THE IMPACT OF SUPERCRITICAL FLUIDS PROCESSING ON MINOCYCLINE ANTIBACTERIAL ACTIVITY

L. Pinheiro[1], A. Bettencourt[1], A. Duarte[1], K. Bettencourt[1], A. J. Almeida[1], M. Castro[1], L. Padrela[2], M. A. Rodrigues[2] and H. A. Matos[2]

[1] iMed.UL – Research Institute for Medicines and Pharmaceutical Sciences, Faculty of Pharmacy, University of Lisbon, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal

[2] Department of Chemical and Biological Engineering, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

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Topic: Material processing in SCFs

ABSTRACT

Controlled release and drug targeting of therapeutic agents like antibiotics, have shown to be promising when administered as micro- or nanoparticles, leading to an increase of the pharmaceuticals bioavailability. Supercritical Fluid (SCF) technology gained significant attention in this field by not only being a powerful tool in the improvement of the pharmaceuticals biological performance, but also accommodating the principles of green chemistry. The main goal of the present contribution is to investigate the impact of the supercritical conditions on the bioactivity of minocycline nano/microparticles, a crucial property in the technological production and clinical application. Two SCF techniques, the Supercritical AntiSolvent (SAS) and the Supercritical Enhanced Atomization (SEA) were used to produce minocycline nano/microparticles with controlled particle size and particle size distributions. The morphologies of the antibiotic particles were analyzed by a Scanning Electron Microscope (SEM) Hitachi S2400. Particle size distributions were obtained by analysis of SEM images using the Sigma Scan Software. The antimicrobial potential of the processed antibiotics was evaluated by standard microbiological assays against a collection of reference antibiotic-susceptible and multidrug clinical (producing multiresistant mechanisms) bacterial strains. The antibiotic nano/microparticles, were successfully evaluated for their antibacterial activity against reference and multidrug resistant isolates.

INTRODUCTION

During the last decade Supercritical Fluid (SCF) technology has gained significant attention as an exquisite tool for processing pharmaceuticals into nano- or micro-scale particles with narrow size distribution. This technology provides a platform for particle design leading to innovative formulations with improved drugs bioactivity and bioavailability, controlled-release systems and to replace parenteral drug delivery by less invasive delivery routes [1–3].
The production of antibiotic-laden particles enables the targeted delivery of these pharmaceutical compounds to infected cells and the foci of bacterial infection, contributing to the high chemotherapeutic effect [4–9]. In addition to issues relating to acquired antibiotic resistance, many antibiotics are generally less effective in treating infections in fatty tissue, biofilms, or along the surfaces of bone and artificial devices where drug-resistant bacterial infections are so often found. As the particulate technologies allow the enhancing

performance of antibacterial drugs, micro/nanoparticle-based antibiotics could be potential treatments for life-threatening bacterial infections [10].
Minocycline is a broad-spectrum long-acting semi-synthetic derivative of the antibiotic tetracycline, with antibacterial, anti-inflammatory and neuroprotective effects [11]. Considering the bacteriostatic activity, its tissue-penetration characteristics are important in community- and hospital-acquired methicillin-resistant *Staphylococcus aureus* (MRSA).
There are several reports of antibiotic particles produced using supercritical fluids [1, 12–15]. However, the outcome of supercritical conditions on the bioactivity of these pharmaceutical compounds has not been consigned so far. The stresses experimented by the pharmaceuticals in supercritical processing depend on the process nature. For instance, Supercritical Anti-Solvent (SAS), where the liquid solution is sprayed into a dense supercritical media, it may cause the formation of polymorph crystalline forms and therefore interfere with the bioactivity. If processed using intense shear atomization by liquid jet dispersion with an assisting SCF as in Supercritical Enhanced Atomization (SEA), the mechanic energy associated to the jet break-up and the high gas-liquid interface of the resulting sub-micron scale droplets has been described as a source of activity loss for protein therapeutics. Thereby, the present study aims to assess the antibacterial activity of minocycline, submitted to supercritical fluid conditions. In this work, commercial minocycline was submitted to a supercritical media for extended time or further processed into particles by SAS and SEA. The particles were characterized by SEM and their antibacterial activity was evaluated against reference and multidrug resistant isolates.

MATERIALS AND METHODS

Minocycline was purchased at microbiological grade from Sigma-Aldrich and was used without further purification. Absolute ethanol (99.5%) was supplied by Panreac, Carbon dioxide (99.998%) was supplied by Ar Líquido (Portugal).

Minocycline exposure to SC-CO_2

Minocycline was put inside a stainless steel cylinder (with an internal diameter of 24 mm, external diameter of 60 mm, and length of 168 mm). This apparatus is described elsewhere [16]. The high-pressure cylinder was pressurized with SC-CO_2 at 8.0 and 12.0 MPa (t = 50 ^{o}C) for 24h. The exposure of the antibiotic to SC-CO_2 served as a preliminary experiment to ensure that no loss of antibiotic activity would result from contact with the supercritical fluid.

Production of Particles by SEA

Figure 1 describes schematically the SEA setup used in this work.

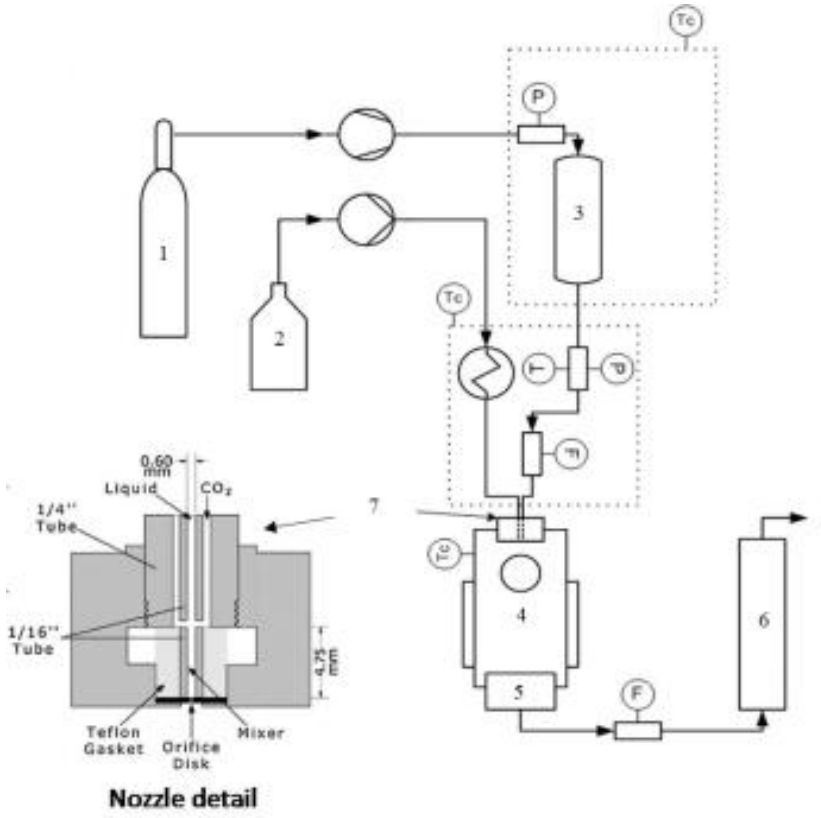


Figure 1 – Schematic diagram of the SEA (and SAS) apparatus. 1: CO_2 cylinder; 2: liquid solution flask; 3: temperature controlled CO_2 storage cylinder; 4: precipitator; 5: filter; 6: cyclone; 7: detail of the nozzle cap.

Minocycline was dissolved in a mixture composed of 50% water and 50% ethanol (300 mg of minocycline in 14 g of solvent). This solution was pumped (by a TSP metering pump, model 2396-74) through a coaxial nozzle where it mixes with the supercritical fluid before depressurization into a precipitator vessel. The nozzle setup can be divided in three sections: the inlet (where the lines for each phase contact), the mixer and the orifice disk. The mixer consists in a 0.5 cm long 1/16″ tube where both phases contact. The orifice disk consists in a 0.25 mm tick disk with a centered orifice of 100 micrometer diameter.

The droplets formed during the spray atomization were dried inside a temperature controlled precipitator near atmospheric pressure (0.1 MPa to 0.5 MPa) and 323 K. The solution flow rate was 0.7 g/min while the SC-CO_2 flow rate was 18 g/min. The CO_2 was compressed by a Newport Compressor (model 46-13421-2) to an atomization pressure of 8 MPa, and the CO_2 mass flow through the nozzle was measured by a Rheonik flowmeter (model RHM007). Pressures are measured by PX603 transducers from Omega. Temperatures inside the air chamber or in a water bath are controlled by T-type thermocouples and Ero Electronic controllers (model LDS). The particles were recovered from the precipitator walls and from a cyclone (Separex, France).

Production of particles by SAS

The SAS setup consists essentially in the same parts of the SEA setup described above with a few exceptions. The precipitator is a high pressure vessel, and the particles were collected in a filter at the precipitator exit. The precipitator pressure (10 MPa) was controlled by a back

pressure regulator (Tescom, model 26-1722-24). The operating temperature was 50 ºC and the flow rates were 30 g/min (SC-CO_2) and 1 g/min for the liquid solution.

Antimicrobial Activity

The studied bacterial strains were a collection of reference antibiotic-susceptible isolates *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and multidrug resistant isolates (identified from different Portuguese hospitals and producing multiresistant mechanisms): *Klebsiella pneumoniae* producing CTX-M-15 beta lactamase; *P. aeruginosa* producing a metallo beta lactamase IMP-5; *E. coli* with a class 1 integron carrying the *bla*CTX-M-9 gene; *Acinetobacter baumannii*, the European clone II, producing an oxacillinase OXA-40 and methicillin resistant *S. aureus* (MRSA). Bacterial suspensions with turbidity equivalent to McFarland standard were prepared. The susceptibilities of the bacterial strains to minocycline nano/microparticles were determined by the Mueller-Hinton agar diffusion method. Stock solutions of minocycline nano/microparticles processed by the two SCF techniques were prepared at a concentration of 1mg/mL. For each bacterial culture, paper discs (Whatman, diameter 6 mm) were soaked with 15 µL of the antibiotic sample and placed on the surface of the agar in sterile Petri plates. After incubation under anaerobic conditions at 37 ºC for 24h, culture plates were read visually by measuring the clear zones around the sample discs with a micrometer. The diameters of inhibition zones of bacterial growth (in millimetres) indicated the bactericidal activity. Commercial discs of minocycline and the non-processed minocycline were used as controls. All plates were done in duplicate for each bacterial strain and each SCF processed minocycline.

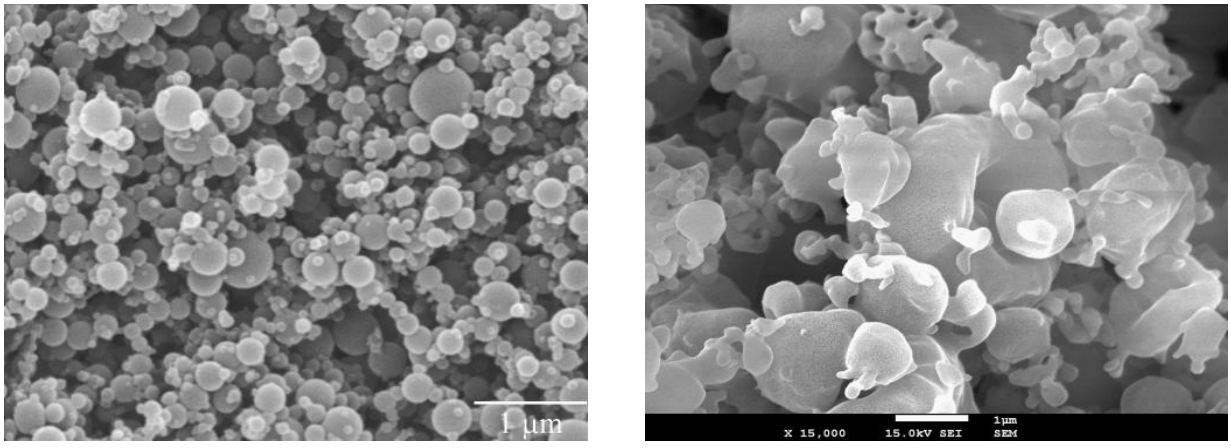
SEM and Particle Size Distribution

The particle size and morphology were analyzed by a Scanning Electron Microscope (SEM) Hitachi S2400. Particle samples were coated prior to measurement with a gold film by electrodeposition in vacuum.

RESULTS

Particle Morphology

The size and morphology of minocycline particles produced by SEA and SAS techniques are represented by Figure 2.



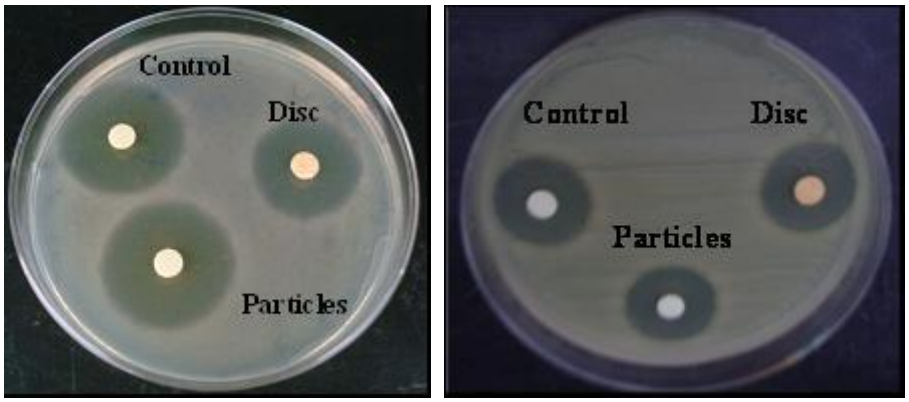
(a) (b)

Figure 2 – SEM images of minocycline processed by (a) supercritical enhanced atomization (SEA) and (b) supercritical anti-solvent (SAS).

As can be seen by Figure 2, minocycline microparticles produced by SEA are slightly smaller than those produced by SAS.

Antimicrobial activity

The sustenance of the antimicrobial characteristics of minocycline nano/microparticles processed by SAS and SEA techniques against reference and multidrug resistant isolates was evaluated through microbiological assays using the agar disk-diffusion procedure. In the study reported herein, the diameters of the bacterial growth inhibition zones were suggestive for the minocycline efficacy, whatever the supercritical fluids methodology employed. Accordingly, the minocycline samples pressurized by SC-CO_2 revealed similar results with those of SAS and SEA. Figure 3 illustrates some of the results obtained from the *in vitro* microbiological experiments. The bacteria inhibition study revealed that minocycline particles retained their biological activity.



(a) (b)

Figure 3 – Bacteria inhibition zones in the agar assays for the antibacterial activity of Minocycline processed by SEA (a) and SAS (b) on *E. coli* ATCC 25922 (*Control*: antibiotic at 1mg/mL; *Disc*: commercial disc; *Particles*: antibiotic nanoparticles at 1mg/mL).

CONCLUSION

Overall, it can be concluded that nano/microparticles of minocycline processed by any of the two SCF techniques were able to inhibit the growth of the selected bacterial strains. As a decisive property in the technological production and clinical application, the antimicrobial preservation of the studied antibiotic particles shows that both SCF methods do not modify its microbiological activity.

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