# ANTIBACTERIAL ACTIVITY OF MINOCYCLINE, TOBRAMYCIN AND VANCOMYCIN PROCESSED BY SUPERCRITICAL FLUIDS

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Sustained/controlled release and drug targeting of antibiotics, have shown to be promising when administered as micro- or nano-sized particles, leading to an increase of these therapeutic agents bioavailability. In this context, Supercritical Fluid (SCF) technology has emerged as a powerful tool in the improvement of the pharmaceuticals biological performance. The key objective of this study is to evaluate the impact of the supercritical conditions on the bioactivity of minocycline, tobramycin and vancomycin micro/nanoparticles, which can be decisive in terms of technological production and clinical application. Two SCF techniques, the Supercritical AntiSolvent (SAS) and the Supercritical Enhanced Atomization (SEA) were used to produce the antimicrobial micro/nanoparticles with controlled particle size and particle size distributions. The morphologies of the antibiotics particles were analyzed by a Scanning Electron Microscope (SEM) and particle size distributions were obtained by analysis of SEM images. The antibiotics micro/nanoparticles were successfully evaluated for their antibacterial activity against reference and multidrug resistant isolates.

**Keywords:** supercritical fluids, atomization, anti-solvent, antibacterial activity

## INTRODUCTION

During the last decade Supercritical Fluid (SCF) technology has gained significant attention as an exquisite tool for processing pharmaceutical compounds 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, sustained/controlled-release systems and to replace parenteral drug delivery by less invasive delivery routes [1–4].

Even though the therapeutic efficacy of antibiotics has been well established, inefficient delivery could result in inadequate therapeutic index and local systemic side effects. 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 and the decrease of therapeutic dosage (thus improving efficiency). 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 [5–11].
Several authors have demonstrated the cominution of antibiotics by SCFs into nanometric ou micrometric particles with relative success [1,4,10,12–16]. However, the physical characterization is usually insufficient to evaluate the process feasibility when processing pharmaceutically active substances. SCFs are known to cause stresses to pharmaceuticals depending of the process nature. Therefore the SCFs interference with the antibiotics bioactivity should not be disregarded. The present study aims to assess the antibacterial activity of the three abovementioned antibiotics, submitted to supercritical fluid conditions. Commercial minocycline, tobramycin and vancomycin were 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

### *Materials*

Minocycline, tobramycin and vancomycin were purchased at microbiological grade from Sigma-Aldrich, Labesfal and Generis Farmacêutica SA, respectively, and were used as received. Absolute ethanol (purity 99.5%) was supplied by Panreac and used without further purification, Carbon dioxide (purity 99.98%) was supplied by Ar Líquido (Portugal). Bacterial species and Mueller-Hinton broth (MH, Oxoid) were employed for microbiological tests. Commercial available discs (Oxoid, 6 mm diameter) containing minocycline, tobramycin and vancomycin were applied. Blank paper discs (diameter 6 mm) were purchased from Whatman.

### *Production of Particles by SEA*

The SEA setup is described schematically in Figure 1. This process exploits the high density and low viscosity of supercritical fluids to enhance the break-up of liquid jets into sub-micron droplets. The nozzle consists in a coaxial setup (described in detail in the Figure 2) where the liquid solution is fed though the inner 1/16’ tube and the SCF trough the outside 1/4’ tube.
In SEA both fluids mix immediately before the atomization in a 0.1 $cm^3$ mixer (see Figure 2). The mixing is too fast for dissolution of the SCF in the liquid, although, it is important to potentiate the liquid jet breakup by the intense shear forces associated to both fluids (liquid and supercritical) simultaneous depressurization.
To facilitate the drying step in the precipitator (at near atmospheric conditions) ethanol was added to the aqueous solutions of the antibiotics.
Each antibiotic (minocycline, tobramycin and vancomycin) was dissolved in a mixture composed of 50% water and 50% ethanol (300 mg of minocycline in 14 g of solvent; 200 mg of tobramycin in 10 g of solvent; 300 mg of vancomycin in 15 g of solvent). This solution was pumped (by a TSP metering pump, model 2396-74) through the coaxial nozzle where it mixed with the supercritical fluid before depressurization through a 100 micromiter laser drilled orifice disk (0.25 mm tick) into a jacketed wall precipitator vessel.

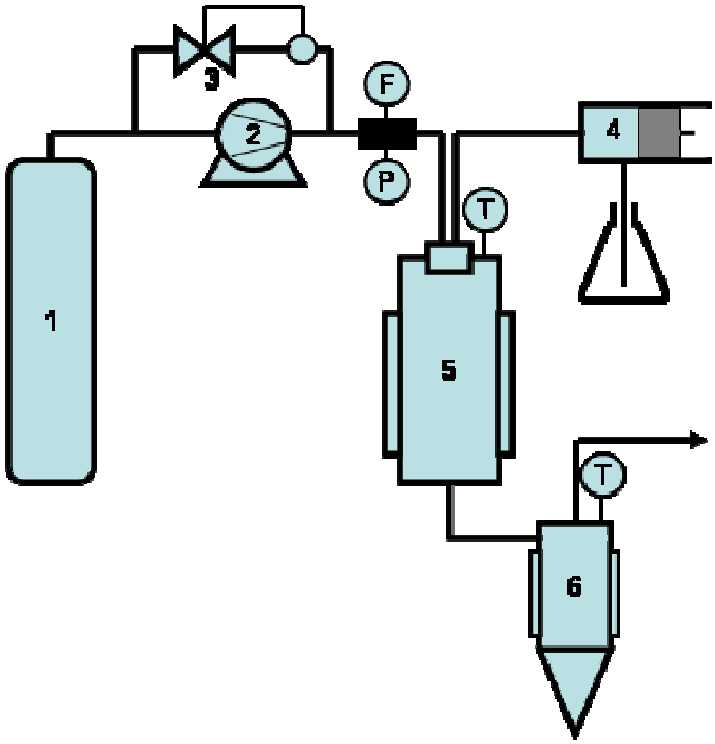


**Figure 1**: SEA setup: 1 - $CO_2$ cylinder; 2 – Gas compressor; 3 – Back pressure controller; 4 – Piston liquid pump; 5 - Jacketed wall precipitator; 6 – Jacketed wall cyclone.

The droplets formed during the spray atomization were dried inside a temperature controlled precipitator near atmospheric pressure (0.1 MPa to 0.2 MPa) and 323 K. The solution flow rate and the SC-$CO_2$ flow rate were, respectively, 0.2 g/min and 18 g/min (minocycline), 0.2 g/min and 25 g/min (tobramycin), 0,2 g/min and 15 g/min (vancomycin).
In this work the liquid/SCF mass flow ratio (*R*) was selected for direct drying of the hidro-alcoolic solution by the expanding gas, i.e. without using a hot gas current to dry the spray, as in conventional spray drying. In SEA the SCF depressurization to normal pressure results in an intense flow of gaseous $CO_2$. The precipitator working temperature selected was 50 ºC which corresponds to a maximum R of 0,07 liquid/gas (mass) at 0.2 MPa. In all experiments the experimental R was set to 0.01 g liquid/g $CO_2$, to ensure direct drying of the spray at 50 ºC and precipitator pressure (0.1 MPa to 0.2 MPa).
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 were 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) and electric resistances. Particularly, two cartridge electric resistances of 80 Watts were used to control the nozzle temperature which otherwise would drop due to Joule-Thompson effect. The particles were recovered from the precipitator walls and from a stainless steel cyclone made by Separex, France.

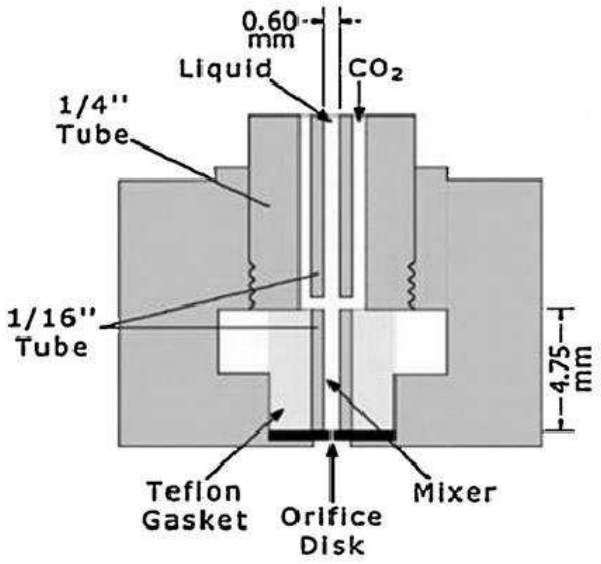


**Figure 2 :** Detailed description of the nozzle setup

***Production of particles by SAS***

The SAS setup (Figure 3) consists essentially in the same parts of the SEA setup described above with a few exceptions. The precipitator is a high pressure (200 $cm^3$) 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 0.2 g/min for the liquid solution (R =0.007).

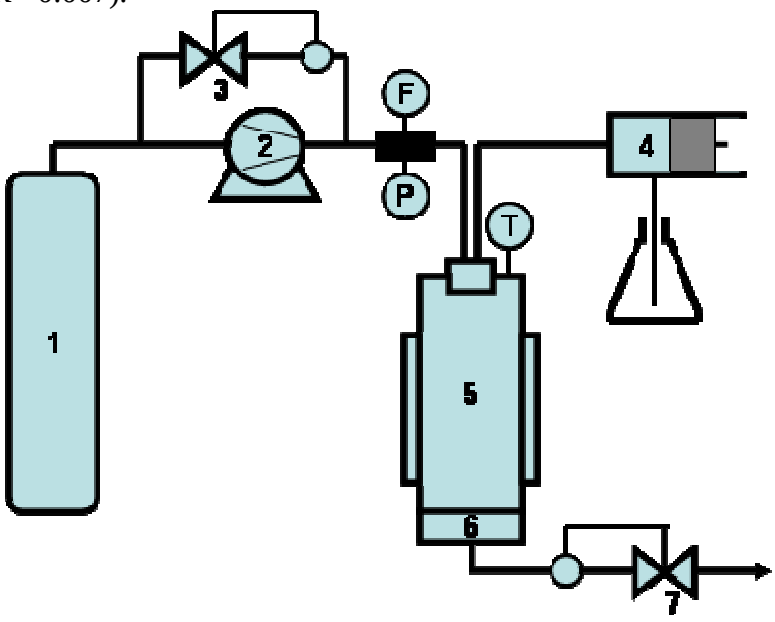


**Figure 3**: SAS setup: 1 - $CO_2$ cylinder; 2 – Gas compressor; 3 – Back pressure controller; 4 – Piston liquid pump; 5 - Jacketed wall precipitator; 6 – Filter; 7 – Back pressure controller.

### *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 0.5 McFarland standard were prepared (bacterial concentration was adjusted to $2–3\times10^5$ colony-forming units per mililiter, CFU/mL). The susceptibilities of the bacterial strains to minocycline, tobramycin and vancomycin micro/nanoparticles were determined by the Mueller-Hinton agar diffusion method. Stock solutions of every antibiotic micro/nanoparticles processed by the two SCF techniques were prepared at a concentration of 1mg/mL. For each bacterial culture, blank paper discs were soaked with 15 µL of the antibiotics samples 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 the antibiotics and the non-processed antibiotics were used as controls. All plates were done in duplicate for each bacterial strain and each SCF processed antibiotic.

### *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. Acceleration voltage during observation was 25 kV. Particle size distributions (PSD) were obtained by analysis of SEM images using the Sigma Scan software (Systat Software Inc), for more than 500 minocycline/tobramycin/vancomycin particles.

## RESULTS AND DISCUSSION

### *Particle Morphology*

The size and typical morphology can be observed through the representative SEM images of minocycline particles produced by SEA and SAS techniques and shown in Figure 4. As can be seen, minocycline microparticles produced by SEA are slightly smaller than those produced by SAS.

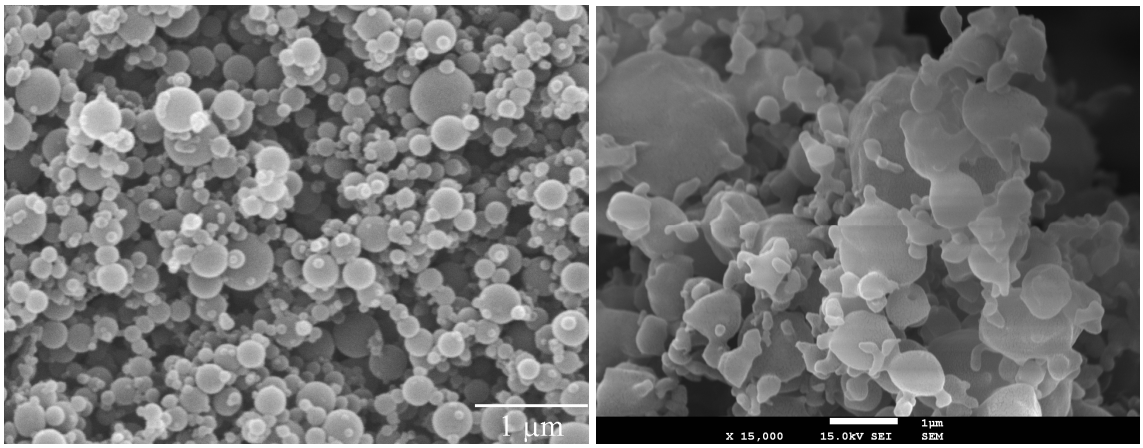


**Figure 4**: SEM images of minocycline particles obtained by SEA processing (left) and SAS processing (right).

***Particle size distributions***

As an example, Figure 5 shows the Feret diameter distribution (PSD) of the minocycline/tobramycin/vancomycin particles processed by SEA, indicating a narrow size distribution (0.1 – 0.4 mm).

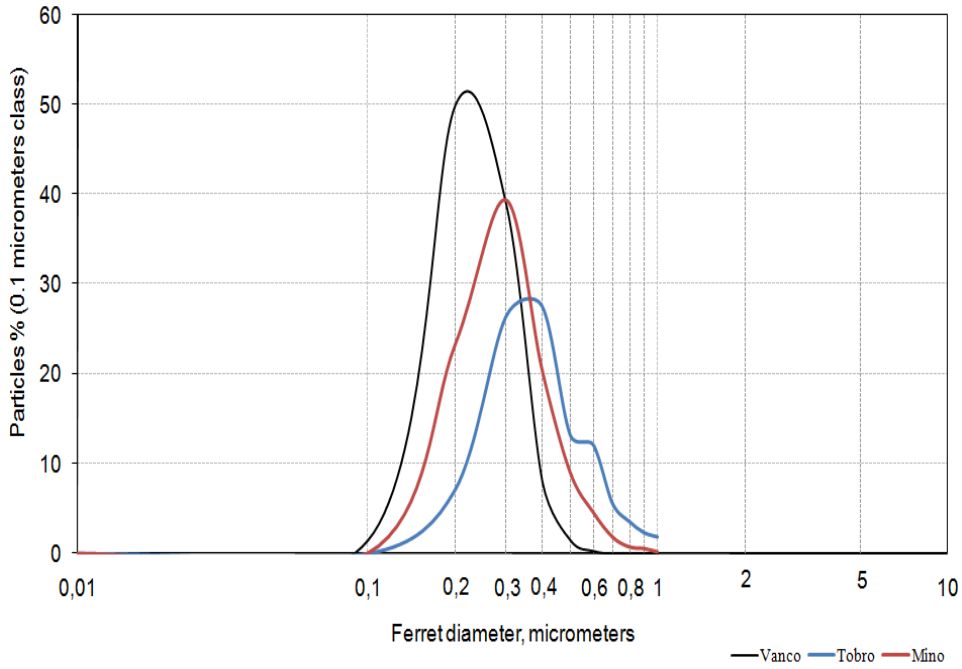


**Figure 5**: Feret diameter distribution of minocycline, tobramycin and vancomycin particles processed by SEA, from corresponding SEM images.

***Antimicrobial activity***

The maintenance of the antimicrobial characteristics of minocycline, tobramycin and vancomycin mino/nanoparticles 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 (very similar for each pair of antibiotic/bacterial strain concerning both SCF techniques) were suggestive for the antibiotics efficacy, whatever the supercritical fluids methodology employed. Figure 6 illustrates some of the results obtained from the *in vitro* microbiological experiments. The bacteria inhibition study revealed that all antibiotics particles retained their biological activity.

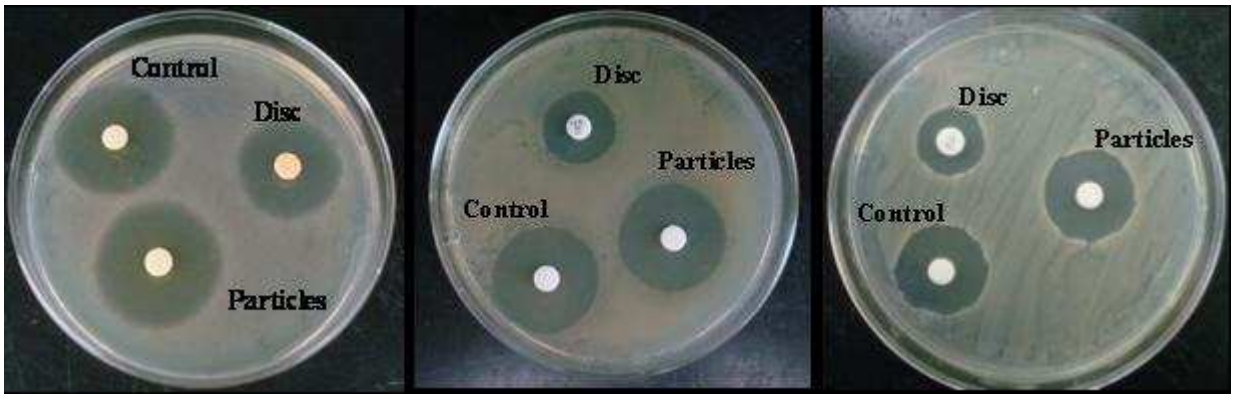


**Figure 6**: Bacteria inhibition zones in the agar assays for the antibacterial activity of minocycline on *E. coli* ATCC 25922 (left), tobramycin on *E. coli* ATCC 25922 (center) and vancomicyn on *S. aureus* ATCC 25923 (right) processed by SEA (*Control*: antibiotic at 1mg/mL; *Disc*: commercial disc; *Particles*: antibiotic particles at 1mg/mL).

## CONCLUSIONS

Antibiotic micro/nanoparticles present a versatile potential for efficient exploitation of different delivery formulations and routes because of the properties provided by their ultra small and controllable size. The ease of controlling particle size and particle size distribution makes supercritical fluid processing particularly attractive in the improvement of the antibiotics formulations characteristics, where the microbiological factor is decisive. Overall, it can be concluded that micro/nanoparticles of minocycline, tobramycin and vancomycin processed by any of the two SCFs techniques were able to inhibit the growth of the selected bacterial strains. As a crucial property in the technological production and clinical application, the antimicrobial preservation of the studied antibiotics particles shows that both SCF methods do not modify its microbiological activity.

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