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Abstract: In this work different nebulisers were investigated in order to assess their efficiency in combination with colistimethate sodium (CMS) inhalation products. Four nebulisers, namely I-neb®, Aeroneb® Go, eFlow® rapid and PARI LC® Sprint were studied in terms of delivered dose (DD), drug delivery rate (DDR) and respirable dose (RD) of CMS. The goal was to provide scientific data to physicians for prescribing the most appropriate nebuliser for the CMS specific user.
All the apparatuses nebulised ColiFin 1MIU/3ml solution (80 mg of CMS) with delivered doses between 31% and 41% of the loaded amount. Aeroneb Go showed the longest nebulisation time (more than 20 min). When ColiFin 2MIU/4ml was nebulised with eFlow rapid or PARI LC Sprint, the CMS respirable dose was 45.3 mg and 39.2 mg, in times of 5.6 and 10.8 min, respectively. I-neb, having a medication cup capacity limited to 0.4 mL, loaded with Promixin 0.4 MIU/0.4 ml (32 mg of CMS), provided in a time of 9 min a RD of 21.5 mg, a value slightly higher than those obtained by nebulising ColiFin 1MIU/3ml with the other nebulisers.
The results illustrate that the clinical outcome depends on the comparative analysis of nebulisation efficiency (respirable dose) and convenience (time), not disregarding the ratios between the amount loaded, delivered and deposited at lung level.

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Editor-in-Chief
Professor Alexander T Florence
International Journal of Pharmaceutics

Parma, January 25th, 2015

Object: Submission of revised manuscript #IJP-D-15-02039R1

Dear Professor Florence,

I wish to thank the Editor and the Board for the important work done in reviewing the paper we submitted. The comments have been addressed and guided the manuscript toward a substantial improvement.
The suggestions of the reviewer have been taken into account as well as additional experimental data have been added in order to explain the differences in performance of the various nebulizer used with the aim to better manifest the clinical relevance of the result obtained. However, the authors did not rank the combinations between nebulizers and solution, leaving to the physician the choice on the most appropriate combination in dependence on the needs of the user.

I look forward to having our manuscript reconsidered for publication in International Journal of Pharmaceutics

Yours sincerely,

Francesca Buttini

IJP AUTHOR CHECKLIST

Combinations of Colistin Solutions and Nebulisers for Lung Infection Management in Cystic Fibrosis Patients

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***Response to Reviewers**

RESPONSES TO REVIEWERS of the manuscript entitled *"Combinations of Colistin Solutions and Nebulisers for Lung Infection Management in Cystic Fibrosis Patients"* (Ms. Ref. No.: #IJP-D-15-02039).

The authors wish to thank the reviewer for the useful comments. The results of the review will give a decisive improvement to the quality of the manuscript itself.

The suggestions of the reviewer have been taken into account in order to explain the differences in performance of the various nebulizer used with the aim to better manifest the clinical relevance of the result obtained. However, the authors did not rank the combinations between nebulizers and solution, leaving to the physician the choice on the most appropriate combination in dependence on the needs of the user.

In particular, concerning the suggestions:

1. the statistics in the discussion of the differences has been introduced where the mean values and standard deviations were not immediately clear;
2. the data with Aeroneb Go have been repeated and complemented with 2MIU/4ml;
3. the use of Colifin formulation with I-neb has not been performed since this nebulizer requires a more concentrated solution for the limited volume of the reservoir;
4. the pediatric breathing pattern was not explored due to the three levels of the specifications reported in the Pharmacopoeia. We preferred to use the breathing profile indicated for adults of Eu.Pharm, leaving to the physician the decision to compensate the dose for the different breathing capability. The recommended dose for the patients older than two years has been specified according to the products leaflet that is from 4 MIU up to 6 MIU per day.

5. the F1 and F2 filters were checked visually for saturation, in particular for the higher rate nebulizers. The amount of aerosol collected was always not more than 40% of the total volume of loaded solution, due to the large amount dispersed or remaining into the nebulizer.

6. the missed nebulization with 2 MIU in Figure 4 (now 3) have been included, except the I-neb for the previous mentioned reasons. The aim was to give the values of mg deposited and not to rank the product/nebulizer combination.

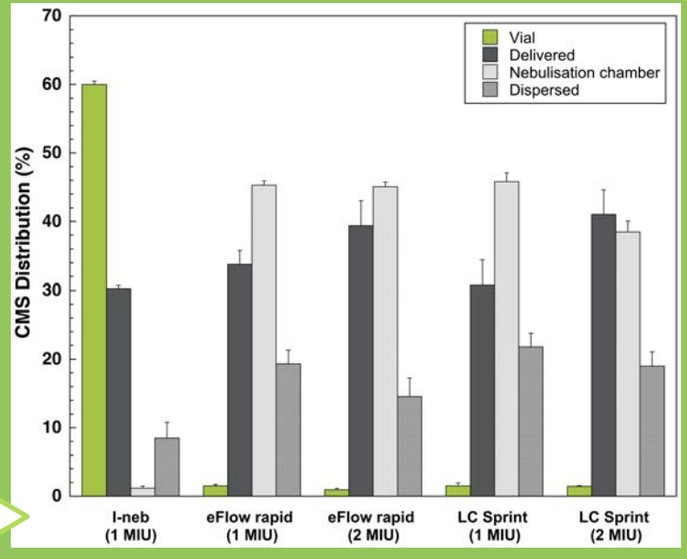
Finally, we tried to include in various section of the manuscript references in terms of mechanistic explanation of the studied nebulisers and their effect on CMS solution aerosolisation.

*Graphical Abstract (for review)

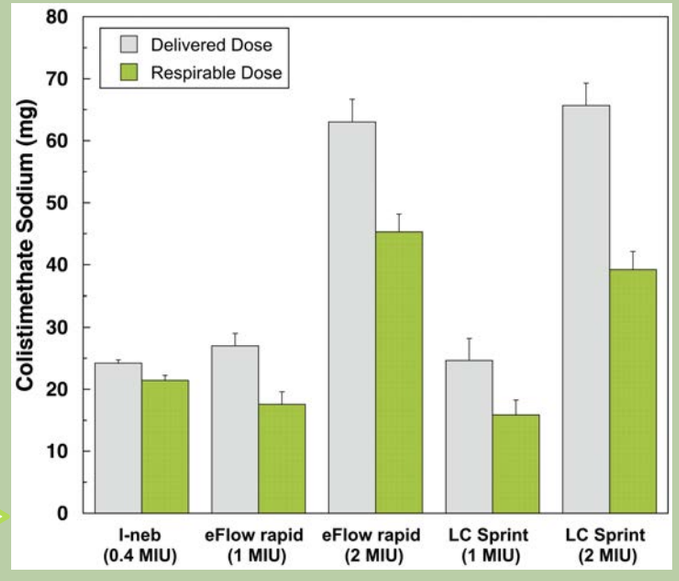
Product/Nebuliser Combination

Colistimethate Sodium
1 MIU (80 mg)
2 MIU (160 mg)

Drug Distribution after Nebulisation



Delivered and Respirable Dose



Combinations of Colistin Solutions and Nebulisers for Lung Infection Management in Cystic Fibrosis Patients

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Abstract

In this work different nebulisers were investigated in order to assess their efficiency in combination with colistimethate sodium (CMS) inhalation products. Four nebulisers, namely I-neb®, Aeroneb® Go, eFlow® *rapid* and PARI LC® Sprint were studied in terms of delivered dose (DD), drug delivery rate (DDR) and respirable dose (RD) of CMS. The goal was to provide scientific data to physicians for prescribing the most appropriate nebuliser for the CMS specific user.

All the apparatuses nebulised ColiFin 1MIU/3ml solution (80 mg of CMS) with delivered doses between 31% and 41% of the loaded amount. Aeroneb Go showed the longest nebulisation time (more than 20 min). When ColiFin 2MIU/4ml was nebulised with eFlow *rapid* or PARI LC Sprint, the CMS respirable dose was 45.3 mg and 39.2 mg, in times of 5.6 and 10.8 min, respectively. I-neb, having a medication cup capacity limited to 0.4 mL, loaded with Promixin 0.4 MIU/0.4 ml **(**32 mg of CMS), provided in a time of 9 min a RD of 21.5 mg, a value slightly higher than those obtained by nebulising ColiFin 1MIU/3ml with the other nebulisers.

The results illustrate that the clinical outcome depends on the comparative analysis of nebulisation efficiency (respirable dose) and convenience (time), not disregarding the ratios between the amount loaded, delivered and deposited at lung level.

Keywords: Colistimethate sodium; Promixin; ColiFin; membrane nebuliser; jet nebuliser; aerosol.

Abbreviation Section

AAD	Adaptive Aerosol Delivery
CF	Cystic fibrosis
CMS	Colistimethate sodium
DD	Delivered Dose
DDR	Drug Delivery Rate
DPI	Dry Powder Inhaler
$D_{(v,50)}$	Median Volume Diameter
GSD	Geometric Standard Deviation
RD	Respirable Dose
RF	Respirable Fraction
MIU	Million International Units
MMAD	Mass Median Aerodynamic Diameter
NGI	Next Generation Impactor
PA	*Pseudomonas aeruginosa*

1. Introduction

Cystic fibrosis is a genetic disease caused by a mutation of the gene coding for cystic fibrosis transmembrane conductance regulator (CFTR) protein that controls the transport of chloride and sodium ions across epithelial membranes. One of the major hallmarks of CF disease is the lung infections by *Pseudomonas aeruginosa* (PA) occurring as early as the first year of life (Salvatore et al., 2012). Pulmonary infection exacerbations of CF patients are commonly treated with systemic antibiotics. The pulmonary administration of antibacterial drugs in form of aerosol is prescribed to manage and control the infection, with the aim to prevent exacerbations (Heijerman et al., 2009). Inhalation route delivers antibiotics directly to the bronchial site of PA infection, while decreasing the systemic exposure.

The doses of pulmonary antibiotics to achieve the infection management are relatively high for the lung administration (from 75 to 300 mg). Consequently, antibiotics are formulated as

77 solution for nebulisation or powder for inhalation, both products capable of delivering high
78 payloads (Balducci et al., 2015; Belotti et al., 2014; Belotti et al., 2015).
79 Colistin for inhalation (administered as colistimethate sodium, CMS) is a cationic polypeptide
80 antibiotic obtained from *Bacillus Polymyxa*; its mechanism of action is the destruction of the
81 outer membrane of the Gram negative bacteria, leading to leakage of intracellular contents
82 and bacterial death (Nation and Li, 2009). Its parenteral use is limited by systemic toxicity;
83 consequently, it has been proposed as inhalation solution for nebulisation and recently, as dry
84 powder for inhalation (Colobreathe®). The twice-daily nebulisation of CMS solution is a time
85 consuming procedure that affects the patient quality of life, leading to lower compliance.
86 Colobreathe DPI is used two times per day as well, breathing in the device as deeply as
87 possible until the capsule containing 125 mg of drug is emptied.
88 (http://www.medicines.org.uk/emc/PIL.27801.latest.pdf)
89 As a reaction to the DPI concurrence, the nebuliser producers focused on the dose
90 nebulisation time by improving the existing devices. Today, CMS solutions for nebulisation
91 are administered with a variety of nebulisers, despite the producers of drug product declare in
92 the leaflet the apparatus tested in the clinical trials. This often creates uncertainty in terms of
93 performance of product/device combination or appropriateness of use with different patients.
94 As example, Promixin® is recommended with the smart membrane device (I-neb) and
95 ColiFin® with the eFlow *rapid* membrane nebuliser. Successful delivery to patients of colistin
96 aerosol is technique-dependent. Therefore, clinicians need to know the performance of
97 nebulisers available for CMS aerosol therapy and the technique to be used in clinical practice
98 with each type of nebuliser (Ari, 2014).
99 In this work, the *in vitro* respirability of the aerosols generated by different nebulisers for the
100 CMS therapy by inhalation was assessed. The study was devoted to solution/device
101 combinations, using three registered CMS solutions for nebulisation having different

concentrations. Four nebulisers, three vibrating membrane and one jet nebuliser, namely I-neb®, Aeroneb® GO, eFlow® *rapid* and PARI LC® Sprint, respectively, were compared. In particular, the pharmaceutical characteristics, i.e, delivered dose and delivery rate and the clinically relevant parameter respirable dose (droplets of size below 5 μm) were measured and compared. The aim was to provide to physicians and nurses the correct knowledge of doses for a specific user by selecting the appropriate nebulising system to combine with CMS solutions.

2. Materials and methods

2.1 Materials

Three different formulations for inhalation of colistimethate sodium were tested: ColiFin® 1MIU/3ml (Batch 1751691) and ColiFin® 2MIU/4ml (Batch 1832597) (PARI Pharma GmbH, Starnberg, DE) and Promixin® 1MIU/1ml (Batch 3K08PM-IT) (Profile Pharma Ltd, Chichester, UK). 1MIU corresponds to 80 mg of CSM. Physiologic saline solution (sodium chloride 0.9% w/v) was obtained by Eurospital Spa (Trieste, IT); purified water was produced by reverse osmosis (Milli-Q Gradient system, Millipore, Molsheim, FR). Analytical grade acetonitrile and trifluoracetic acid were purchased from Sigma Aldrich (Milan, IT).

Three different electronic vibrating membrane nebulisers and one breath enhanced jet system were employed in this work to nebulise CMS solutions. eFlow® *rapid* (PARI Pharma GmbH, Munich, DE) and Aeroneb® Go (Aerogen, Galway, IE) were selected as active membrane nebulisers. I-neb®, the smart nebuliser working with Adaptive Aerosol Delivery (AAD) system (Philips Respironics, Chichester, UK) was used with the grey medication chamber (0.3 mL volume) recommended for Promixin.

The CMS solutions were also tested with PARI LC® Sprint breath enhanced jet nebuliser,

hereafter LC Sprint, that releases more aerosol during inhalation through one-way valves in the mouthpiece, powered by a PARI BOY S® compressor (PARI Pharma GmbH, Munich, DE) working at flow rate of 18.5 L/min.

2.2 Nebulisation procedure

All tests were conducted dissolving the CMS freeze-dried powders with the appropriate volume of saline, accurately pipetted into the vial that was slightly shaken and allowed to stand until the powder was dissolved. The solutions were considered ready for the experiments when the foam layer disappeared.

The devices Aeroneb Go, eFlow *rapid* and LC Sprint were tested using two different drug strengths, i.e., ColiFin® 1MIU/ 3ml and 2MIU/4 ml. The grey medication chamber of I-neb was filled up to the maximum volume (0.4 mL) using Promixin 1MIU/1ml. The washing of the nebuliser between the repetition tests was performed according to the producer indications.

2.3 Determination of CMS Delivered Dose and Delivery Rate

Delivered dose (DD), drug delivery rate (DDR) and nebulisation time values were determined in accordance with the European Pharmacopoeia 8th ed. (Ph.Eur 2013).

A breathing simulator (Model SRV500CC, VCS Srl, Parma, IT) mimicking an adult breathing pattern (15 breath/min, tidal volume of 500 mL) and an inhalation:expiration ratio of 1:1 was used. The nebulisers were filled with the respective volumes of CMS formulation, as reported in Table 1. Each nebuliser was connected to the sine pump through a filter holder (PARI Pharma GmbH, Munich, DE) containing a filter (Pall Corporation, type A/E Glass diameter 76 mm, NY, US), using rubber adaptors specifically built to connect the nebuliser mouthpiece to the filter holder.

The pump was switched on and 10 seconds later the nebuliser was activated. The nebuliser was run for 60 seconds and then switched off. Five seconds later the pump was stopped. The filter (F1) and its filter holder were removed and substituted with a new filter and holder (F2). The pump was switched on and 10 seconds later the nebuliser was activated. The nebuliser was run until 1 minute after the end of nebulisation. Five seconds later the pump was stopped. The CMS amount deposited on the two filters and holders, the amount remaining inside the nebulisation chamber and the amount left in the glass vial were quantitatively collected using purified water/saline solution (50/50% v/v). The amount of CMS of each sample was determined by HPLC analysis. Each test was executed in triplicate.

The mass of CMS emitted was calculated by summing the CMS collected on F1, F2 and their filter holders. The DDR, representing the mass emitted per minute, was measured by quantifying the CMS collected on the F1. The nebulisation time is the time (min) required to aerosolize the entire loaded dose from the start until the end of aerosol production.

2.4 Median volume diameter determination by laser diffraction

Median volume diameter ($D_{(v,50)}$) was determined by low angle laser light scattering with a Spraytec (Malvern Instruments Ltd, Worcestershire, UK) according to EN 13544-1:2001. CMS reconstituted solution was placed in the nebuliser and an aspiration flow (50 L/min) provided by a vacuum pump was put in front of the mouthpiece of the nebuliser at a distance of 10 cm. The aerosol cloud was arranged to cross the laser beam perpendicularly. The mouthpiece was positioned 2.0 cm from the beam and 1.0 cm at the side of the detector. Each measurement was taken at the first a minute after the start of the nebulisation. Each measurement was performed in triplicate. The result of the analysis was expressed as median volume diameter ($D_{(v,50)}$) i.e., cumulative undersize volume diameter at 50% of particle population and geometrical standard deviation (GSD).

2.5 *In vitro* respirable dose determination

In accordance with Ph. Eur. 8th ed. specifications on aerosols the aerosol aerodynamic characterization was conducted using a next generation impactor (NGI, Copley Scientific Limited, Nottingham, UK). All the parts of the NGI, i.e. the impactor, the induction port and the micro-orifice collector, were pre-cooled in a refrigerator (set at 5°C) for 90 minutes before the analysis. The NGI was coupled with a VP1000 S vacuum pump (Erweka GmbH, Heusenstamm, DE) and a Critical Flow Controller (TPK Copley, Copley Scientific Limited, Nottingham, UK). The air-flow rate was set at 15 L/min by flow meter (DFM 2000, Copley Scientific, Nottingham, UK).

The nebulisers were filled with the respective volumes of CMS solution. The nebuliser was connected to the mouthpiece adapter of the induction port, ensuring that all connections were airtight. The aspiration through the system was turned on for 30 seconds, then the nebuliser was activated for a sampling period between 180-360 s to avoid overloading on the stages. The nebuliser was switched off, followed 5 seconds later by the stopping of the vacuum pump. The amount of CMS deposited on the different parts of the impactor was collected using purified water/saline solution (50/50% v/v). The amount of CMS of each sample was determined by high-performance liquid chromatography (HPLC) analysis. Nebulisation was performed in triplicate at environmental conditions (23 ± 2 °C and 50 ± 5% RH).

The amount of drug deposited in the impactor allowed for the calculation of different aerosol parameters. The respirable fraction (RF%) was the ratio between the mass of drug with aerodynamic diameter lower than 5 μm and the total mass collected inside the NGI multiplied for 100.

The Mass Median Aerodynamic Diameter (MMAD) and the Geometric Standard Deviation (GSD) were determined by plotting the cumulative percentage of mass lower than a stated

diameter (probability scale) versus aerodynamic diameter (log scale).

The mass of drug with aerodynamic diameter lower than 5 μm, calculated by the following formula, represents the respirable dose (RD):

$$RD = \frac{RF\% \times DD}{100} \qquad \text{equation 1}$$

where RF is the respirable fraction as percentage and DD the delivered dose.

2.6 Drug assay

CMS was analysed using a validated HPLC method using an Agilent HP 1260 (Agilent Technologies, CA, USA) equipped with a UV-Visible Detector (G1314B), auto-sampler (G1329A), degassing unit (G1322A) and column oven (G1316A). ChemStation Rev. B.03.02 was the Agilent software used to analyse the data.

The analysis was performed using a Luna C18 reverse-phase stationary column, 150x3.0 mm, 3 μm, 100 A (Phenomenex®, Torrance, CA, USA). The column oven was set at 40 °C. Trifluoroacetic acid 0.05% v/v water solution (Sol A) and acetonitrile (Sol B) mixture were used as mobile phase in a gradient elution according to the following sequence: 0-2 min, 20% Sol B; 2-6 min, 95% Sol B; 6-7 min, 95% Sol B; 7-8 min, 20% Sol B; 8-13 min, 20% Sol B. CMS was analysed at a detector wavelength of 214 nm. The injection volume was 100 μl and a flow rate of 0.45 mL/min was used. CMS retention time was about 7 minutes. The method precision (Relative Standard Deviation calculated following six injections of a 0.49 mg/mL standard solution) was 0.16% and the linearity was in a range from 0.05 to 1 mg/mL (R^2 =0.9965). LOD and LOQ values were 0.01 mg /mL and 0.05 mg/mL respectively and the accuracy of the method expressed as percentage recovered of a CMS working standard solution was 95.88% with a confidence interval of 0.95.

Statistical analysis

All results are expressed as mean and standard deviation of at least three separate determinants. To determine significance between groups, unpaired two-tailed t-tests was performed and quoted at the level of $p<0.05$.

3. Results and discussion

Colistimethate sodium has been demonstrated effective in managing lung infections of CF patients caused by multidrug-resistant *Pseudomonas aeruginosa* and *Klebsiella pneumonie*. Furthermore, in combination with other drugs, CMS is used to attack PA biofilm (Herrmann et al., 2010). CMS for inhalation is supplied as lyophilized powder of sodium methanesulfonate derivative of colistin. Dissolved in water, colistimethate sodium undergoes hydrolysis to a mixture of sulphomethyl derivatives and active colistin A and colistin B (Yahav et al., 2012). The the product leaflet states that colistimethate sodium should be reconstituted not more than 24 h prior to administration.

To treat CF lung chronic infection, CMS products for nebulisation on the market are prescribed with recommended specific nebuliser. These products have different CMS concentration and often CMS solutions for inhalation are randomly combined with nebulisers (Doring et al., 2000). Inhalation by nebulisation is a typical combination therapy and its efficacy depends on the physico-chemical characteristics of drug solution and on the nebuliser used to generate the aerosol (Le et al., 2010). The inhaled dose of CMS may change considerably using different nebulisers with the same drug solution; then, the efficacy of the inhalation product varies with the delivery device. This device variability makes crucial for the therapy the knowledge of the delivered and respirable doses, the last, the amount of drug having size capable to penetrate the lungs and deposit on the target site.

There are two registered CMS medicinal products with recommended specific nebulisers.

Promixin (1MIU/1ml) leaflet reports the use of I-neb, a vibrating membrane nebuliser coupled with adaptive Aerosol Delivery (AAD) technology, designed to deliver small volumes of concentrated solutions. Metering chambers, colour coded for easy use and ranging from 0.25 to 1.4 mL, are available for different drug formulations (Denyer et al., 2010).

ColiFin product is available in two strengths for the nebuliser ampoule filling: 1MIU/3 mL (80 mg of CMS) and the more concentrated 2MIU/4mL (160 mg of CMS). ColiFin recommends eFlow *rapid*, an active vibrating membrane nebuliser (116 kHz) based on a metal perforated membrane with 4000 tapered holes. However, Promixin, ColiFin or CMS generic solutions approved for inhalation could be nebulised with non-specified aerosol generating apparatuses. For this reason, in this study two additional marketed nebulisers, i.e., the vibrating membrane Aeroneb Go, and the breath-enhanced open vent jet nebuliser LC Sprint, have been tested. Figure 1 illustrates the four nebulisers studied.

Using the CMS products, the drug delivery rate, the delivered dose and the nebulisation time were preliminary measured (Table 1). Aeroneb Go, eFlow *rapid* and LC Sprint nebulisers were tested with both the Colistin solution strengths, whereas I-neb was tested only with Promixin concentrated solution.

The I-neb has a limitation in the dose to deliver due to filling chamber volume. As a consequence, using the Promixin 1MIU/1ml solution, 0.6 mL of solution, corresponding to 60% of the nominal dose, remains in the product vial and has to be discarded as specified in the leaflet. This fact imposes to compare the mass balance of the nebulisation with all the apparatuses studied. The distribution of the nebulised solution as per cent of dose delivered, residue in the nebulisation chamber and product vial, and dispersed in the environment (Figure 2) was similar when the 1MIU/3 ml solution was used and delivered by the membrane eFlow *rapid* or the jet LC Sprint. With these nebulisers, 34 and 31% of the vial CMS content, respectively, was delivered. It was significant to observe that 45 and 46% remained in the

ampoules and 19 and 22% was lost in the environment. In both the nebulisers that differ for aerosolisation mechanism, the nebulisation of a higher volume of the more concentrated solution (2MIU/4mL) determined an increased CMS delivered dose to 39 and 41%, and a residual in the ampoule of 45 and 38% (Figure 2 and Table 1).

Aeroneb Go, loaded with either 1MIU/3ml or 2MIU/4ml, showed a significant low residual volume in the reservoir placed above the membrane, but a very consistent amount of drug was dispersed. This has to be assigned to the aerosol impact on the nebuliser mouthpiece and the significantly long nebulisation time that required about 300 inhalation/expiration acts to empty the chamber content.

Analysing the amount of aerosol exiting the nebuliser and available for inhalation (Table 1), eFlow *rapid*, LC Sprint and Aeroneb Go, loaded with Colistin solution 1MIU/3ml (80 mg CMS) provided delivered amount values in nearby interval 27.1, 24.7 and 29.3 mg, respectively.

The nebulisation chamber of I-neb (0.3 mL) filled up to the maximum level with Promixin solution, could contain 0.4MIU/0.4 mL i.e. 32 mg of CMS. Despite this significant lower amount, the dose of CMS delivered with I-neb was 24.2 mg, a value not dissimilar to the other nebulisers. Thus, this device was quite efficient (see Figure 2) in CMS nebulisation. This depended in part from the higher CMS concentration, the very small residual volume and the consistent delivery, since I-neb is equipped with the Adaptive Aerosol Delivery. The AAD system predicts the length of the patient's next inhalation based on the duration of the three previous acts, and delivers a pulse of aerosol into the first 50% of that inhalation (Nikander et al., 2010).

The parameter DDR represents the amount of drug released by the nebuliser in the first minute. The value is related both to nebuliser characteristics and to concentration and volume of loaded drug. The delivery rate can have an important effect on drug deposition. In our

study the rate varied from 1.2 mg/min for Aeroneb Go with Colistin 1MIU/3ml, to 9.6 mg/min with eFlow *rapid* loaded with Colistin 2MIU/4ml. Obviously, the DDR increased when the more concentrated 160mg/4ml solution was loaded in the apparatus. The most concentrated Promixin (80mg/ml) was used only with I-neb nebuliser, since the solution was expressly formulated for this nebuliser, requiring to be diluted to 3 or 4 ml in case of use with the others. In this nebuliser the DDR was 2.7 mg/min.

A typical manner to increase the delivered dose is to load larger amount of CMS, such as 2 MIU. Using the more concentrated solution in the reservoir, i.e., ColiFin 160 mg of CMS dissolved in 4 mL, the delivered amount of CMS rose to 63.0, 65.7 and 58.9 mg for eFlow *rapid*, LC Sprint and Aeroneb Go, respectively. Aeroneb Go (membrane) doubled the delivered dose. However, the increase of concentration and volume of the CMS solution provided more than the double of delivered dose using eFlow *rapid* (membrane) and LC Sprint (jet), that are the fastest nebulisers in the group studied. This performance was not dependent on the mechanism of aerosolisation.

The nebulisation rate could influence the convenience and adherence of patients to the therapy, since it determines the nebulisation time. Compliance in CF patients has been demonstrated to decrease by increasing the treatment time (Latchford et al., 2009). The apparatuses studied completed the nebulisation in different times from 3.7 to 25.1 minutes. For instance, eFlow *rapid* showed the fastest performance when filled with 1MIU/3mL solution. This apparatus belongs to the new-generation nebulisers used to reduce the time for inhalation, the strategy adopted to improve compliance. The significantly shorter nebulisation time of eFlow *rapid* does not affect the antibiotic efficacy. It has been shown that plasma and sputum concentration in CF patients with PA infection supported a comparable pulmonary delivery and safety of antibiotic solution administered using eFlow *rapid* or Pari LC Plus (Govoni et al., 2013). Despite the low volume to deliver in comparison to the other

apparatuses, I-neb nebulised the content in 8.9 min. Aeroneb Go required 20.6 and 25.1 min to nebulise 1MIU/3ml and 2MIU/4 mL of CMS solution, respectively. This active membrane nebuliser has a membrane with only 1000 holes vibrating at 100 kHz.

Completed the analysis of the quantitative delivery parameters (pharmaceutical characteristics), the therapy with inhalation antibiotics requires the deposition of the drug droplets in the lungs in contact with the infected site. This is the clinically relevant aspect that determines the effectiveness of the antibiotic aerosol therapy. The deposition is measured as respirable dose that is the amount of particles/droplets having an aerodynamic size lower than 5 µm, hence entering the lung. With regard to liquid nebulisation, optimal deposition for the treatment of the chronic *PA* infection has been found with a MMAD of 1-5 µm, combined with a respiration air flow rate of approximately 15–30 l/min and the inhalation volume convenient for the subject (Heijerman et al., 2009).

The aerodynamic diameter (d_{ae}) is the parameter expressing the capability of a droplet to follow an air stream, calculated from the equivalent volume diameter by the formula:

$$d_{ae} = d_v \sqrt{\frac{r}{r_0}} \qquad \textit{equation 2}$$

where d_v is the spherical equivalent volume diameter, ρ is the density of the solution, ρ_0 is a density of 1.00 g/mL Regarding the liquid aerosol, d_{ae} is close to d_v since droplets have a spherical shape and density close to 1 g/mL (Beck-Broichsitter et al., 2014; Buttini et al., 2013).

The median volume diameters ($D_{(v,50)}$) was determined by laser diffraction. CMS solutions aerosolised with the different nebulisers gave a droplet range considered functional for inhalation, specifically between 5.3 µm for Aeroneb Go 2MIU/4ml and 3.8 µm for eFlow *rapid* 2MIU/4 ml (Table 2). The aerosols generated were monodisperse around the mean droplet size. This condition is considered very effective for inhalation treatment (Usmani et

al., 2005).

MMAD, the value involved in the respirable dose calculation, was determined from the particle size distribution obtained from the next generation impactor. eFlow *rapid* and LC Sprint presented values close to 4 μm, in good agreement with the geometric value obtained by laser diffraction (Table 3). It was described that with CMS 1MIU/3ml inhalation solution with eFlow *rapid*, the geometric and aerodynamic droplet size distribution showed good correlation (Bitterle et al., 2008). In contrast, the Aeroneb Go produced an aerodynamic droplet size close to 4 μm, significantly smaller than the geometric size for 2MIU/4ml ($p = 0.002$). The increase of solution concentration did not change significantly the aerodynamic diameter. Then, the aerodynamic test leads to MMAD values around 4 μm for all the nebulisers, except for I-neb that was significantly lower.

The knowledge of the MMAD allowed the aerosol respirable fraction calculation for the classical nebulisers with ColiFin 1MIU/3ml or 2MIU/4 ml. The values of RF as percentage were between 56% and 72% (Table 3). The highest value among these nebulisers was obtained with eFlow *rapid* and ColiFin 2MIU/4ml. However, I-neb presented the highest value of respirable fraction with 88.6%. Now, multiplying the respirable fraction with the respective delivered dose, the respirable dose that is the value clinically significant, directly connected with the CMS activity, was calculated.

Figure 3 compares the delivered dose and respirable dose of CMS from the tested nebulisers with different loaded products. In I-neb, that produced an aerosol of small droplets (MMAD = 2.69 μm), despite the low filling volume (32 mg of CMS), the favourable RF contributed to a respirable dose of 21.5 mg. Interestingly, this value was higher than those obtained nebulising 1MIU/3mL with both the other membrane and jet nebulisers.

The possibility to fill the nebuliser with 2MIU/4ml of CMS significantly augmented the dose inhaled by the patient, in particular the dose deposited on the infected site. Considering that

the eradication and suppression of *PA* is dependent on the minimal inhibitory concentration of antibiotic, the amount of drug at the target site should be maximized combining the drug product and nebuliser (Weers, 2015). In our study, there were not largely different respirable doses among the nebulisers, but the top amount was achieved when eFlow *rapid* was filled with 2MIU/4mL (Figure 3). In this case, the loaded dose and delivered dose gave the possibility to obtain up to 45.3 mg of drug expected to enter the upper airways and deposit in the lower airways. In addition, this result could be obtained in a significantly short time of nebulisation. However, taking into account the variability of the determinations, LC Sprint respirable dose was not significantly different from eFlow *rapid* nebuliser ($p = 0.06$) and the amount of colistimethate sodium able to *in vitro* deposit into lungs by Aeroneb Go was not significantly different from LC Sprint ($p = 0.1$).

In summary, despite the different nebulisation structure and mechanism, eFlow *rapid*, LC Sprint and Aeroneb Go nebulising 1MIU/3ml of ColiFin (CMS 80 mg), showed similar *in vitro* performances, providing respirable doses of 17.6 mg, 15.9 and 16.3 mg, respectively. In this case for the three nebulisers, the nebulisation time was 3.7 min, 5.4 min and 20.6 min, respectively. I-neb, with its limited capacity of medication cup (0.4 mL; 32 mg of CMS) provided a RD of 21.5 mg, a value higher than that obtained by the other devices nebulising 1MIU/3mL. The aerosol respirable dose of CMS solutions was maximized when 2 MIU/4ml (CMS 160 mg) were nebulised with either the membrane or jet nebulisers. The most effective administration of CMS solution by inhalation in terms of respirable dose was seen with the combination between 2MIU/4mL of ColiFin with eFlow *rapid* or LC Sprint nebulisers (45.3 mg and 39.2 mg, respectively) (see Table 3).

Now, the effectiveness of the nebulisation of CMS solutions could be metered by combining the dose deposited with the time of nebulisation, taking into account the yield of the process compared to the drug amount used.

Conclusions

The recommended daily dose of CMS nebulisation for patients older than two years ranges from 4 MIU daily up to 6 MIU per day. Thus, Promixin and ColiFin, due to their different drug concentrations, require to be combined with a nebuliser able to support the prescribed dose in a time convenient for the patient. The therapy effect is dependent on the respirable dose, i.e. the amount deposited into lung regarded as the clinically relevant parameter, that is a fraction of both the CMS dose loaded into device and amount delivered to patient. Colistimethate sodium nebulisation performance changed more with drug concentration than with delivery device characteristics. Different solution volumes and doses of ColiFin, aerosolised with the classical nebulisers studied, showed that the performance did not differentiate too much among them, whereas Promixin with the smart nebuliser I-neb presented the highest nebulisation efficiency, but the dose loaded was low in relation to the recommended posology.

Finally, the lung deposition of the aerosol obtained from different concentrated colistimethate sodium solutions and nebulisers dictates the therapy design to the physician for cystic fibrosis patients. The real effectiveness of the nebulisation, intended to improve the clinical outcome, derives from a combined analysis of the respirable dose provided and the respiration time duration, without disregarding the yield of the treatment in relation to the drug amount used.

It was out of the scope of this study to rank the nebulisers but simply to provide experimental data to the users in order to facilitate their choice in terms of apparatus to combine with the different CMS solution strength.

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426 Author Disclosure Statement

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Figure Legends

Figure 1. Nebulisers employed to aerosolise CMS reconstituted solutions. From left to right: Aeroneb® GO, I-neb®, eFlow® *rapid* and LC Sprint®.

Figure 2. Percentage distribution of CMS vial content: amount remaining in the vial, delivered by the nebuliser, entrapped inside the nebulisation chamber and dispersed in the environment during the nebulisation, (n=3, mean value and standard deviation). Drug amount used: 1 MIU of CMS (80 mg) or 2 MIU (160 mg).

Figure 3. Delivered and respirable dose of CMS provided by different nebulisers and solution strengths (n=3, mean values standard deviation).

Table(s)

Tables

Table 1. Aerosol delivery values of CMS from different nebulizers using different CMS strengths and filling volumes, (n=3; mean values ± standard deviation).

	I-neb	**Aeroneb Go**	**Aeroneb Go**	**eFlow *rapid***	**eFlow *rapid***	**LC Sprint**	**LC Sprint**
Loaded Dose/ Filling Volume	**Promixin 0.4 MIU/0.4 ml (32 mg)**	**Colifin 1 MIU/3 ml (80 mg)**	**Colifin 2 MIU/4 ml (160 mg)**	**Colifin 1 MIU/3 ml (80 mg)**	**Colifin 2MIU/4 ml (160 mg)**	**Colifin 1 MIU/3 ml (80 mg)**	**Colifin 2 MIU/4 ml (160 mg)**
Drug delivery rate (mg/min)	2.7 ± 0.2	1.2 ± 0.0	1.3 ± 0.3	3.5 ± 0.2	9.6 ± 0.7	5.2 ± 0.9	6.7 ± 0.5
Neb. time (min)	8.9 ± 0.6	20.6 ± 0.2	25.1 ± 0.7	3.7 ± 0.1	5.6 ± 0.4	5.4 ± 0.4	10.8 ± 0.7
Delivered Dose (mg)	24.2 ± 0.5	29.3 ± 2.3	58.9 ± 1.3	27.1 ± 2.0	63.0 ± 3.7	24.7 ± 3.6	65.7 ± 3.6

Table 2. Median volume diameter ($D_{(v,50)}$) and Geometrical Standard Deviation (GSD) values measured by laser light diffraction from different nebulizers using different filling volumes, (n=3; mean values ± standard deviation).

	I-neb	**Aeroneb Go**	**Aeroneb Go**	**eFlow *rapid***	**eFlow *rapid***	**LC Sprint**	**LC Sprint**
Loaded Dose/Filling Volume	**0.4MIU/0.4 ml (32 mg)**	**1MIU/3ml (80 mg)**	**2MIU/ml (160 mg)**	**1MIU/3ml (80 mg)**	**2MIU/4ml (160 mg)**	**1MIU/3ml (80 mg)**	**2MIU/4ml (160 mg)**
$D_{(v,50)}$	4.3* ± 0.4	4.7 ± 0.1	5.3 ± 0.1	4.1 ± 0.1	3.8 ± 0.1	4.2 ± 0.1	4.4 ± 0.0
GSD	_	1.4 ± 0.0	1.5 ± 0.0	1.6 ± 0.0	1.6 ± 0.0	2.0 ± 0.0	2.1 ± 0.0

*available from (Medicines and Healthcare products Regulatory Agency, 2011)

Table 3. Aerosol aerodynamic characterization of CMS aerosol generated by different nebulizers using different filling volumes, (n=3; mean values ± standard deviation).

	I-neb	**Aeroneb Go**	**Aeroneb Go**	**eFlow *rapid***	**eFlow *rapid***	**LC Sprint**	**LC Sprint**
Loaded Dose/ Filling Volume	**0.4 MIU/0.4 ml (32 mg)**	**1 MIU/3 ml (80 mg)**	**2 MIU/4 ml (160 mg)**	**1 MIU/3 ml (80 mg)**	**2 MIU/4 ml (160 mg)**	**1 MIU/3 ml (80 mg)**	**2 MIU/4 ml (160 mg)**
MMAD	2.69 ± 0.14	4.11 ± 0.26	4.04 ± 0.23	4.13 ± 0.13	3.86 ± 0.05	4.02 ± 0.41	4.10 ± 0.21
GSD	1.56 ± 0.02	2.09 ± 0.04	2.11 ± 0.03	1.6 ± 0.0	1.60 ± 0.01	2.21 ± 0.12	2.14 ± 0.11
RF %	88.6 ± 1.9	55.8 ± 1.3	58.1 ± 1.5	65.1± 1.0	71.9 ± 0.8	64.5 ± 1.3	59.7± 1.2
RD (mg)	21.5 ± 0.8	16.3 ± 0.4	34.3 ± 0.9	17.6 ± 2.0	45.3 ± 2.9	15.9 ± 2.4	39.2 ± 2.9

Figure(s)

Figure 1.

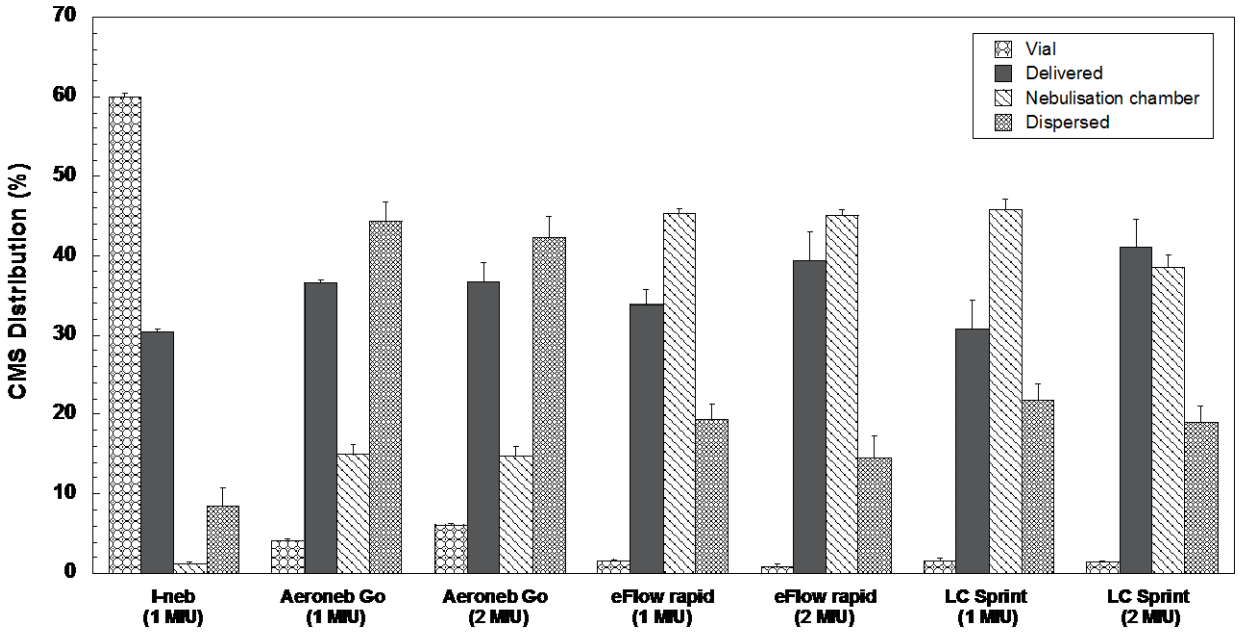
CMS Distribution (%)
70
60
50
40
30
20
10
0
Vial
Delivered
Nebulisation chamber
Dispersed
I-neb (1 MIU)
Aeroneb Go (1 MIU)
Aeroneb Go (2 MIU)
eFlow rapid (1 MIU)
eFlow rapid (2 MIU)
LC Sprint (1 MIU)
LC Sprint (2 MIU)

Figure 2.

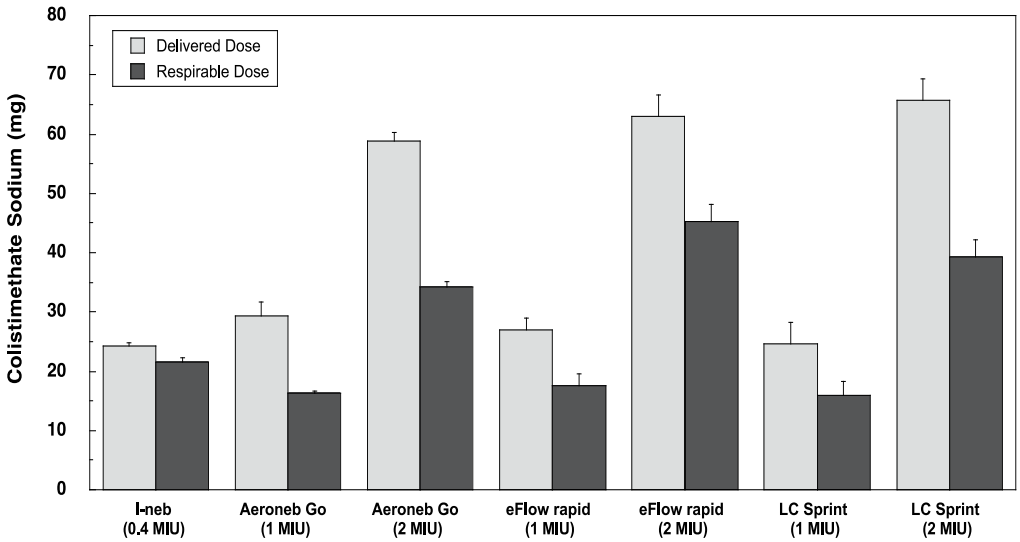
Delivered Dose
Respirable Dose
Colistimethate Sodium (mg)
80
70
60
50
40
30
20
10
0
I-neb (0.4 MIU)
Aeroneb Go (1 MIU)
Aeroneb Go (2 MIU)
eFlow rapid (1 MIU)
eFlow rapid (2 MIU)
LC Sprint (1 MIU)
LC Sprint (2 MIU)

Figure 3