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Physicochemical compatibility of nebulizable drug admixtures containing colistimethate and tobramycin

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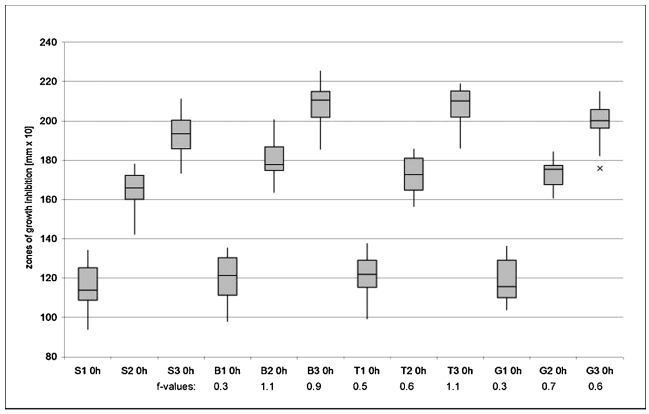
Inhalation therapy with nebulizable antibiotic drugs is a mainstay in treating *Pseudomonas aeruginosa* infections in cystic fibrosis patients. The combination of tobramycin and colistin was found to be superior to monotherapy in killing *P. aeruginosa* in biofilms. The simultaneous inhalation of tobramycin and colistin might be an option to increase the compliance of patients. The objective of this *in-vitro* study was to determine whether admixtures of inhalation solutions containing colistin methanesulfonate (CMS) and tobramycin are physicochemically compatible. Physical compatibility was determined by measuring pH and osmolality. Chemical compatibility was determined by testing the antibiotic activity of the mixtures by the pharmacopoeial microbiological assay and comparing the results to those of standard solutions. Samples were analyzed immediately after mixing and after 24 h. Values of pH and osmolality remained unchanged and in physiologically acceptable ranges. Neither for colistin methanesulfonate (CMS) nor for tobramycin losses of antibiotic potency were registered at any time. Admixtures of nebulizer solutions containing CMS and tobramycin were shown to be physicochemically compatible. Further investigations are needed to determine whether drug delivery is affected by mixing the nebulizer solutions to ensure that simultaneous inhalation is recommendable.

1. Introduction

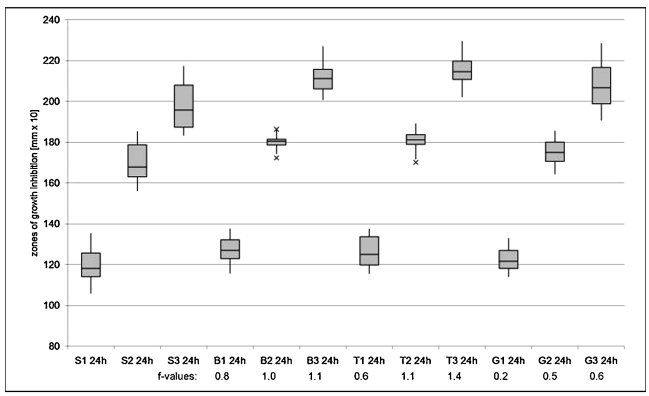
Chronic airway infection is the hallmark of cystic fibrosis (CF) lung disease which may start very early in the life of CF patients (Emerson et al. 2002). Pulmonary insufficiency is responsible for at least 80% of cystic fibrosis-related deaths (O'Sullivan and Freedman 2009). The most common airway pathogen in patients with CF is *Pseudomonas aeruginosa* (Flume et al. 2007; Doring et al. 2000). Respiratory infection with *P. aeruginosa* was found to be a major predictor of morbidity and mortality (Emerson et al. 2002). Soon after onset of infection, *P. aeruginosa* evolves into variants forming mucoid, biofilm like macrocolonies (Doring et al. 2000; Herrmann et al. 2010). The mucoid variant requires 100–1,000 times the concentration of a certain antibiotic to be effective compared with its non-mucoid variant (Doring et al. 2000).

Inhalation therapy with nebulized antibiotics is a mainstay in the treatment of *P. aeruginosa* associated lung infections (O'Sullivan and Freedman 2009; Flume et al. 2007; Hodson et al. 2002; Touw et al. 1995). Potential advantages of aerosol delivery of antibiotics are high drug concentrations in the lung, decreased toxicity as systematic absorption is limited, reduced costs, and less disruption to patient's life especially when compared with intravenous administration (Doring et al. 2000; Campbell and Saiman 1999). Tobramycin, a member of the aminoglycoside antibiotic drug category, is highly recommended for chronic use to improve lung function and reduce exacerbations (Flume et al. 2007). The long-term use of tobramycin is common all over Europe which causes a remarkable increase in resistance (Li et al. 2006; Prince 1986).

Due to the difficulty of treating *P. aeruginosa* infections and the increasing resistance, combining two antibiotics with different modes of actions is obvious. The well-known polymyxin antibiotic colistin has particular importance (Li et al. 2006). Nebulized colistin is used frequently in treating *P. aeruginosa* infections in CF patients although there is poor evidence for colistin monotherapy to improve lung function and reduce exacerbations (Flume et al. 2007). Studies of Herrmann et al. (2010) showed that combination therapy with colistin and tobramycin is superior to monotherapy for killing *P. aeruginosa* biofilm cells in a rat lung infection model. Furthermore consecutive inhalation of the two antibiotics resulted in a significant decrease in *P. aeruginosa* colony-forming units in sputum of CF patients (Herrmann et al. 2010). The prolonged time period needed for consecutive inhalation of the two antibiotics was mentioned as a disadvantage in this study. This prompted us to investigate the physicochemical compatibility of admixtures of inhalation solutions containing colistin methanesulfonate (CMS) and tobramycin, since compatibility is regarded as a prerequisite for effective simultaneous inhalation of different nebulized drugs (Kamin et al. 2006).



(a)



(b)

Fig. 1: Antibiotic activity of CMS expressed as the diameters of growth inhibition zones (N = 24), a) immediately after mixing and b) after 24 h of storage. The whiskers indicate the 1.5 x interquartile range (IQR), the crosses indicate outliers (outside of 1.5 x IQR). S = Colistin CF Standard, B = mixture of Colistin CF with Bramitob®, G = mixture of Colistin CF with Gernebcin®, T = mixture of Colistin CF with TOBI®; 1 = 11 µg CMS/µl, 2 = 55 µg CMS/µl, 3 = 111 µg CMS/µl.

2. Investigations and results

2.1. Physical compatibility of inhalation admixtures

Mixing the hypotonic inhalation solutions TOBI®, Gernebcin® and Bramitob® with Colistin CF initially resulted in one less hypotonic (TOBI® + Colistin CF) and two isotonic solutions (Gernebcin® + Colistin CF and Bramitob® + Colistin CF). In the mixtures containing Colistin CF and one of the three different tobramycin containing inhalation solutions no significant changes in pH and osmolality were registered over the test period of 24 hrs (compare Table 1). No colour changes, particulate matter or turbidity were observed.

2.2. Microbiological potency

In Fig. 1 the diameters of inhibition zones determined in the CMS potency assays are illustrated as box plots. With regard to CMS no loss of antibiotic activity was observed in the inhalation mixtures compared to unmixed components. The results rather indicate a slight increase in antibiotic activity in comparison to the standard solutions. However the calculated f-values, expressing the differences between the mean values in no case reached twofold the combined standard deviation thereby indicating that the microbial activities of the CMS test solutions are not different from the standard solutions immediately after mixing of the components (compare Fig. 1). The same results were noticed after 24 h of storage. There was a trend towards increased antimicrobial activity over the storage time and the same trend when comparing test solutions to standard solutions. The differences did not exceed twofold the combined standard deviation (compare Fig. 1).

As an example for the different tobramycin containing mixtures antimicrobial potency of tobramycin was determined for the mixture of Bramitob® and Colistin CF. Tobramycin potencies were not different when potency in the mixture and the standard solutions were compared neither initially after mixing nor after 24 h of storage (compare Fig. 2).

The results of the compatibility studies prove that all admixtures of Colistin CF with Bramitob®, TOBI®, or Gernebcin® are physicochemically stable over a period of 24 h.

Clinical studies regarding the treatment of *P. aeruginosa* infections by simultaneous inhalation of nebulized tobramycin and colistin are necessary to control our findings *in vivo* and to further improve antibiotic inhalation therapy in CF patients.

3. Discussion

Osmolality and pH are important factors influencing the tolerability of nebulizable drugs. Values outside of the pH limits set by the pharmacopoeia (4.5 to 8.7) and an osmolality outside the range of 150 – 550 mOsmol/kg can cause airway hyperreactivity, manifesting as cough or bronchoconstriction or both (Law 2011; Pharmacopoeia Europaea 2011). Osmolality and pH of the mixtures stayed within the ranges of the mentioned specifications.

Microbiological assays were chosen for the determination of chemical compatibility of nebulizable drug products containing tobramycin and CMS. The reason is, that CMS hydrolyzes in aqueous solution and forms an extremely complex mixture of partial sulphomethylated derivates and the active metabolite colistin (Li et al. 2003a). By microbiological assays the antibiotic activity of CMS and its degradation products is assayed in sum. Even though specific HPLC assays have been developed to determine CMS and colistin in parallel in pharmaceutical products (Li et al. 2003b; Wallace et al. 2008), partially hydrolyzed CMS derivatives are not captured by these methods and quantification is problematic. Although the variance of results is bigger in bioassays, microbiological assays were considered as the most appropriate ones to detect the overall antibiotic activity and changes due to mixing. The bioassay was reported elsewhere to be accurate and sensitive (Wootton et al. 2005). The two different strains used as indicator organisms differed from those recommended in the Pharmacopoeia because the mixtures contained two antibiotic drugs and selective resistance of the strains against CMS and tobramycin was inevitable to achieve valid results. Appropriateness and sensitivity of the indicator organisms were given.

The trend towards increased antibiotic activity of mixed inhalation solutions in comparison to the standard solution could be a result of the combination of two antibiotics. The combination of colistin and tobramycin was reported to be more effective in killing *P. aeruginosa* in biofilms (Herrmann et al. 2010). Considering the degradation pattern of CMS, increased potency can also result from higher concentrations of hydrolyzed CMS. CMS is the inactive prodrug of colistin (Bergen et al. 2006) and the formation of colistin and partially hydrolyzed CMS is a prerequisite for antibacterial activity against *P. aeruginosa*. CMS was developed because it is less nephrotoxic and neurotoxic than colistin when administered parenterally. Most common potential side effects with inhalation are chest tightness and bronchospasm whereas CMS is associated with lower adverse effects than colistin (Li et al. 2006). Due to the very low systemic exposure after inhalation of CMS/colistin, systemic side effects are unlikely.

Our results show that there is no initial loss of antibiotic activity due to mixing. A potential increase of activity due to the rea-

Table 1: pH values and osmolality of mixtures of CMS with tobramycin containing inhalation solutions and of unmixed drug products.

Method	pH values ± SD		Osmolality [mOsmol/kg] ± SD	
	Initially after mixing	After 24 hrs	Initially after mixing	After 24 hrs
Test solution				
Colistin CF + Bramitob® (n = 9)	5.10 ± 0.04	4.88 ± 0.04	316.8 ± 13.0	330.0 ± 8.1
Colistin CF + TOBI® (n = 9)	5.85 ± 0.01	5.57 ± 0.01	263.6 ± 6.3	277.2 ± 3.2
Colistin CF + Gernebcin® (n = 9)	5.42 ± 0.02	5.37 ± 0.01	303.3 ± 3.2	307.3 ± 2.6
Bramitob® (n = 3)	5.38 ± 0.03	n.d.	244.0 ± 1.0	n.d.
TOBI® (n = 3)	6.09 ± 0.02	n.d.	171.0 ± 1.5	n.d.
Gernebcin® (n = 3)	5.62 ± 0.01	n.d.	97.3 ± 0.6	n.d.
Colistin CF (n = 3)	7.26 ± 0.02	n.d.	368.7 ± 0.6	n.d.

SD = Standard Deviation; n.d. = not determined.

sons mentioned above is particular small because differences in activity, compared to the CMS standard solutions, lay within the 2-fold variance range. According to the HPLC-studies of Wallace et al. (2008), hydrolysis of CMS in pharmaceutical products proceeds faster in diluted solutions and at higher temperatures. Concentrations of the mixtures investigated here varied from 10 to 16 mg/ml and less than 4% of the total CMS is expected to be converted to colistin. The results of the microbiological assays correspond to the results of the HPLC-assays reported by Wallace et al. Furthermore, hydrolysis of CMS to colistin would be expected to be accompanied by an increase in the number of osmotically active species (Wallace et al. 2008). The unchanged osmolality over the entire test period supports our results of the bioassay indicating no or minimal hydrolysis of CMS to colistin. Tobramycin is known to be very stable in aqueous solution (Brandl and Gu 1992). Oxidation is the major degradation pathway for tobramycin at pH values between 5.8 and 7.4 and leads to decreased antibiotic activity. The rate of degradation depends on temperature and time and relevant changes were not to be expected in our setting (Brandl and Gu 1992).

Mixing the antibiotic drugs offers the possibility to benefit from combination therapy without additional expenditure of time for the patients. Several surveys showed that patients tend to mix inhalation solutions anyway to shorten the time consuming inhalation procedure (Bergen et al. 2006; Brandl and Gu 1992). Simultaneous nebulization of inhalation solutions can affect drug delivery, e.g. drug output and aerosolized droplet size (Kamin et al. 2007; Berlinski and Waltrep 2006). Due to the increased charge volume and the constant dead volume of the nebulizer, nebulization of admixtures can be more efficient. If nebulization is continued until the nebulizer runs dry, total mass output and mass of nebulized drug is increased (Kamin et al. 2007). However future studies should address the effect of mixing colistin and tobramycin nebulizer solutions on aerosol characteristics and drug output. Regarding microbiological aspects, mixing should only take place immediately before administration and remaining drug admixture should be discarded.

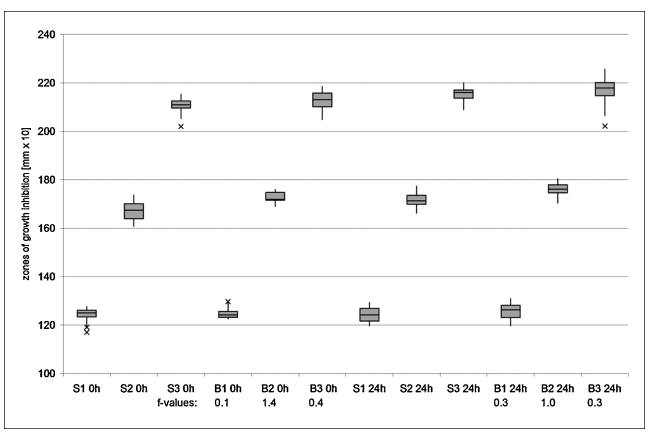


Fig. 2: Antibiotic activity of tobramycin expressed as the diameters of growth inhibition zones (N = 24), immediately after mixing and after 24 h of storage. The whiskers indicate the 1.5 x interquartile range (IQR), the crosses indicate outliers (outside of 1.5 x IQR). S = Bramitob® Standard, B = mixture of Bramitob® with Colistin CF; 1 = 21.4 µg tobramycin/µl, 2 = 42.8 µg tobramycin/µl, 3 = 214 µg tobramycin/µl.

4. Experimental

All tests were performed with the commercially available nebulizer solutions Colistin CF (80 mg colistin methanesulfonate/3 ml, Grünenthal, Aachen, Germany, Lot 907M02), Bramitob® (300 mg tobramycin/4 ml, Asche Chiesi, Hamburg, Germany, Lot IG015), TOBI® (300 mg tobramycin/5 ml, Novartis, Nürnberg, Germany, Lot 06K6AI2), and Gernebcin® (80 mg tobramycin/2 ml, Infectopharm, Heppenheim, Germany, Lot G030904). Colistin CF powder was reconstituted with 3 mL 0.9% NaCl solution for injection resulting in a CMS solution containing 1 Mio I.U./3 mL.

Mixtures were prepared in triplicate by mixing 3.0 ml CMS solution with either 4.0 ml Bramitob®, 5.0 ml TOBI® or 2.0 ml Gernebcin® in 10 ml polypropylene tubes with cap. Mixtures were stored at room temperature and under ambient light conditions over a testing period of 24 h.

4.1. Physical compatibility

Samples of the admixtures were analyzed in triplicate immediately after mixing and after 24 h of storage. Values of pH were measured with a digital pH-meter (pH 2010 Microprocessor, HANNAH Instruments, Kehl am Rhein, Germany) with glass electrode. Osmolality was determined *via* the freezing-point depression method with an osmometer (Osmomat 030 Cryoscopic Osmometer, Gonotec GmbH, Berlin, Germany). Whenever samples were withdrawn, the test solutions were visually inspected with the unaided eye for colour changes, turbidity or particular matter.

4.2. Microbiological assays of CMS and tobramycin

Potency of tobramycin and CMS in the admixtures were determined by microbiological assays according to the Ph. Eur. monograph 2.7.2 "Microbiological Assay of Antibiotics" (Phar-

macopoiea Europaea 2011). The inhibitory effect of a known concentration of an antibiotic drug on sensitive microorganisms is compared to the inhibitory effect of known concentration of a reference standard. The procedures were carried out according to the EUCAST guidelines (EUCAST 2009).

Two different bacteria strains were required for testing admixtures of tobramycin and CMS. The strain used in the CMS assay, needed to be sensitive to CMS or rather colistin and resistant to tobramycin and the strain used for testing tobramycin *vice versa*. According to the literature *Pseudoxanthomonas mexicana* ATCC 700993 was selected to determine the microbial potency of colistin (Thierry et al. 2004). A suitable strain to assay the potency of tobramycin was identified by testing the Minimum Inhibitory Concentrations (MIC) of tobramycin and CMS against several bacteria strains via Etest® (Biomérieux, Marcy-lÈtoile, France, Etest® Colistin Lot 1000103390, Etest® Tobramycin BK1085). *Staphylococcus aureus* ATCC 29213 turned out as most suitable.

4.3. Sample preparation for tobramycin assays

Tobramycin standard solutions were prepared in triplicate by diluting Bramitob® with 0.9% sodium chloride solution to nominal concentrations of 21.4 µg/ml, 42.8 µg/ml and 214 µg/ml. Aliquots withdrawn from the mixtures of Colistin CF and Bramitob® were diluted with 0.9% sodium chloride solution to nominal concentrations according to the standard solutions. Samples were prepared in duplicate.

4.4. Sample preparation for CMS assays

CMS standard solutions were prepared in triplicate by diluting Colistin CF solution with 0.9% sodium chloride solution to nominal concentrations of 11 µg/ml, 55 µg/ml and 111 µg/ml. Aliquots withdrawn from the mixtures consisting of Colistin CF with Bramitob®, TOBI® or Gernebcin® were diluted with 0.9% sodium chloride solution to nominal concentrations according to the standard solutions. Samples were prepared in duplicate.

Under aseptic conditions 40 µl aliquots of the samples prepared were transferred to blank BD sensi disks (BD Diagnostics, Heidelberg, Germany, Lot 0272065) and air dried for 10 to 15 min. The discs were placed on inoculated rectangular agar plates; the antibiotic concentrations were taken into account by placing discs with high concentrations not next to each other resulting in 16 discs per plate. The inoculum was prepared from overnight cultures (on blood agar, incubated 18 – 20 h at 37 °C) of *Pseudoxanthomonas mexicana* or *Staphylococcus aureus* which were suspended in sterile 0.9% sodium chloride solution and diluted to a standardized density using McFarland standard 0.5. The inoculum suspension was streaked on commercially available Mueller-Hinton II agar plates 12x12 cm (BD Diagnostics, Heidelberg, Germany, Lot 1144322) and incubated for 20 ± 4 h at 35 ± 2 °C. The resulting zones of inhibition were measured in duplicate to the nearest 0.1 mm using vernier callipers. Each sample disc with one of the three concentrations of test samples or standard samples was prepared and assayed in duplicate (in total 12 discs per concentration).

To compare the antibiotic activity of the test solutions and the standard solutions, mean values and standard deviations of diameters of growth inhibition zones were calculated. Differences between the mean values of corresponding concentrations were evaluated using the following equation, where t means test solution, s means standard solution and SD is the standard deviation:

$$f = \text{mean (t)} - \text{mean (s)} \,/\, \sqrt{(SD(t)^2 + SD(s)^2)}$$

Where f represents the coefficient of the combined standard deviation which expresses the difference between the two mean values; 0 means there is no difference and e.g. 2 means the difference between the mean values is within the range of twofold the combined standard deviation. Additionally, median diameters of growth inhibition zones were calculated and illustrated as box plots.

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