European Journal of Pharmaceutical Sciences 73 (2015) 88–92

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Drug release from an oromucosal paste for the selective decontamination of the oropharynx (in ICU patients and healthy volunteers)

Johannes Rood [a,1], Haif Nawzad [a,1], Raween Kalicharan [a,b], Mies van Steenbergen [b], Herman Vromans [a,b,*]

[a] Department of Clinical and Compounding Pharmacy, Compounding University Medical Centre Utrecht, PO Box 85500, 3508 GA Utrecht, The Netherlands
[b] Department of Pharmaceutics, Utrecht University, PO Box 80082, TB Utrecht, The Netherlands

ARTICLE INFO

Article history:
Received 26 January 2015
Received in revised form 19 March 2015
Accepted 25 March 2015
Available online 3 April 2015

Keywords:
Selective oropharyngeal decontamination (SOD)
Oromucosal adhesive paste
Biopharmaceutical justification
Local concentration antibiotics

ABSTRACT

Selective oropharyngeal decontamination (SOD) is used in many ICUs in the Netherlands and some other European countries. While its clinical effect has been studied intensively, no studies have been done to assess the biopharmaceutical aspects of the paste, i.e. it is not known which local concentrations exist. For this study, five healthy volunteers were subjected to 400 mg of the generally used paste. Ten ICU patients were treated according to the normal standard in the ICU of the University Medical Center Utrecht. Salivary levels of the various substances were measured over time using two separate analytical methods. Also the microbial burden of the oropharynx was assessed. The results show significant variation in release, both ICU patients and healthy volunteers. The antimicrobials tobramycin and colistin showed a relatively fast release, while nystatin exhibited a controlled release-like pattern. Amphotericin B is hardly released from the formulation. The concentration of the antimicrobial agents drop to sub-MIC levels relatively fast. From a biopharmaceutical perspective, amphotericin B should be replaced by nystatin. The application of the mouth paste is subject to massive variation in daily practice; each nurse applies a different amount, in a different way. In addition, the formulation is hard to apply and unpleasant with regards to the taste and feel for the conscious patients.

This is not a clinical study, but a study that aimed to give a biopharmaceutical justification for SOD Both the clinical practice and the clinically determined levels of drugs enable critical evaluation of the outcome of clinical studies performed until now.

1. Introduction

In 1983, the idea of selective oropharyngeal decontamination was opted by Stoutenbeek et al. (1983). They based the concept on the observation that infections in critically ill patients often originate from pathogenic microorganisms in patients own digestive- and respiratory tract (Stoutenbeek et al., 1983; Park, 2005). The idea of selective decontamination is that a mixture of non-resorbable antimicrobial and antifungal drugs would selectively kill these potentially pathogenic organisms. During its use, the regimen-composition has varied over time. Various antibiotics and antifungals have been used in the products. In the beginning, the treatment consisted of two antibiotics and one antimycotic processed in a mucoadhesive paste and a suspension. Some hospitals add intravenous cefotaxim to improve the decontamination. Currently, both selective oropharyngeal decontamination (SOD) and selective decontamination of the digestive tract (SDD) are considered to be standard care in Intensive Care Units (ICUs) in the Netherlands. The benefit of the therapy has been studied intensively by Stoutenbeek et al. (1987), Pugin et al. (1991), Abele-Horn et al. (1997), De Smet et al. (2009), de Smet et al. (2011) and recently Oostdijk et al. (2014). Current drug products contain tobramycin and colistin to kill bacteria (mainly *Pseudomonas aeruginosa*, *Staphylococcus aureus* and enterobacteria (Park, 2005)) and amphotericin B or nystatin to eliminate Candida. A mucoadhesive paste is used for oropharyngeal decontamination. Until now, no studies have been published on drug release and no effort has been put into the biopharmaceutical quality of the formulations used, which means that no relationship between clinical outcomes and existing drug levels has been demonstrated. The aim of the present study was to evaluate the bio-pharmaceutical aspects of

* Corresponding author at: Department of Clinical and Compounding Pharmacy, Compounding University Medical Centre Utrecht, PO Box 85500, 3508 GA Utrecht, The Netherlands. Tel.: +31 30 253 7306.
E-mail address: h.vromans@uu.nl (H. Vromans).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.ejps.2015.03.025
0928-0987/

the SOD paste by measuring levels of the active compounds in saliva. Additionally, the replacement of amphotericin B by nystatin is evaluated. The reason for this alteration is the poor availability of amphotericin B in the Dutch hospital pharmacy. The study in ICU patients was expanded with a microbiological assay to determine the microbiological burden in the oropharynx.

2. Materials and methods

The study was performed in accordance with the principles of the Declaration of Helsinki (World Medical Association, 2013). The Medical Ethics Evaluation Commission of the University Medical Center Utrecht (UMCU) approved this study under research protocol number 12-499. As this is not a clinical study, a limited amount of patients was included, sufficient to determine the level of drugs in the oral cavity. It was not the aim to determine clinical outcomes. All included subjects, or in case of incapable patients their relatives, were informed about the study and signed an informed consent. Inclusion criteria for healthy volunteers: 18–85 years old, non-smoker, no known diseases, no drug administration one week prior to the study (excluding oral contraceptives), willing to fast in the hour preceding the study and six hours during and good compliance with the study regime. We included patients 18–85 years old. Exclusion criteria were: head or mouth trauma and known colonization of resistant strains. For the study in ICU patients, the regular SOD-paste of UMCU was used. One gram of the standard SOD-paste is composed of a mucoadhesive base consisting of 20 mg amphotericin B (AB), 30.5 mg tobramycin sulfate (TS), equivalent to 20 mg tobramycin base (TB), 20.0 mg colistin sulfate (CS), 50.0 mg liquid paraffin, 170.7 mg hypromellose, and 682.8 mg white Vaseline. The basis of this paste is in fact a generally applied mucoadhesive ointment, which is e.g. found in the dutch formulary (FNA). For the healthy volunteers amphotericin B was replaced by 46.1 mg/g nystatin (NY), equivalent to 250,000 IU/g. In ICU setting, the paste is applied every six hours, in healthy subjects the same time-period was evaluated. The application of the paste in ICU patients was done by the nurses. This was done according to the daily practice. For the healthy volunteers an accurate amount of 400 mg was pre-weighed on cellophane, and was applied using a nitrile glove. This quantity of 400 mg equals the amount that has been proposed at the start of the application of this paste in the early 80's.

2.1. Sample collection

It is only possible to measure the in vivo release of the paste when all saliva is collected. This is of course not possible in patients and therefore this was done with volunteers. They were asked to collect the total saliva production in time-frames up to six hours. The total saliva fractions of ten minutes (0–60), 15 min (60–90) or 30 min (90–360) were collected in standard 15 mL polypropylene tubes. This method was not applicable for ICU patient, therefore a different method was used: At fixed moments, cotton rolls (Salivette®) were placed under the tongue. The roll was removed and placed back into the container when saturation was occurred or after a maximum of five minutes. After the collection of saliva, the Salivettes® were stored at 4 °C for a maximum of two hours. To extract the saliva from the cotton roll, the Salivette® was centrifuged at 4000*g* for 10 min at 4 °C. The saliva was stored for a maximum of 4 h at 4 °C until analysis.

2.2. Analytical method

2.2.1. Tobramycin and colistin

A method using 9-fluorenylmethylchloroformate (FMOC-Cl) derivatization was used, based on the method described by Li et al. (2001) to analyze CS in human plasma. Samples were prepared in the following way: 50.0 µl sample was pre-treated by adding 20.0 µl methanol/10.0% trichloroacetic acid (mixed in equal parts) and after vortex-mixing centrifuged at 22.000*g* for 15 min at 4 °C. 50.0 µl of the supernatant was added to 50 µl 1% carbonate buffer set to pH 10 with a sodium hydroxide solution. 5.0 µl 100 mM FMOC-Cl in acetonitrile was added, and the samples were directly vortex-mixed for one minute at 2200 RPM. After 10 min of reaction time, 70.0 µl of the derivatized sample was transferred to a polypropylene (PP) HPLC vial with septa-less PP caps (Waters Inc.). The derivatives of TS and CS were separated via gradient HPLC (see Table 1) consisting of acetonitrile, water, tetrahydrofuran and trifluoracetic acid (A = 86.2/10.0/3.8/0.1 and B = 91.2/5.0/3.8/0.1). The chromatographic system consisted of a Waters 2695 separation module, and a Waters 2475 fluorescent detector. The fluorescence of FMOC-Cl was detected at λ_{ex} = 260 nm and λ_{em} = 315 nm. Compounds were separated by a GraceSmart RP C18 (5 mm, 250 × 4.6 ID), kept at 30 °C with a flow of 1 mL/min. Auto sampler temperature: 4 °C. Data collection and processing: Empower 3 (Waters Inc.). The lower limit of quantification (LLOQ) was set at 0.5 µg/mL. The upper limit of quantification was 50.0 µg/mL. The calibration curves covered a range of 0.5–50.0 µg/mL in undiluted samples. Outliers were identified using the ROUT-method (Q = 5) (GraphPad Prism 6).

Table 1
HPLC Gradient timetable for the analysis of TS and CS in saliva.

Time	% *A*	% *B*
0	75	25
3.5	75	25
5.5	0	100
10	0	100
11	75	25
15	75	25

2.2.2. Amphotericin B and nystatin

The assay of AB and NY was performed via isocratic Normal-Phase HPLC, based on the method described by Llabot et al. (2007). Sample pre-treatment was done by adding 30.0 µl methanol–acetonitrile (mixed in equal parts) to 30.0 µl sample. After vortex mixing the samples were centrifuged at 22.000*g* for 15 min at 4 °C. 40.0 µl of the supernatant was transferred to a PP HPLC vial with septa-less PP caps (Waters Inc.). The eluent consisted of methanol, water and dimethyl sulfoxide (DMSO) (70/20/10). The chromatographic system consisted of a Waters 2695 separation module, Waters 2487 dual channel UV/VIS detector, λ 305 nm and 405 nm. Compounds were separated by an Alltima Silica (5 mm, 250 × 4.6 ID) kept at 30 °C, with a flow of 1 mL/min. The auto sampler was kept at 20 °C. A standard solution of 200.0 µg/mL for both NY and AB was prepared in DMSO and diluted with demi water. The LLOQ was set at 0.1 µg/mL. The calibration curves covered a range of 0.1–20.0 µg/mL in undiluted samples. When a level lower than the LLOQ was found, the 1/2 LLOQ was used in the representation of the data.

2.2.3. Microbiological analysis

Microbiological analysis was performed by a standard throat-swab using a sterile cotton swab. The microbiological assessment was done by standard microbiological assay. Obtained throat-swabs were analyzed for the presence of (potentially) pathogenic microorganisms. The microbiological assay was performed to assess the effectiveness of the paste.

2.2.4. Mass of the applied paste

To determine the amount that is applied in practice, 11 nurses were asked to apply SOD-paste to a cellophane-sheet (duplo) with

the tools they would use in daily practice. The tools used were gloves, cue-tips, syringes and DentaSwabs®. Nett average mass per gift was calculated using IBM SPSS 12.

3. Results

Five healthy volunteers were enrolled in this study. The characteristics of this group were 23.9 ± 3.1 years of age; the male to female ratio was 60:40. In this group the normal saliva-production was found to be 20.5 ± 8.8 mL per hour. Ten eligible and consenting ICU patients were included for this study. The characteristics of this group were 67.1 ± 15.5 years of age; the male to female ratio was 60:40. The reasons for admission: high energetic trauma: 2; neurological trauma: 2; respiratory distress: 1; cardial trauma: 4. Saliva production in these patients varied considerably between almost no production to overproduction. With regard to ICU patients, this study was purely observational and therefore reflects the daily hospital practice. Patient 4 was put on a different regime, in which the SOD-paste was applied eight times per day due to an elevated CFU count.

3.1. Release of active substances

3.1.1. ICU-patients

A major inter-patient variation in release was found in both ICU patients and healthy volunteers. For instance, the Cmax for TS in ICU patients varied between 0.7 and 153.5 μg/mL, see large standard deviation at Tmax in Fig. 1A. For the further analysis, the CS-data of ICU patients six and eight were excluded. In these patients the levels of CS would suggest that several grams of CS were applied, which was not the case. The TS release did not show the same results, indicating the possibility of an interfering substance.

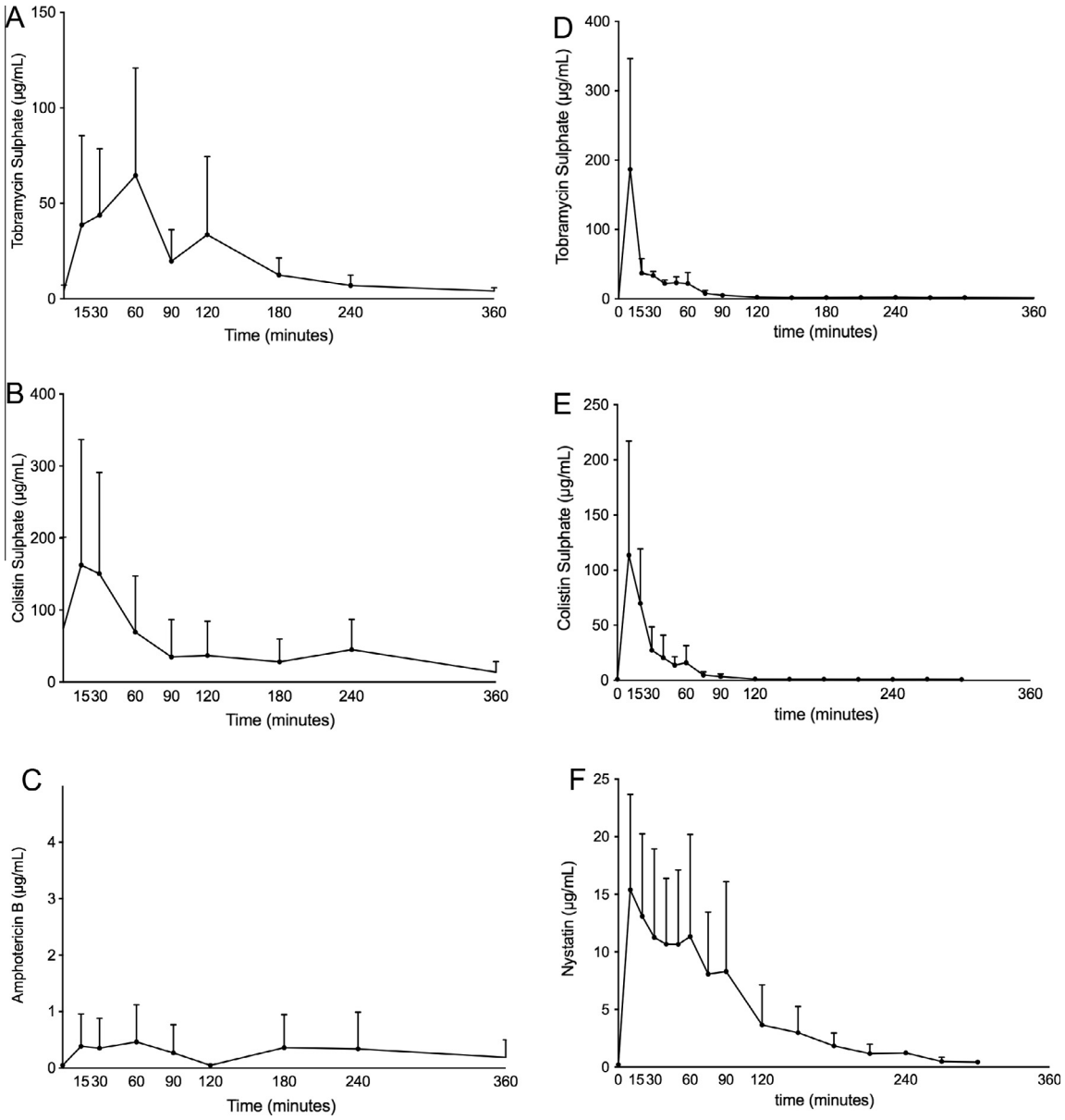

Fig. 1. The concentration of TS (A), CS (B) and AB (C) in the saliva of ICU patients after application of the SOD-paste, containing TS, CS and AB and of TS (D), CS (E) and NY (F) in the saliva of five healthy volunteers after application of the SOD-paste, containing TS, CS and NY. The concentration was measured on multiple time-points for a total of six hours.

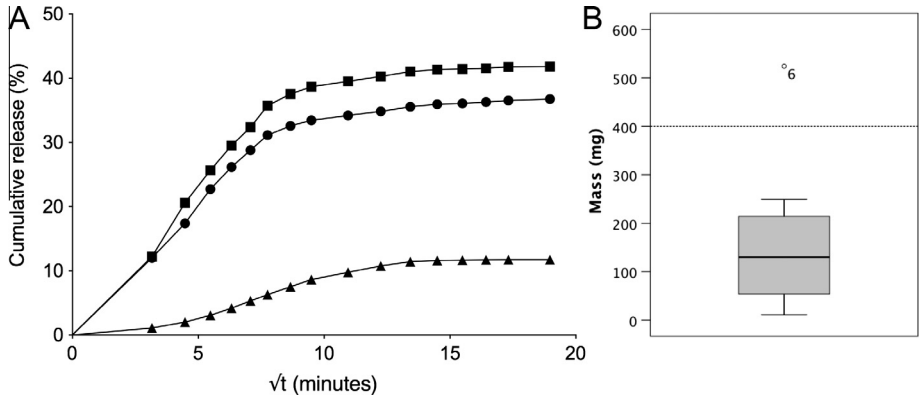

Fig. 2. (A) The cumulative release of compounds TS (●), CS (■) and NY (▲) in the saliva of five healthy volunteers after application of the SOD-paste, containing TS, CS and NY. The concentration was measured on multiple time-points for a total of six hours. (B) Box plot of mass of SOD-paste, applied by 11 nurses (median 130 mg). All data-points were measured in two-fold, the dotted line represents the preferred amount of paste (400 mg).

3.1.2. Healthy volunteers

Due to the sample-size, no outlier-tests were performed on the healthy volunteer data. When data of the healthy volunteers was observed, one could suggest that a higher quantity of TS is released than CS (see Fig. 1D and E respectively). This difference is explained by the higher concentration of TS in the paste, roughly one third higher (30.5 vs 20.0 mg/gram), so relatively speaking, the release properties of both substances are comparable. NY shows a more controlled-like release pattern, with above MIC levels for more than three hours after application. This is substantially longer than TS and CS, which were above MIC for approximately two hours. Fig. 2A depicts the cumulative release of the compounds as obtained from the collected saliva. As can be seen, CS and TS show a higher yield than NY. Principally, this reflects an underestimation of true values, taking into account that swallowing of some saliva is unavoidable. Yet, it is clear that release seems to remain incomplete.

3.1.3. Microbiological data

In ICU-patients an assay was performed to investigate the microbiological growth. Patients 1, 2, 3, 7 and 8 did not show any bacterial or fungal growth in the oral cavity (Table 2). When combining the data, there does not seem to be a correlation between the concentration of antibiotics and the prevalence of microorganisms. As was mentioned earlier, patient 4 received an alternative regime of eight doses per day. This was done in response to an elevated microbial count. We found that in this patient a high fungal count was present. In this particular patient, we did not measure any AB concentration above the detection-level of 0.1 μg/mL.

Table 2
Microbiological data of ten critically ill ICU-patients after multiple applications of the SOD-paste, containing TS, CS and AB.

Time	Patient									
–	1	2	3	4	5	6	7	8	9	10
0	0/0	0/0	0/0	0/++	0/0	0/++	0/0	0/0	0/0	NA
15	0/0	0/0	0/0	0/++	0/⩽5	0/+	0/0	0/0	0/0	$+^{2}$/⩽5
30	0/0	0/0	0/0	0/++	0/0	0/++	0/0	0/0	0/0	$++^{1}$/+
60	0/0	0/0	0/0	0/+++	0/0	0/+	0/0	0/0	0/⩽5	$++^{1}$/⩽5
90	0/0	0/0	0/0	0/+++	0/0	0/⩽5	0/0	0/0	0/0	$+^{2}$/⩽5
120	0/0	0/0	0/0	0/+++	0/0	0/⩽5	0/0	0/0	0/⩽5	$+^{2}$/0
180	0/0	0/0	0/+	–	0/0	0/⩽5	0/0	0/0	0/0	$+^{1}$/0
240	0/0	0/0	0/0	–	0/0	0/+	0/0	0/0	0/⩽5	0/⩽5
360	0/0	0/0	0/+	–	0/0	0/++	0/0	0/0	0/0	$+^{1}$/0

Results shown as 'bacteria/fungi'. 0: negative result, no pathogens found; ⩽5: less than 5 CFUs; +: 1–10 CFUs; ++: 10–100 CFUs; +++: >100 CFUs. Notes: 1: Gram negative bacteria; 2: Enterobacer spp.

3.2. Mass of the applied paste

A large spread of paste application was noticed during the treatment of patients. A small study was conducted to determine the applied quantity. The true applied amount of paste was measured, revealing that too little paste was applied (see Fig. 2). On average, 65% less paste than the required 400 mg was applied (median: 139.0 mg). Furthermore, there is a considerable variation in applied amount. Another observation was, that when using DentaSwabs®, hardly any paste was administered to the oral mucosa. Here, a large amount of paste was pressed into the sponge.

4. Discussion

This study presents the in vivo values of active substances released from SOD-paste. Since the introduction of this therapy, this has not been published so far. We demonstrate that, even in a strictly controlled setting, a major variation in drug concentration was found in the oropharyngeal cavity. It should be noted that the therapeutic SOD protocol may vary between different centers. This may complicate the sound comparison of clinical data gathered in different treatment centers. In future research, the quantitative application of paste should be guaranteed in order to draw a reliable conclusion. One of our findings is that far less than the recommended 400 mg paste was applied. For this reason, an additional investigation was done with the amount of paste that is meant to be applied to patients. Healthy volunteers were given a fixed dose of paste, so that dose variation was reduced to a minimum. Even then, the concentrations measured exhibit a considerable variation. The release of TS and CS in patients is initially well above the MIC values of those compounds. However, after approximately 2 h this is not the case anymore. This means that levels are sub-MIC for the next 4 h. The original formulation was designed in 1983, but until now no biopharmaceutical studies were performed. The classical thoughts on the dosing of tobramycin is based on the intravenous method, in which it is given in a hit and run fashion, mainly because of its toxicity in long term high levels. In non-systemic administration, the post antibiotic or sub-MIC-effect approach might be less relevant. Even when the hit and run strategy is favorable, colistin and nystatin might not favor such an approach. In volunteers, the concentrations are somewhat higher, which is partly due to the larger amount of paste administered. Concentrations drop quicker than in patients. Remarkably, the

release of the paste is not complete (Fig. 2A). Of course, this may be an underestimation as some saliva may be swallowed. Anyway, it can be estimated that at least 40% of TS and CS will reach the intestine within two hours after administration. Surprisingly, NY is released to a far greater extent than AB. An explanation might be that AB is very poorly water-soluble and lipophilic. This result would justify the pending replacement of AB with NY. Even though NY is not nearly released in the amounts that TS and CS are released, the concentration would be enough to achieve a kill-concentration (Arikan et al., 2002). The poor availability, the insufficient quality and high price of amphotericin B for compounding purposes were the reasons to change the antifungal to NY.

Since the introduction of SOD/SDD, a number of clinical studies have been evaluated which overall demonstrate the value of the treatment. These clinical effects are relatively small (Stoutenbeek et al., 1987; Pugin et al., 1991; Abele-Horn et al., 1997; De Smet et al., 2009; de Smet et al., 2011). Recently, Oostdijk et al. (2014) concluded from a randomized clinical trial that there is no difference in day-28 mortality when SOD is compared with SDD. The positive effect of SOD on mortality can be related to the concentrations measured in the present study. It can be speculated that the effect would have been more pronounced when the appropriate amounts of paste would have been applied. Unfortunately, data are not available, however. An improved formulation that maintains super MIC values longer could further improve the clinical outcomes. Currently, predicting antimicrobial effectivity is based on an inhibitory concentration in a closed system, in which there is the assumption that there is no fresh supply of micro-organisms. In the ICU, there are continuous sources of infection, together with an impaired mechanical barrier, such as an altered airway anatomy, decreased mucociliary clearance and an impaired cough-reflex. The effectiveness of antibiotics on cultured microorganisms is only indicative of the effectiveness on colonized bacteria in a microfilm. The extracellular DNA creates an environment surrounding the bacteria that is not easily penetrated by antibiotics. The addition of DNases could aid in the destruction of such a layer, improving the penetration of antimicrobial agents (Kaplan et al., 2011). In summary, our study shows that the current SOD paste is in need for an update. Our findings that TS and CS are only above MIC for a relatively short period and that AB is not released at all support this. Furthermore, the administration of the paste should be unambiguous in method and amount.

5. Conclusion

The current SOD medication is in need of an update. The formulation has been roughly the same as proposed some thirty years ago, is hard to apply and unpleasant with regards to the taste and feel for the conscious patients. The current formulation yield adequate oropharyngeal concentrations of TS and CS which however drop to sub-MIC relatively fast. AB should be replaced by NY as it does not reach MIC at all. An improved formulation should reflect an adequate controlled release mechanism, providing a long lasting barrier against harmful bacteria and fungi.

Conflict of interest

The authors declare that there were no conflicts of interest.

Author contributions

J.R, H.N, M.v.S, R.K and H.V. wrote the manuscript; J.R and H.N designed the study; J.R, H.N and R.K analyzed the data.

Acknowledgements

We acknowledge the support of the University Medical Center Utrecht Intensive Care Center. The authors dedicate this work to the remembrance of Koos Rood.

References

Abele-Horn, M., Dauber, A., Bauernfeind, A., Russwurm, W., Seyfarth-Metzger, I., Gleich, P., Ruckdeschel, G., 1997. Decrease in nosocomial pneumonia in ventilated patients by selective oropharyngeal decontamination (SOD). Intensive Care Med. 23 (2), 187–195.

Arikan, S., Ostrosky-Zeichner, L., Lozano-Chiu, M., Paetznick, V., Gordon, D., Wallace, T., Rex, J.H., 2002. In vitro activity of nystatin compared with those of liposomal nystatin, amphotericin B, and fluconazole against clinical Candida isolates. J. Clin. Microbiol. 40 (4), 1406–1412.

de Smet, A.M.G.A., Kluytmans, J.A.J.W., Blok, H.E.M., Mascini, E.M., Benus, R.F.J., Bernards, A.T., Kuijper, E.J., Leverstein-van Hall, M.A., Jansz, A.R., de Jongh, B.M., et al., 2011. Selective digestive tract decontamination and selective oropharyngeal decontamination and antibiotic resistance in patients in intensive-care units: an open-label, clustered group-randomised, crossover study. Lancet Infect. Dis. 11 (5), 372–380.

De Smet, A.M.G.A., Kluytmans, J.A.J.W., Cooper, B.S., Mascini, E.M., Benus, R.F., der Werf, T.S., der Hoeven, J.G., Pickkers, P., Bogaers-Hofman, D., der Meer, N.J., et al., 2009. Decontamination of the digestive tract and oropharynx in ICU patients. N. Engl. J. Med. 360 (1), 20.

Kaplan, J.B., LoVetri, K., Cardona, S.T., Madhyastha, S., Sadovskaya, I., Jabbouri, S., Izano, E.A., 2011. Recombinant human DNase I decreases biofilm and increases antimicrobial susceptibility in staphylococci. J. Antibiot. (Tokyo) 65 (2), 73–77.

Li, J., Milne, R.W., Nation, R.L., Turnidge, J.D., Coulthard, K., Johnson, D.W., 2001. A simple method for the assay of colistin in human plasma, using pre-column derivatization with 9-uorenylmethyl chloroformate in solid-phase extraction cartridges and reversed-phase high-performance liquid chromatography. J. Chromatogr. B Biomed. Sci. Appl. 761 (2), 167–175.

Llabot, J.M., Allemandi, D.A., Manzo, R.H., Longhi, M.R., 2007. HPLC method for the determination of nystatin in saliva for application in clinical studies. J. Pharm. Biomed. Anal. 45 (3), 526–530.

Oostdijk, E.A.N., Kesecioglu, J., Schultz, M.J., Visser, C.E., de Jonge, E., van Essen, E.H.R., Bernards, A.T., Purmer, I., Brimicombe, R., Bergmans, D., et al., 2014. Effects of decontamination of the oropharynx and intestinal tract on antibiotic resistance in ICUs: a randomized clinical trial. JAMA 312 (14), 1429–1437.

Park, D.R., 2005. The microbiology of ventilator-associated pneumonia. Respir. Care 50 (6), 742–765.

Pugin, J., Auckenthaler, R., Lew, D.P., Suter, P.M., 1991. Oropharyngeal decontamination decreases incidence of ventilator-associated pneumonia: a randomized, placebo-controlled, double-blind clinical trial. JAMA 265 (20), 2704–2710.

Stoutenbeek, C.P., van Saene, H.K.F., Miranda, D.R., Langrehr, D., et al., 1987. The effect of oropharyngeal decontamination using topical nonabsorbable antibiotics on the incidence of nosocomial respiratory tract infections in multiple trauma patients. J. Trauma-Injury, Infect. Crit. Care 27 (4), 357–364.

Stoutenbeek, C.P., Van Saene, H.K.F., Miranda, D.R., Zandstra, D.F., 1983. A new technique of infection prevention in the intensive care unit by selective decontamination of the digestive tract. Acta Anaesth. Belg. 34 (3), 209–221.

World Medical Association, 2013. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. JAMA J. Am. Med. Assoc. 310 (20), 2191.