [2]. For hydrophobic drugs belonging to the BCS Class II, the dissolution process of the drug acts as the rate-controlling step and, therefore, determines the rate and degree of absorption [2]. For this reason, many researchers have attempted to improve the bioavailability of BCS Class II drugs by developing new formulations with enhanced drug solubility [3], based on physical and chemical modifications of the drug, which include nano/micronization, nanosuspension, dendrimers, niosomes, drug-cyclodextrin inclusion complexes, solid dispersions, lipid-based drug delivery systems, polymeric nanoparticles, salt forms of drugs, and amorphization of the drug [2, 4, 5].

Nano/micronization or polymeric nanoparticles are highly used methods to decrease particle size, increase effective surface area and increase solubility for improved bioavailability. However, micronization does not effectively increase the bioavailability in many of the new challenging drugs [6], and in those cases nanoparticle technology is required. These processes can be carried out by precipitation approaches, such as spray drying, solvent evaporation, nanoprecipitation or supercritical processes [6]. However, some disadvantages inhibit their widespread adoption, such as the use of high temperature, a broad particle size distribution due to agglomeration between nanoparticles, and/or their limited implementation in industry due to a challenging scale-up [7, 8].

Another widely used method for promoting solubility, dissolution rate and consequently bioavailability of poorly soluble drugs is the formation of solid dispersions in a proper carrier
Solid dispersions are defined as pharmaceutical forms in which a drug is dispersed in a biologically inert matrix, either in molecular, amorphous particulate form or microcrystalline particulate form [9, 10]. Solid dispersions increase the solubility via several contributing mechanisms, such as increased porosity, reduction in agglomeration and particle size, improved wettability and solvent access due to increased surface area [9]. As the soluble carrier dissolves, the insoluble drug is exposed to the dissolution medium in the form of very fine particles for quick dissolution and absorption [11]. Hitherto, solvent dispersions could be prepared by solvent evaporation, fusion method or melt-solvent methods [5]. However, several drawbacks limit their applicability, such as the use of high temperatures, the lack of control of the drug particle size, low drug loading efficiencies and the toxicity of the large amounts of carriers required.

Electrohydrodynamic processing, such as electrospinning and electrospraying, is an novel encapsulation technology, used for both hydrophilic and hydrophobic bioactive compounds [12]. The fundament of this technology is based on an electrically charged polymer solution, which lead to ultrafine structures, generating after drying nano- or micro-sized fibers or particles. This technology is performed at room temperature, which reduces denaturation of bioactives, and it does not require an additional drying-step to separate the obtained structures from the medium. The use of high voltage results in high encapsulation efficiency, and control of the size distribution [13]. In addition, the fast and efficient drying of the microdroplets favors the precipitation in an amorphous state of the bioactive compound [14]. Other advantage of this technology is the wide range of polymeric wall materials that can be used [12]. However, the main disadvantage of the electrohydrodynamic processing has typically been its low productivity, which has limited its widespread use in industrial applications [15].

The research group of Lagaron et al. has tried to solve this challenge by developing a novel high-throughput encapsulation technique which combines electrospraying process
with pneumatic atomization [16]. This innovative technology called electrospraying assisted by pressurized gas (EAPG) uses a pneumatic injector that nebulizes thanks to compressed air within a high electric field. During this process, the particles are dried at room temperature in an evaporation chamber and are then collected as a free-flowing powder. The potential of this high throughput electrospraying technology has been proved for the encapsulation of omega-3 rich oils in different encapsulants [15-18]. However, this technology could be an alternative to produce new pharmaceutical forms solving the limitations of solid dispersions and nanoparticle technology. Thanks to solution and process parameters, this technology allows to produce controlled nanonizations of drugs at room temperature, obtaining nano-within-micro amorphous structures with control of the drug particle size distribution and avoiding agglomeration, which allows to modulate dissolution rate.

Carvedilol

((2RS)-1-(9H-carbazol-4-yloxy)-3-[[2-(2-methoxyphenoxy)ethyl]amino]propan-2-ol) is one example of this BCS Class II drug candidates [19]. This challenging drug is a lipid soluble compound, practically insoluble in water and poorly absorbed from the gastrointestinal tract, exhibiting a pH-dependent solubility [20-22]. It is widely used to treat a variety of cardiovascular ailments, including hypertension, heart failure and left ventricular dysfunction following myocardial infarction [23]. This drug is commercially available in the form of tablets for oral administration with a low oral dose (6.25-25 mg) [24]; however, its systemic bioavailability is only 25-35% due to extensive hepatic first-pass metabolism by cytochrome P450, and also has a short plasma half-life [25]. Several approaches have been developed to enhance the solubility of carvedilol as reviewed Fernandes et al. [5]. Hitherto, several works were performed enhancing carvedilol bioavailability by electrospinning with very promising results [24, 26-30]; however, to the best of our knowledge encapsulation of carvedilol has not been performed by electrospraying or EAPG techniques, so far.
This paper reports on the preparation of carvedilol nano-within-micro structures by the EAPG technology to improve bioavailability of this drug using the fast dissolving hydrophilic polymeric carrier, polyvinylpyrrolidone K30 (PVP K30) [11] as encapsulating matrix. Characterization of the produced formulations in terms of morphology was performed by scanning electron microscopy (SEM) and dynamic light scattering (DLS). Changes in the molecular state of carvedilol were investigated by differential scanning calorimetry (DSC), confocal Raman spectroscopy, attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) and wide angle x-ray scattering (WAXS). The in vitro carvedilol’s dissolution behavior was also characterized. Finally, in vitro Caco-2 cells transport studies and in vivo pharmacokinetics in rats were also performed.

2. Materials and Methods

2.1 Materials

Carvedilol was used as a BCS Class II model drug and it was obtained from Inke (Castellbisbal, Spain). Polyvinylpyrrolidone K30 (PVP K30) was obtained from JH Nanhang (Shanghai, China). Acetone (Ph. Eur.) was purchased from Panreac (Castellar del Vallès, Spain). Caco-2 cell line, EMEM medium, fetal bovine serum (FBS), Hank's balanced salt solution (HBSS) and trypsin-EDTA solution were purchased from ATCC (Manassas, VA, USA). Span 20, penicillin, streptomycin, amphotericin B, phosphate buffered saline (PBS), HEPES solution, nimodipine, acetonitrile and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Corning® Transwell® polycarbonate membrane cell culture inserts were purchased from Corning (Corning, NY, USA). Size 9 gelatin capsules (8.4 mm × 2.7 mm, volume of 0.025 mL) were purchased from Torpac® (Fairfield, NJ, USA). Polyurethane #9 capsule dosers were purchased from Instech (Plymouth Meeting, PA, USA). Deionized water was used throughout the study.
2.2 Solution preparation

Carvedilol and PVP K30 were dissolved in aqueous solution of acetone 70% (v/v.), at room temperature. Carvedilol was used at a concentration of 23.1 mg/mL, and PVP K30 at a concentration of 6 mg/mL. The drug-to-polymer ratio was 80:20. Two formulations were used with (SD1) or without surfactant (SD2), in this case, Span 20, which was used at a concentration of 1 mg/mL, in order to compare the effect of the surfactant in the dissolution rate, permeability and bioavailability.

2.3 EAPG process

The EAPG process took place in a patented Capsultek™ pilot plant from Bioinicia S.L. (Valencia, Spain). This pilot installation comprises a nebulizer, connected to a voltage source (0-30 kV), a drying chamber, and a cyclonic collector as described elsewhere [16, 31]. The application of an electric field to the drops generated by the nebulizer reduces their size, which facilitates the drying process, the encapsulation, and helps to control the final particle size in their travel towards the collection unit. The experiments were performed at 10 kV, with a solution flowrate of 4 mL/min and an air flowrate of 10 L/min, under controlled environmental conditions, i.e. 25°C and 30% relative humidity (RH).

2.4 Morphology

A Hitachi S-4800 FE-SEM (Hitachi High Technologies Corp., Tokyo, Japan) with an electron beam acceleration of 10 kV was used to analyze the morphological characteristics of the obtained particles. Samples were sputtered with a gold/palladium layer before the scanning electron microscopy (SEM). Image J Launcher v1.41 software (National Institutes of Health, Bethesda, USA) was used to determine particle diameter, which was obtained averaging a minimum of 100 particles.
2.5 Carvedilol particle size distribution

Nano-within-micro refers to drug nanoparticles dispersed and distributed within microparticles, in which the drug nanoparticles with controlled size, due to the process and solution parameters, are separated by a polymeric excipient. The average particle size of the carvedilol nanoparticles inside the PVP K30 matrix was measured by dynamic light scattering (DLS) (Zetasizer Nano–ZS, Malvern Instruments Ltd., Malvern, UK.). To proceed to these measurements, the microparticles were dissolved in PBS (pH 7.4) at a concentration of 40 mg/L, at room temperature, with slow magnetic stirring for 30 s. After that, 1 mL of the solution was taken and measured. The stirring time of 30 s was selected, as it was sufficient for the dissolution of the PVP K30 that allowed the nanoparticles to be released, without favoring the dissolution of the carvedilol nanoparticles according to the dissolution curves. Measurements were carried out at 25 °C, in triplicate. The DLS expert report generated indicated that the measurements were not optimal suggesting that during the short time lapse used to analyze the particle size, bigger particles may have still be present. It could not have been possible to let the measurements to be optimal; otherwise the carvedilol nanoparticles would have been dissolved. The particle sizes were confirmed by transmission electron microscopy (TEM). For that, one droplet of microparticles solution in PBS after 30 s of stirring was deposited over a carbon-coated copper grid, and observed in a Jeol Jem 1010 (Tokyo, Japan) under an accelerating voltage of 80 kV.

2.6 Attenuated total reflection – Fourier transform infrared spectroscopy (ATR-FTIR)

The infrared spectra of approximately 50 mg of particles were scanned using a Bruker Tensor 37 FT-IR Spectrometer (Bruker, Ettlingen, Germany) coupled with the ATR sampling accessory Golden Gate (Specac Ltd., Orpington, UK) at 4 cm\(^{-1}\) resolution in a wavenumber range between 4000-600 cm\(^{-1}\), averaging 10 scans. Measurements were
performed in triplicate. OPUS 4.0 software (Bruker, Ettlingen, Germany) was used for data analysis.

2.7 Differential scanning calorimetry (DSC)
Differential scanning calorimetry analysis were performed to characterize the physical state of the carvedilol in the commercial and here-obtained formulations. Thermal characteristics of each sample were studied on a DSC-8000 analyzer from PerkinElmer Inc. (Waltham, MA, USA), equipped with a cooling accessory, Intracooler 2 also from PerkinElmer Inc. A heating program was applied from 35 and 240 °C. The heating rate was set at 10 °C/min under nitrogen atmosphere with a flow-rate of 20 mL/min. The sample weight was around three milligrams, while an empty aluminum pan was used as reference. Calibration was performed using an indium sample. All tests were carried out, at least, in duplicate. Thermograms were analyzed using the Pyris Manager software (PerkinElmer Inc., Waltham, MA, USA).

2.8 Confocal Raman spectrophotometry
The micro-Raman experiments were carried out with a confocal micro-Raman spectrometer NRS-3100 (Jasco Corporation, Tokyo, Japan), equipped with a diode laser excitation source illuminating at 532 nm, providing ca. 80 mW at the sample position. A few milligrams of the samples were put on a glass slide and thinly spread, and measured with an objective lens of x20 for total 60 seconds. Under these conditions, the error in wavenumbers was 17.3 cm\(^{-1}\).

2.9 Wide angle X-ray scattering (WAXS)
The crystallographic structural analysis was performed by wide angle X-ray scattering in a Bruker AXS D4 Endeavor diffractometer (Bruker, Ettlingen, Germany). Samples were
scanned at scattering angles (2Θ) comprised between 5-30°, at ambient conditions, in reflection mode, applying incident Cu K-alpha radiation (Cu Ka =1.54 Å) running at 40 kV and 40 mA.

2.10 In vitro dissolution testing

The dissolution rate profiles of commercial carvedilol, and here-obtained formulations were obtained in enzyme-free simulated intestine fluid (SIF) (pH 6.8). In vitro dissolution testing was conducted using a United States Pharmacopeia method II dissolution tester (Dissolution System 2100C, Distek Inc., North Brunswick, NJ, USA). The carvedilol samples equivalent to 10 mg of carvedilol were placed in dissolution vessels containing 900 mL of dissolution medium, maintained at 37.0 ± 0.1 °C and stirred at 50 rpm. This concentration was selected according to literature [11, 32], and under the intrinsic solubility of carvedilol in water [32]. The temperature and stirring speed of this analysis were also selected according to the literature [11, 32, 33]. Enzyme-free SIF (pH 6.8) was prepared by adding 6.8 g of potassium dihydrogen phosphate in deionized water and adjusting the pH with 0.1 M sodium hydroxide solution to obtain 1 L of enzyme-free SIF (pH 6.8). 5 mL aliquots of dissolution medium were withdrawn between 0 and 630 min and were filtered through 0.45 µm PTFE syringe filters. At each sampling time, an equal volume of dissolution medium was added to the dissolution vessel. The carvedilol content was determined using a UV/VIS spectrophotometer (UV4000, Dinko Instruments, Barcelona, Spain) at a detection wavelength of 284 nm.

2.11 Caco-2 cell model for in vitro transport study

The human colorectal adenocarcinoma cell line Caco-2 is widely used as an in vitro model of the human small intestinal mucosa to estimate the absorption of drug administered orally. Cells were cultured in EMEM medium added with 10% heat inactivated FBS,
penicillin G (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (0.25 μg/mL). Cells were seeded (passages between 15 and 25) at 1 × 10^5 cells/well onto 12 well Transwell® plate containing 12 mm polycarbonate inserts (0.4 μm pore size) and experiments were performed after 21 days post-confluence, a necessary period to allow the formation of fully polarized monolayers. The complete medium was totally replaced every three days. Every day, the monolayer integrity was checked by measuring the transepithelial electrical resistance (TEER) with a Millicell-ERS-2 volt-ohmmeter (Merck-Millipore, Darmstadt, Germany). All experiments were performed with TEER measurements between 300 and 600 Ω·cm² in HBSS (donor and receptor compartment). The well plates were incubated in sterile conditions at 37 °C in a 5% CO₂ humidified atmosphere. Commercial carvedilol, SD1, SD2, and physical mixtures with same composition as formulations SD1 and SD2 (Mix SD1 and Mix SD2, respectively) were used at 20 μg/mL in HBSS. Physical mixtures were prepared to assess the hypothetical incidence of the polymer matrix or of the surfactant in the enhancement of the drug transport across the Caco-2 monolayer. Samples were collected at different time points: 0, 15, 30, 60 minutes.

The concentration of carvedilol in the collected samples was estimated using liquid chromatography tandem mass spectrometry (LC/MS/MS) (ACQUITY® TQD, Waters, Milford, MA, USA). The chromatographic separation was carried out using an ACQUITY UPLC BEH C18 column (Waters, Milford, MA, USA, particle size 1.7 μm; 2.1 mm × 50 mm). The mobile phase was constituted of acetonitrile and 0.1% formic acid in water (80:20). The flow rate used was 0.4 mL/min. The mass spectrometer was equipped with a Z-spray electrospray ionization source and the samples were analyzed with the following conditions: capillary: 3.5 kV, extractor: 5 V, RF Lens: 0.3 V, source temperature: 120 °C, desolvation temperature: 300 °C, cone gas: 25 L/h, desolvation gas: 680 L/h. MS1 parameters were: LM resolution: 13; HM resolution: 13; ion energy: 1. MS2 parameters were: LM resolution: 13; HM resolution: 13; ion energy: 0.7; multiplier: 650 V. Spectra were
acquired in positive ionization multiple reaction monitoring mode with inter-channel delay of 0.03 s and the transition of carvedilol was 407.00 – 100.00.

The apparent permeability coefficient \( P_{app} \) from the donor part to the receptor part was estimated according to equation 1.

\[
P_{app} = \frac{V}{A \times T} \times \frac{[dr]}{[dd]}
\]  
(Eq.1)

Where \( V \) is the volume (mL) of the receptor compartment, \( A \) is the membrane area (cm\(^2\)), \([dr]\) is the concentration of the drug in the receptor compartment and \([dd]\) is the concentration of the drug in the donor compartment, \( T \) is the time (s).

2.12 Pharmacokinetic study in rats

The animal study was conducted in compliance with the University of Valencia (Valencia, Spain) guidelines for the care and use of laboratory animals and it was approved by the Ethics Committee of the same university. Fifteen male Sprague-Dawley rats (250 ± 20 g body weight) were divided randomly into three groups of 5 rats. Rats were kept under standard laboratory conditions at 25 ± 0.2 °C, 55 ± 5% RH and 12 h light schedule. The animals were housed in polypropylene cages with free access to standard laboratory diet and water ad libitum. The rats were fasted for 5 hours prior to the study with free access to water.

The duration of the fasted conditions was selected according to the Ethics Committee and the veterinary responsible of the University of Valencia to avoid an excessive stress to the rats during the study. Prior to the administration of the capsules, each rat was anesthetized by inhalation of isoflurane and a catheter was inserted directly into the tail vein for blood sampling. Each animal received a single gelatin capsule (0.025 mL capsule volume and 1.25 mg/capsule of carvedilol loading), and soon after a gavage of 1 mL of water. Animals in group one received pure commercial carvedilol at a dose of 5 mg/kg; group two and group three were administered respectively with SD1 and SD2 in an equal dose. The dose was
selected according to the literature [25]. All through the procedure, each rat was housed in a single cage with free access to standard chow and water. Blood samples (approximately 0.25 mL) were collected in heparinized tubes from the tail vein catheter at specified time points during 24 h: 0 – 0.25 – 0.5 – 1 – 2 – 3 – 6 – 24 h. Plasma samples for LC/MS/MS analytics were obtained by centrifugation at 13000 rpm at 4 °C for 5 min. 0.1 mL of plasma were mixed with 0.2 mL of acetonitrile for protein precipitation and vortexed for 3 min. All samples were centrifuged at 13000 rpm at 4 °C for 10 min and the supernatants were collected and stored in LC/MS/MS plates at -80 °C until the drug concentration were measured. The calibration curve was built up using the peak area in comparison with the nominal concentration in the range of 0.1 – 500 ng/mL ($r^2 = 0.986$). Nimodipine was used as internal standard. The area under the curve (AUC$_{0→24h}$), the maximum concentration of carvedilol after oral administration ($C_{\text{max}}$) and the time to reach the maximum concentration ($T_{\text{max}}$) were determined from the experimental data.

2.13 Statistical analysis

Statistical analyses of the Caco-2 experiments and of the in vivo pharmacokinetics using Statgraphics Centurion XVI software (StatPoint Inc., Warrenton, Va, USA) were performed by one-way ANOVA, and Tukey’s multiple comparison tests was used as the post-hoc test. A $p$ value less than 0.05 was considered statistically significant.

3. Results and discussion

Carvedilol was formulated via EAPG into nano-within-micro structures, which were characterized in terms of morphology, carvedilol particle size distribution, DSC, WAXS, confocal Raman spectroscopy, and ATR-FTIR. In addition, in vitro dissolution tests, in vitro transport study in Caco-2 cells, and in vivo pharmacokinetic in rats test were carried out.
3.1. Morphology

Figure 1 shows a comparison of the morphology of the carvedilol commercial microparticles (Figure 1a) and the carvedilol produced formulations processed by the EAPG technology (Figure 1b, c). While the commercial carvedilol has rectangular shape with smooth surface; acicular or spherical microparticles with size around 4 µm were obtained by EAPG depending on the presence of Span 20 in the formulation. It seems that the increase in the viscosity of the solution due to the presence of the Span 20 in the formulation SD1, promoted the formation of acicular particles as shown in Figure 1b. Some aggregation between microparticles could occur during sample preparation prior to SEM observation, due to the high hygroscopicity of PVP K30. However, the powder was collected from the equipment at controlled ambient conditions as free-flowing powder and stored in desiccators until further analysis.
Whereas spherical particles were obtained by EAPG at a drug:carrier ratio of 80:20, Lee et al. produced carvedilol crystals coated by PVP K30 and Tween 80 in a carvedilol-to-carrier ratio of 67:33 by spray drying [9]. It was required to increase the ratio of PVP K30 to 50:50 or 25:75 to obtain spherical solid dispersions of carvedilol in PVP K30 by spray drying, as reported by Oh et al. [33]. Tapas et al. reported the formation of spherical particles with a rough surface and high porosity by solvent diffusion at a carvedilol loading of 40% [32]. However, via EAPG it was possible to obtain spherical particles with a lower concentration of carrier, which could favor the economic viability of the process.

3.2 Carvedilol particle size distribution

The particle size distribution of the drug inside the microparticles is an important factor to take into consideration to increase the solubility and bioavailability of a BCS Class II drug, such as carvedilol. For that, it was taken advantage of the fact that PVP K30 dissolves quickly when it comes into contact with water, releasing the carvedilol irregularly shaped nanoparticles. A stirring time of 30 s for the microparticles in PBS solution was selected, as it was sufficient for the dissolution of the PVP K30 that allowed the nanoparticles to be released, without favoring the dissolution of the carvedilol nanoparticles.

The comparison of the carvedilol particle size distribution between the two formulations herein produced, SD1 and SD2, is shown in Figure A in supplementary data. The size of the carvedilol particles inside of the microparticle from SD1 and SD2 confirms that carvedilol was released into the buffer solution in the form of nanoparticles. SD1 generated
nanoparticles of 100 nm, and SD2 generated nanoparticles of 200 nm. Despite the
measurements were not of optimum quality, mainly due to the presence of settling
microparticles, as evidenced the expert advice and a high polydispersity index close to 1,
these results provide an idea of the difference in size between both samples, since it is
possible to observe a reduced particle size due to the presence of the surfactant, which could
enhance the solubilization rate. However, the quality of the DLS measurement could not be
improved since the sample evolves over time, and longer stirring times would have favored
the disappearance of the microparticles, but also the dissolution of the carvedilol
nanoparticles. Nevertheless, these results were validated by observing the nanoparticles
suspensions after 30 s of microparticles stirring in PBS solution by TEM as shown in Figure
2, which confirmed the results obtained by DLS. At this point, it is required to evaluate if
these carvedilol nanoparticles are in amorphous or crystalline state.
Figure 2. TEM images of the carvedilol nanoparticles suspensions after 30 s of microparticles stirring in PBS solution. (A) SD1 and (B) SD2. Scale bar corresponds to 200 nm in A, and to 100 nm in B.
In order to investigate the physical state of the drug in the microparticles, DSC measurements were performed. The drug melting peak in the thermograms was used as an indicator of the crystalline form of the drug in the sample. If the drug is in an amorphous state, no melting peak could be observed. Figure 3 shows the thermograms for the carvedilol samples. The commercial carvedilol thermogram exhibited a single sharp endothermic peak at 121°C, which corresponded to its melting point, being the enthalpy of fusion of 115 J/g. Similar melting points were reported by other authors [33]. The absence of melting peak in the DSC thermograms of the formulations here-in obtained, SD1 and SD2, suggests that carvedilol is in an amorphous state in both samples. The amorphous form of the drug is a high-energy state that exhibits enhanced solubility and dissolution rate and, thus, increased bioavailability compared to the crystalline state [2, 30]. The amorphization of the drug could be due to the fast solidification process that occurs during electrohydrodynamic processing as stated by other authors [14]. Samples SD1 and SD2 showed water loss upon heating between 35-75°C due to sample dehydration, as a consequence of the high hygroscopicity of PVP K30, which also show a peak between 50-120°C. The range of temperatures decreases due to the low content of PVP K30 in the here-prepared samples. Sharma and Jain reported partial amorphization of carvedilol in a 50:50 mixture of carvedilol and PVP K30 prepared by solvent evaporation, and complete amorphization of carvedilol when the PVP K30 ratio was further increased (25:75 and 17:83) [11]. Lee et al. obtained solid dispersions with crystalline behavior with ratios 67:33 by spray drying [9]. Similarly to microparticle morphology, the amount of carrier seems to be a key factor in the amorphization of the drug by conventional technologies; however, by EAPG, the amount of carrier could be reduced.
3.4 WAXS

The WAXS patterns of the carvedilol samples were recorded to determine if a crystalline phase could be detected. The diffractograms of commercial carvedilol and SD1 and SD2 samples are shown in Figure 4. The diffractogram of commercial carvedilol showed numerous intense peaks due to its highly crystalline nature. Peaks at 5.7°, 11.7°, 13.0°, 14.9°, 17.6°, 14.5°, 24.4°, 26.2° were particularly distinctive. On the contrary, on SD1 and SD2 diffractograms did not reveal any distinctive diffraction peaks, only the amorphous background or halo could be observed, being similar to the patterns of the pure polymer. Therefore, these results suggest that carvedilol in SD1 and SD2 was in an amorphous state.
These results are also in concordance with the ones obtained by DSC, confirming that the carvedilol formed an amorphous nanodispersion inside of the microparticle.

Figure 4. Wide angle X-ray scattering diffractogram of (a) commercial carvedilol, (b) PVP K30, (c) SD1 with Span 20, (d) SD2 without Span 20.

3.5 Confocal Raman spectroscopy

Commercial carvedilol, pure PVP K30 and SD2 were characterized by confocal Raman spectroscopy. This technique can be used as a “non-invasive” method to study, among other things, the composition and molecular order of materials. The Raman spectra of commercial carvedilol shown in Figure 5, presents the bands of the main chemical groups of the molecule namely, CC stretching (phenyl ring breathing) at ca. 1012 cm\(^{-1}\); the stretching of CC/CO at ca. 1333 cm\(^{-1}\), 1240 cm\(^{-1}\); the COC deformation at ca. 576 cm\(^{-1}\); the stretching of CN at ca.
1285 cm\(^{-1}\), 1223 cm\(^{-1}\), 1124 cm\(^{-1}\); the CCC and CNC deformation at ca. 865 cm\(^{-1}\); the NH perpendicular bending at ca. 726 cm\(^{-1}\); the CC/CN/C-OH stretching at ca. 1064 cm\(^{-1}\); CC stretching at ca. 1632 cm\(^{-1}\); and CH symmetric stretching at ca. 3066 cm\(^{-1}\) [34]. PVP K30 has broad characteristic peak at around 970 cm\(^{-1}\) reported to display the ring breathing of the pyrrolidone function [35].

**Figure 5.** Raman spectra of commercial carvedilol (a), PVP K30 (b), and SD2 without Span 20(c).

Arrows indicate the main spectral changes in comparison with the commercial carvedilol.

Some differences could be observed between the Raman spectra of the commercial carvedilol and the spectra of SD2 as shown in Figure 5. The arrows in Figure 5 indicate the main spectral changes between SD2 and the commercial carvedilol. The encapsulation procedure did not cause measurable chemical changes in the carvedilol molecule, since for
SD2, the characteristic Raman spectroscopy peaks maintain the peak position; however, the variation in the relative intensity of some bands between 500 and 1500 cm\(^{-1}\) could be due to sample fluorescence. The width of some of the characteristic peaks of carvedilol in SD2, such as 3066, 1632, 1285 and 726 cm\(^{-1}\), are seen broader and their intensity and sharpness decreased compared to the ones of commercial carvedilol, which could be ascribed to difference in crystallinity, being in concordance with the observations made by DSC and WAXS.

3.6 Attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR)

The ATR-FTIR spectra of commercial carvedilol, PVP K30 and produced formulations are presented in Figure 6. ATR-FTIR spectra of commercial carvedilol showed intense and well defined infrared absorption bands at ca. 3337 cm\(^{-1}\) corresponding to the O-H and N-H stretching vibration peaks merged together; 2926 cm\(^{-1}\) were medium intensity bands assigned to C-H stretching vibration in –CH\(_3$/CH\(_2$-; 1590, 1502 and 1444 cm\(^{-1}\) were low intensity bands from C=C stretching bond in benzene ring; 1255, 1215 and 1098 cm\(^{-1}\) bands were assigned to C-O and C-N stretching; and 745 and 716 cm\(^{-1}\) were assigned to the out of plane C-H [29]. The most distinct peak in the ATR-FTIR spectrum of PVP K30 was the stretching vibration of the carbonyl group that would typically appear around 1655 cm\(^{-1}\). Additionally, a broad peak was displayed at about 3400 cm\(^{-1}\) due to O-H stretching vibrations of absorbed water, bands at ca. 2800-3000 cm\(^{-1}\) were assigned to C-H stretching vibration, the band at ca. 1422 cm\(^{-1}\) was assigned to C–H bending, and band at ca. 1287 cm\(^{-1}\) was assigned to C-N stretching [29, 33].

The ATR-FTIR spectra of the produced formulations clearly showed the main absorption bands corresponding to carvedilol and PVP K30; however, a change in relative
intensity and broadening, see the band at ca. 1590 cm\(^{-1}\) identified with a dotted line, was observed as shown in Figure 6, which could be attributed to the amorphization of the drug in the microstructures as observed in DSC and WAXS. Contribution of PVP K30 in the spectra of SD1 and SD2 samples was also identified in Figure 6.

**Figure 6.** ATR-FTIR spectra of commercial carvedilol (a), PVP K30 (b), SD1 (c), SD2 (d). Squares identify the PVP K30 contribution in the spectra of samples SD1 and SD2. The dotted line identifies the band at 1590 cm\(^{-1}\).

### 3.7 In vitro dissolution rate

The dissolution rate profiles of commercial carvedilol and SD1 and SD2 in enzyme-free SIF (pH 6.8) are shown in Figure 7. The observed dissolution rate of commercial carvedilol
was quite low in SIF. The amount dissolved of commercial carvedilol in 30 min reached no
more than 17%. Regarding the produced formulations, it seems that carvedilol was released
in a biphasic model with an initial fast rate followed by a slower one. This faster release could
be attributed to a burst release of carvedilol loaded near the particles surface.
Correspondingly, the amount of carvedilol released in the first 30 min was of 39 % for the
SD2. Remarkably, the amount of dissolved carvedilol for SD1 during the first 30 min was of
70%, this increase could be due to the presence of Span 20 which could favor humectation.
This means that the dissolution rate of SD1 was 4-fold faster than the dissolution rate of
commercial carvedilol. The dissolution improvement may be attributed to a combination of
several factors. First, the difference in particle size, being larger for commercial carvedilol
and SD2. Furthermore, the amorphous state help to increase the dissolution rate, and
consequently SD1 and SD2 are faster than commercial carvedilol. In addition, the presence
of Span 20 could help to the wettability of the SD1 sample.
Comparing these results with the provided by other authors, Tapas et al. got 80% of
dissolution in 30 min by encapsulating carvedilol in PVP K30 and Aerosil 200 via solvent
diffusion. Those particles showed a different polymorph crystal and increased porosity [32].
Sharma et al. got 70% after 30 min by solvent evaporation using a high amount of PVP K30
to get amorphization [11]. Lee et al. got 25% of dissolution after 30 min by encapsulating
carvedilol in PVP K30 via spray drying, but carvedilol maintained the crystalline state [9].
Figure 7. Dissolution rate profiles of carvedilol from (a) commercial carvedilol, (b) SD1 with Span 20, (c) SD2 without Span 20.

3.8 In vitro permeation through Caco-2 cells

The in vitro permeation through the gastrointestinal barrier was assessed using Caco-2 cells monolayer. SD1 exhibited an important and significant enhancement of permeability across the Caco-2 cell membrane in comparison to the commercial product in the first 30 min after the treatment, as shown in Figure 8, even if the error at 60 min was high. This effect could be due to the presence of Span 20 that has been reported of acting as a permeability enhancer [36]. SD2 showed a similar trend to the commercial drug curve during the first 15 min and then gradually increased to clearly exceed it within 30 min of the permeability assay. If the results obtained with the physical mixtures, i.e. Mix SD1 and Mix SD2, are compared with the results of the formulations SD1 and SD2, it is possible to conclude that the presence of polymer and surfactant was not the main reason of the enhancement in the permeability rate. However, the reduced drug particle size within the microparticle could be another
factor, which could explain the enhancement in permeability of formulations SD1 and SD2 in comparison with the commercial drug.

**Figure 8.** Caco-2 permeability profile of carvedilol produced formulations (SD1 and SD2), physical mixtures with same composition of SD1 and SD2 (Mix SD1 and Mix SD2) and commercial drug. The concentration of carvedilol used was 20 µg/mL. Each point represents the mean ± SD (n=3).

The apparent permeability values ($P_{app}$) for each sample are presented in the Table 2. The apparent permeability of the SD1 was nearly 2.5-fold higher than the apparent permeability of the commercial carvedilol, showing a significant difference between them according to the statistical analysis shown in Table 2. As shown in Table 2, the similarity of the values between the commercial carvedilol and the physical mixtures corroborate that the presence of polymer and surfactant are not directly related to the permeability rate increase in the *in vitro* experiments with cells. Up to our knowledge, there is no many literature about carvedilol permeability studies in Caco-2 cells, most of the studies were performed with other kind of permeability tests such as corneal membrane or sublingual mucosa [37, 38]. Tian *et al.* obtained larger $P_{app}$ values of carvedilol in Caco-2 transport experiments around $10 \times 10^{-6}$
cm/s; however, the study was performed in an incubator with shaking to avoid the unstirred water layer (UWL) effect of drug transport [39]. As well, Berben et al. obtained an apparent permeability for carvedilol in Caco-2 cells of $6.2 \times 10^{-6}$ cm/s by incubating the cells with shaking and in a fasted state human intestinal fluids as dissolution medium [40]. However, despite the absence of shaking in our study, SD1 formulation reached similar $P_{\text{app}}$ values, as shown in Table 2.

**Table 2.** Apparent permeability ($P_{\text{app}}$) evaluation of commercial carvedilol, formulations SD1 and SD2 and physical mixtures Mix SD1 and Mix SD2 using the Transwell® model with Caco-2 cell line.

Different letters indicate significant differences ($p < 0.05$).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>$P_{\text{app}}$ for Caco-2 Permeability ($\times 10^{-6}$cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial carvedilol</td>
<td>2.59 ± 0.75a</td>
</tr>
<tr>
<td>SD1 with Span 20</td>
<td>6.36 ± 2.82b</td>
</tr>
<tr>
<td>SD2 without Span 20</td>
<td>4.40 ± 0.11a</td>
</tr>
<tr>
<td>Mix SD1</td>
<td>3.65 ± 1.24a</td>
</tr>
<tr>
<td>Mix SD2</td>
<td>3.29 ± 0.26a</td>
</tr>
</tbody>
</table>

3.9 Preliminary pharmacokinetic study in rats

In this study, the pharmacokinetic (PK) profiles of three different formulations of carvedilol, pure commercial drug, SD1 and SD2, were evaluated to confirm the in vitro improved dissolution rate and Caco-2 permeability. Plasma drug concentration results and the PK parameters ($T_{\text{max}}, C_{\text{max}}$ and $AUC_{0-24}$) obtained from the study are shown in Figure 9 and Table 3, respectively. The results showed a relatively high variability between subjects indicating that although, there is a trend of increased bioavailability for SD1 and SD2
compared to the pure commercial drug, further confirmation studies using a large batch of animals than initially estimated by the inferential statistical modelling approved by the Ethical Committee, need to be carried out. Thus, the in vivo assay of the developed formulations indicates there is a trend in decreasing the time to reach the maximum concentration of carvedilol in the blood stream in comparison with the commercial product according to Figure 9. Additionally, the results suggest that the developed formulations could present a higher systematic exposure (AUC$_{0\rightarrow24}$) in comparison to the commercial drug. However, as a result of the variability among the animals tested, the results do not show a significant difference. For this reason, it would be beneficial in future works to perform additional in vivo experiments with a large quantity of animals to further confirm the trends observed.

Figure 9. Mean plasma concentration of carvedilol formulations (SD1 and SD2) and commercial drug following oral administration (mean ± SD, n = 5).

Several strategies were proposed to improve the oral bioavailability of poorly water-soluble drug carvedilol. Rueda et al. demonstrated the effectiveness of solid
dispersions of carvedilol in combination with specific surfactants to increase the plasma concentration of the drug after oral administration in Sprague-Dawley rats but without reducing the time to reach the maximum concentration [41, 42]. As well, Li et al. suggested hollow mesoporous silica nanoparticles as promising carriers for carvedilol delivery; they showed a great improvement of the systematic exposure during 48 hours but also an 8-hours delay in reaching the highest concentration of the drug in the blood stream [43]. Nevertheless, the here-obtained results are very promising, and the EAPG technology shows a great potential to improve the oral bioavailability of BCS Class II drugs.

Table 3. Pharmacokinetic parameters of commercial carvedilol, and formulations SD1 and SD2 in rats (mean ± SD, n=5). Different letters in the same row indicate significant differences (p < 0.05).

<table>
<thead>
<tr>
<th>PK Parameters</th>
<th>Commercial carvedilol</th>
<th>SD1</th>
<th>SD2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>3.00</td>
<td>0.25</td>
<td>1.00</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng/mL)</td>
<td>339.75 ± 323.07a</td>
<td>277.78 ± 489.20a</td>
<td>520.91 ± 783.38a</td>
</tr>
<tr>
<td>$\text{AUC}_{0-24}$ (ng·h/mL)</td>
<td>2854 ± 757a</td>
<td>4719 ± 1946a</td>
<td>3205± 1138a</td>
</tr>
</tbody>
</table>

Therefore, taking into account all the results presented above, a correlation was observed between the carvedilol particle size inside the microparticles and the dissolution rate. This fact together with the presence of surfactant help to increase the permeability through the Caco-2 cells and consequently the observed trend of increased bioavailability in rats.

4. Conclusions
Approximately 40% of newly developed drug candidates are categorized as BCS Classes II and IV. This imposes a big challenge to the pharmaceutical industry that has made great research efforts to develop formulation strategies in order to improve the bioavailability of drugs with limited solubility in water. This work demonstrates that it is possible to produce amorphous nano-within-micro structures that can potentially increase the bioavailability of a BCS Class II model drug via the disruptive high throughput electrospraying assisted by pressurized gas processing technology. According to the obtained results this process solves the limitations of solid dispersions and nanoparticle technology, presenting advantages such as a high drug loading capacity and a low requirement for excipients to stabilize the drug, which reduces the possibility of toxicity and makes the process more economically viable. On the other hand, the process presents the possibility of nanostructuring the microparticles by controlling the size of the drug, so the particles have the advantages of the nanoparticles such as solubilization rate, but without the risk of agglomeration or safety problems in their handling, since nanoparticles are gathered inside microparticles, which are collected in the form of free-flowing powder. In addition, the process has the ability to amorphize the drug, favoring its rapid dissolution and gut absorption. Finally, the process takes place at room temperature, which reduces the possibility of degradation of the drug, and it is also available on an industrial scale.

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