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Nebulization and anti-*Pseudomonas aeruginosa* activity of colistin

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ABSTRACT: Colistin aerosols are frequently administered to patients with cystic fibrosis. However, questions arise concerning the effect of both jet and ultrasonic nebulizers on the properties of the drug. The aim of this study was to characterize the anti-*Pseudomonas aeruginosa* (PA) activity of colistin after jet (Pari LL®) and ultrasonic (DP100®) nebulization.

A bench study was performed by capturing the aerosols, determining the drug mass, and assessing its anti-PA activity. Because the inhaled mass of colistin had to be entirely recovered for the bacteriological study, it was assessed by isotopic methods, mixing the drug with a ^{99m}Tc-labelled tracer and demonstrating that ^{99m}Tc activity accurately predicted the mass of colistin. Colistin was extracted from the filters and its antibiotic activity was determined using the method employed for the study of the bacteriostatic and bactericidal power of serum on the ATCC 27853 PA strain.

The postnebulization minimum inhibitory concentrations (MIC) were 1.9 µg·mL^{-1} with DP100® and 0.5 µg·mL^{-1} with Pari LL®. These values were less than two dilutions different from the 1 µg·mL^{-1} MIC of non-nebulized colistin.

We conclude that neither jet nebulization nor ultrasonic nebulization alter the antibiotic properties of colistin and that both systems can be used to nebulize colistin.
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Colistin methane sulphonate (colistin) is a major anti-*Pseudomonas aeruginosa* (PA) antibiotic; it is a polypeptide and has been developed for parenteral administration. However, colistin is frequently administered as an aerosol to treat PA colonization in patients with cystic fibrosis (CF) [1]. We have demonstrated that both jet and ultrasonic nebulizers are effective for generation of colistin aerosols that can deposit in the lungs in CF patients [2]. However, questions arise concerning the effect of both systems on the physicochemical, and therefore antibiotic, properties of the drug. It is well known that some ultrasonic nebulizers may alter the therapeutic properties of certain polypeptides, such as recombinant human deoxyribonuclease (rhDNase) I [3] and insulin [4]. In the case of colistin, the gas flow which generates the aerosol in jet nebulizers induces a foamy appearance to the solution in the nebulizer reservoir. For some physicians, this physicochemical effect is of sufficient concern to prevent them using jet nebulizers. Although colistin aerosols seem to be effective *in vivo* [4, 5], the hypothesis that the antibiotic properties of colistin might be altered by the different processes of nebulization has not, to our knowledge, been formally tested.

The aim of this study was to verify that jet (Pari LL®; Pari, Starnberg, Germany) and ultrasonic (DP100®; DP Medical, Meyland, France) nebulization does not alter the antibiotic activity of colistin. Under laboratory conditions, the inhalation procedure was standardized, and the aerosol was filtered and extracted from the filter. The drug mass was then measured, its antibiotic properties on a *P. aeruginosa* strain were assessed using bacteriological methods, and this activity was compared to the equivalent mass of non-nebulized colistin. It was essential that the drug mass and activity be determined in the same run with methods that did not interfere with each other. Indeed, the whole drug mass had to be recovered after measuring to test its antibiotic activity. Therefore, colistin was mixed with a tracer (human serum albumin (HSA) labelled with ^{99m}Tc), which was used to assess the colistin mass. The accuracy of the method was established in the laboratory by demonstrating that the radioactivity truly reflected the colistin mass in the aerosol.

Methods

Nebulizers

Two nebulizers were tested: 1) DP100®, an ultrasonic nebulizer that operates at a frequency of 2.4 MHz and was used at the maximum nebulization and medium flow

rates; and 2) Pari LL®, a jet nebulizer equipped with a 1.45 Bar pressure Pari Master® compressor.

Preparation of the nebulizer charge

A batch (No. 5673) of 1 million unit (33,300 µg) vials of powdered colistin was obtained from Laboratoires Roger Bellon (France). For each experiment, one million units of colistin were dissolved in 5.7 mL saline, and mixed with 0.3 mL HSA (kit TCK 2; CIS Bio-International, France) labelled with 370 MBq ^{99m}Tc.

Mass-activity relationship and particle size distribution

In order to determine the mass-activity relationship, a sample of the aerosol was collected in an impactor, the activity counted and the mass of colistin impacted at each stage measured. The nebulizer was charged with 6 mL colistin mixed with ^{99m}Tc-labelled HSA. A 10 stage GS1 cascade impactor (California measurement, USA) was connected to a piston pump (Harvard, USA), used at a frequency of 20 breaths·min^{-1} with 750 mL tidal volume and 0.5 inspiration/expiration (I/E) time ratio, and to the nebulizer. The aerosol was sampled isokinetically at a flow rate of 1 L·min^{-1} over 1 min. Each of the slides of the cascade impactor was then washed with 1 mL sterile water, which was collected in separate vials to be counted (isotopic method) and assayed (high performance liquid chromatography (HPLC) method).

Isotopic method. The activity present in each of the vials was counted in a 10 cm diameter NaI (Tl) solid scintillator coupled to a TISA spectroanalyser device (ARIES, France). It was expressed as a percentage of the total activity recovered in the impactor.

HPLC method. Reagents and standards. HPLC grade methanol and hydrochloric acid were purchased from Merck (Darmstadt, Germany). Diethylamine was obtained from Aldrich and was of analysis grade. A stock standard solution of colistin was prepared. Working standard solutions (2,500, 5,000, 10,000 and 20,000 U·mL^{-1}) were prepared by dilution of the stock solution with the mobile phase.

Albumin extraction. The albumin had to be extracted from the solution of ^{99m}Tc-labelled HSA colistin because of the risk of saturating the column. In view of the difference in molecular weight of both molecules, and the fact that they were not bound to each other, we used an ultrafiltration unit, AMICON model 15 (AMICON, Bedford, USA), with a filter, DIAFLO® AMICON 25 mm, at a nitrogen pressure of 4 Bars and with a cut-off of 10,000 Da. The filter was washed under pressure with 3 mL saline between each assay.

Chromatography and extraction procedure. Analyses were performed using HPLC (Waters Assoc., Milford, MA, USA) equipped with a 600 E Waters pump and a 712 wisp injector thermostatically controlled at 30°C. The detector was a Waters 991 photodiode assay model. The column was a VYDAC 218 TP C 18.

The mobile phase was a mixture of methanol (30%) diethylamine (0.05%), adjusted to a pH of 2.2 with dilute hydrochloric acid (69.95%).

The amount of colistin present in each of the vials collected by washing the slides was assayed. The response of the assay, tested with standard solutions, was linear until the 10,000 U·mL^{-1} concentration with a slope of 1.292. At concentrations higher than 10,000 U·mL^{-1} there was a curve inflexion, and the peak at 20,000 U·mL^{-1} concentration was 85% of the expected value if the response had remained linear. Reliability was assessed by five subsequent assays performed the same day on each standard working solution. The variation coefficients for the 2,500, 5,000, 10,000 and 20,000 U·mL^{-1} solutions were 2.15, 2.02, 1.14 and 2.6%, respectively. The mass of colistin recovered from each stage was expressed as a percentage of the total mass recovered in the impactor.

Data analysis. The experiment was performed with both nebulizers and the results were pooled for analysis. The amounts of colistin measured by HPLC and estimated from ^{99m}Tc countings were plotted on a graph with HPLC results on the abscissa and ^{99m}Tc countings on the ordinate. Linear regression analysis was used to assess the relationship between drug and activity data. Particle size distribution, mass median aerodynamic diameter (MMAD) and geometric standard deviation (σg) of the colistin aerosols generated by both nebulizers were also determined from these experiments, based on HPLC measurement data.

Bacteriological study

The determination of the antibiotic activity of the colistin aerosols produced by the nebulizers necessitated capturing the aerosols on filters, determination of the mass of drug captured, and verification that the postnebulization antibiotic activity of colistin was similar to the activity of the equivalent mass of non-nebulized colistin.

Determination of the inhaled mass. The nebulizer charge was assessed before beginning inhalation. It was determined by counting the activity placed in the nebulizer in a standard 4 π well counter. The nebulizer was then connected to the piston pump in the same conditions as described above. An absolute filter of low resistance (Gelman Sciences, Ann Harbor, Michigan, USA) was interposed between the nebulizer and the ventilator. The DP100®) nebulizer was used at a continuous medium flow rate, whereas Pari LL® was activated during the inspiratory phase of the ventilator. Nebulization was considered to be complete when 2 min had elapsed without production of aerosol by the nebulizer [6]. The radioactivity deposited on the filter was then counted. The inhaled mass was determined after decay correction, taking into account the nebulizer charge, which was 1,000,000 U (33,300 µg) in all cases. For each nebulizer, the experiment was performed with the ^{99m}Tc-labelled HSA colistin solution in duplicate. The inhaled mass was measured on both filters but only one filter was processed further for the bacteriological study.

Antibiotic activity. The filters were put into 25 mL saline and squeezed to extract colistin. After extraction from

the filters, the content of the tubes was filtered on a Millex GS 0.22 µm millipore filter, and 2 mL aliquots of each were put into different vials. Three filters, on which known amounts of 50,000, 100,000 and 500,000 U colistin, respectively, had been deposited, served as references and were processed using the same method. To determine the antibiotic activity of the colistin extracted from these filters, we applied the method employed for the study of bacteriostatic and bactericidal power of serum [7] on the ATCC 27853 *P. aeruginosa* strain, whose minimal inhibitory concentration (MIC) to colistin is 1 µg·mL^{-1}. Two blind observers each determined in triplicate the maximum inhibitory dilution (MID) and the maximum bactericidal dilution (MBD) of each solution. The most frequent MID among the six values (three experiments by two observers) was used to assess the postnebulization minimum MIC. Taking into account the previously determined inhaled masses and the dilution in 25 mL saline, the concentration of colistin in each solution collected was C (µg·mL^{-1}) = inhaled mass (µg)/25 (mL). The MIC for each solution, obtained from DP100® and Pari LL®, was then calculated by the ratio C/MID. Finally, the postnebulization MIC was compared to the 1 µg·mL^{-1} known MIC of non-nebulized colistin on the ATCC 27853 *P. aeruginosa* strain for each nebulizer. A difference of less than two dilutions was considered to be nonsignificant.

Results

Mass-activity relationship and particle size distribution

The relationship between the mass of colistin measured by HPLC, expressed as a percentage of the total mass recovered during the experiment, and the ^{99m}Tc activity expressed as a percentage of the total activity recovered during the experiment was assessed by linear regression analysis. There was a close correlation ($r=0.99$; $p<0.000001$) between HPLC and gamma countings. The linear regression slope was 0.865.

The particle size distributions of the ^{99m}Tc-labelled HSA colistin aerosols were determined with both nebulizers. With DP100®, the MMAD was l.9 µm and σg was 2.8. With Pari LL®, the MMAD was 1.4 µm and σg was 2.6.

Bacteriological study

The inhaled masses, determined in duplicate by the isotopic method with both nebulizers, were 23.6 and 27% of the nebulizer charge with Pari LL® and 10.5 and 8.4% of the nebulizer charge with DP100®. For the bacteriological study, we used the inhaled mass filters corresponding to 23.6% of the nebulizer charge with Pari LL® and 10.5% of the nebulizer charge with DP100®. Taking into account the 0.865 slope of the linear regression between mass and activity and the 33,300 µg nebulizer charge, these inhaled masses corresponded to 6798 µg for Pari LL® and 3024 µg for DP100®.

The results of MID and MBD obtained from reference filters and from DP100® and Pari LL® experiments are presented in tables 1 and 2, respectively, and expressed as the inverse of the dilutions. For each solution, there was not more than two dilutions' difference between experiments or between observers. Of the six values for the 50,000, 100,000 and 500,000 reference solutions, the most frequent MID and MBD were: 64 and 32; 128 and 64; 1,024 and 256, respectively. These values corresponded to the actual mass of colistin deposited on the filters. Of the six values for the DP100® and Pari LL® solutions, the most frequent MID and MBD were: 64 and 16; and 512 and 128, respectively. The estimated concentrations of colistin with DP100® and Pari LL®, based on the dilution in 25 mL saline for filter extraction, were 121 and 272 µg·mL^{-1}, respectively. The ratios between these concentrations and the corresponding MID provided a postnebulization MIC of 1.9 µg·mL^{-1} with DP100® and 0.5 µg·mL^{-1} with Pari LL® These postnebulization MIC values were less than two dilutions different from the 1 µg·mL^{-1} MIC of colistin on the ATCC 27853 *P. aeruginosa* strain.

Table 1. – Maximum inhibitory dilution obtained from colistin solutions extracted from reference filters (50,000, 100,000 and 500,000 U) and extracted from inhaled mass filters with Pari LL® and DP100® nebulizers

	Minimum inhibitory dilution					
	Run 1		Run 2		Run 3	
	Obs 1	Obs 2	Obs 1	Obs 2	Obs 1	Obs 2
Reference 50,000 U	64	64	64	64	128	128
Reference 100,000 U	128	128	64	128	256	128
Reference 500,000 U	1024	1024	512	512	1024	1024
DP100®	64	64	32	32	64	64
Pari LL®	512	512	256	256	512	512

Each measurement was determined in triplicate by two different observers (Obs).

Table 2. – Maximum bactericidal dilution obtained from colistin solutions extracted from reference filters (50,000, 100,000 and 500,000 U) and extracted from inhaled mass filters with Pari LL® and DP100® nebulizers

	Maximum bactercidal dilution					
	Run 1		Run 2		Run 3	
	Obs 1	Obs 2	Obs 1	Obs 2	Obs 1	Obs 2
Reference 50,000 U	32	32	32	32	32	32
Reference 100,000 U	64	32	64	32	64	64
Reference 500,000 U	256	128	128	256	256	512
DP100®	16	16	16	16	16	32
Pari LL®	128	128	64	64	128	256

Each measurement was determined by two different observers (Obs).

Discussion

This study demonstrates that the antibiotic properties of colistin are not altered after nebulization with the Pari LL® jet nebulizer and the DP100® ultrasonic nebulizer, and that both jet and ultrasonic nebulizers can be used to generate colistin aerosols. The solution has a foamy appearance with jet nebulizers and the possible effects on the nebulization and antibiotic properties of the drug had not previously been studied. With ultrasonic nebulization, the problem was to exclude the degradation of the drug that has been described for other peptides, such as insulin and rhDNase.

There were several reasons for the choice of Pari LL® as an example of a jet nebulizer and DP100® as an example of an ultrasonic nebulizer to test the hypothesis that nebulization processes might alter the antibiotic properties of the drug. Pari LL® is an efficient jet nebulizer and we have demonstrated in the present study that it produces a colistin aerosol with a high inhaled mass of about 25% of the nebulizer charge, and an MMAD of 1.4 µm. DP100® is an ultrasonic nebulizer and the reservoir is coupled to the crystal through a water interface, thus preventing heating of the nebulizer charge during nebulization [8]. The fact that there are no variations in temperature of solutions placed in the DP100® reservoir during nebulization allowed us to focus our evaluation on the effects of ultrasound (and not heat) on the activity of colistin.

The inhaled mass of DP100® in this study was about 10% of the nebulizer charge. The efficiency of DP100® has recently been demonstrated to be very sensitive to volume fill [9] and the optimal volume fill is between 12 mL [10] and 18 mL [11]. In this study, we did not use the "control dose" which was designed by the nebulizer company to improve the efficiency of DP100® for small fill volumes. DP100® was, therefore, not used in our study in optimal conditions with regard to the inhaled mass. However, our goal was not to optimize the way nebulizers should be used but to obtain enough drug on a filter to test its antibiotic activity, and this was achieved with both nebulizers. Furthermore, both nebulizers produced an aerosol with a particle size distribution that would make it suitable for peripheral lung deposition.

In order to assess the antibiotic activity of colistin after nebulization, we had to capture the drug, measure the amount of drug deposited on the filter, extract it and compare its antibiotic properties with a similar mass of reference drug, *i.e.* non-nebulized drug. Because the validity of the bacteriological study required the avoidance of any loss of drug, the inhaled mass was measured before filter processing using isotopic methods. For this purpose, the colistin was mixed with a tracer (^{99m}Tc-labelled HSA) and it was demonstrated that the distribution of the ^{99m}Tc activity in the aerosol correlated with the distribution of the drug itself. Because the regression slope between the mass of colistin and the activity of ^{99m}Tc-labelled HSA was 0.865, the data were corrected by dividing them by this value to determine the true inhaled masses. The quality of colistin extraction was also verified by studying reference filters on which known amounts of non-nebulized colistin had been deposited. Indeed, using bacteriological methods similar to those applied to the inhaled mass filters, the antibiotic activity measured after extraction of the drug from the reference filters was the expected activity taking into account the mass of drug deposited.

Two different observers each established the postnebulization antibacterial activity indices, MID and MBD, in triplicate. The difference between observers and/or experiments was never more than two dilutions. These results demonstrate the relevance and reproducibility of the method in the present experimental conditions. The most frequent MID was used to calculate the postnebulization MIC, chosen to assess the hypothetical effect of nebulization on colistin antibiotic because the MIC of the ATCC 27853 *P. aeruginosa* strain to colistin is known to be 1 $\mu g \cdot mL^{-1}$. The fact that after nebulization the MIC was less than two dilutions different from the reference colistin with both nebulizers demonstrates that neither jet nor ultrasonic nebulization processes alter the antibiotic properties of colistin. There is, therefore, no loss in terms of drug activity when colistin is administered by aerosol.

In conclusion, colistin can be prescribed as an aerosol generated either by jet or ultrasonic nebulization to treat *Pseudomonas aeruginosa* infection, without any risk of degradation of the drug by the nebulization process.

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