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Chlorhexidine Digluconate Formulations Used for Skin Antisepsis

S. Salvatico[1], D. Pereira[1], C. Feuillolay[1], N. Bégeault[2], C. Roques[1,3*]

[1]Fonderephar, Faculté des Sciences Pharmaceutiques, Toulouse, France
[2]Pierre Fabre Dermatologie, Lavaur, France
[3]Laboratoire de Génie Chimique UMR 5503, Faculté des Sciences Pharmaceutiques, Université de Toulouse, CNRS, INPT, UPS, Toulouse, France
Email: *ch.roques@wanadoo.fr

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Abstract

Aims: The representativeness of European standards phase 2, step 1 regarding bactericidal and yeasticidal activities was used for the comparison of two marketed antiseptic solutions, one containing chlorhexidine digluconate (0.5%) and the other combining chlorhexidine digluconate (0.25%), benzalkonium chloride (0.025%) and benzylic alcohol (4%). **Methods**: The bactericidal activity of the antiseptic solutions used pure or diluted was assessed according to the European standards NF EN 13727 and NF EN 13624 for the bactericidal and yeasticidal activity respectively. The contact time was 1 min at 20˚C. Interfering substances used correspond to soiling conditions *i.e.* bovine serum albumin and sheep erythrocytes. A reduction of colony-forming units by ≥5 log10 was deemed to meet the requirements to conclude bactericidal activity and ≥4 log10 for yeasticidal activity. **Results**: Regarding all the mandatory strains, both solutions are bactericidal and yeasticidal even after a 40% dilution and even under "dirty" conditions. **Conclusions**: The present study demonstrated the efficient bactericidal and yeasticidal activity of aqueous solutions containing chlorhexidine digluconate either alone at a concentration of 0.5% (w/v) or at a concentration of 0.25% (w/v) when combined with benzalkonium chloride at 0.025% (w/v) and benzylic alcohol 4%. These results have to be considered regarding the respective formulations and potent allergy risks.

Keywords

Antiseptic, *In Vitro* Bactericidal Activity, *In Vitro* Yeasticidal Activity, Chlorhexidine, Benzalkonium Chloride, NF EN 13624, NF EN 13727, NF EN 14885

1. Introduction

Over the past decade, antiseptics have been increasingly used for prophylactic or therapeutic purposes, in healthcare setting [1]. When used appropriately, antiseptics act by reducing the transient then commensal flora [2] [3] permitting infection control.

The choice of an antiseptic and the optimal conditions of use should be guided by its known antimicrobial spectrum [2], the targeted situations/indications and the related risk of potential adverse health effects of each ingredient and of the formulation regarding the population of concern.

There are currently various cutaneous solutions being used for antisepsis, including soaps, alcohol-based solutions, iodine-based solutions, and chlorhexidine agents [4] with the aim to control cutaneous or mucocutaneous microbial colonization resulting in a reduction of the transient or commensal flora [3]. Chlorhexidine (CHX) is frequently considered as gold standard for cutaneous and mucosal antisepsis in many situations regarding its superior microbiological and clinical effects [4] [5] [6] [7].

The antiseptic activity is primarily examined using *in vitro* tests carried out according to internationally accepted standards and generally consisting of the determination of the reduction of the number of viable test microorganisms by pure or diluted solutions. The NF EN 14885 [8] describes how European Standards apply for chemical antiseptics with the distinction of standards without (phase 1) and with interfering substances (phase 2). Organic (proteins) or inorganic (electrolytes, divalent cations) substances found in biological fluids may inhibit activity of active ingredients as well as marketed solutions [9]-[16]. Regarding that, the sensitivity of various agents to interfering substances is taken into account for the standards to be as close as possible to routine practice conditions. The NF EN 13727 [17] and NF EN 13624 [18] standards are phase 2 step 1 standards required for respectively the *in vitro* bactericidal and yeasticidal assessment of final formulations/to-be-marketed antiseptic products with the aim of establishing the bactericidal/yeasticidal activity under simulated practical conditions appropriate to its intended use. Practical conditions correspond to dilutions and time points relevant to the actual use pattern of the final antiseptic preparation and in the presence of adapted interfering substances.

The current NF EN 13727 [17] requires the use of four different test organisms: two Gram-positive *cocci*: *Staphylococcus aureus* CIP 4.83 and *Enterococcus hirae* CIP 58.55, and two Gram-negative *bacilli*: *Escherichia coli* K12 CIP 54117 and *Pseudomonas aeruginosa* CIP 103467 representative of the most clinically relevant organisms found in health care settings, in terms of spectrum and pathophysiological involvement [19] [20] [21]. In the same time, specific contact times for hygienic/surgical hand wash/hand rub uses are indicated as well as the interfering substances regarding the pre-cleaning (clean conditions) or not (dirty conditions). For cutaneous antisepsis, NF EN 13624 [18] concerns *Candida albicans* DSM 1386 which represents the most opportunistic yeast implicated in

cutaneous and mucosal colonization or infection [22] [23].

The objective of the experimentation was to compare two marketed products, one containing only chlorhexidine digluconate at a 0.5% concentration (w/v) and the other containing a lower chlorhexidine digluconate concentration of 0.25% (w/v) but combined with benzalkonium chloride and benzylic alcohol regarding the *in vitro* bactericidal NF EN 13727 [17] and yeasticidal NF EN 13624 [18] activities in soiling conditions.

2. Material and Methods

2.1. Solutions

1) Chlorhexidine digluconate 0.5% (w/v) ready to use solution for cutaneous application (w/v) (Diaseptyl® 0.5%, Pierre Fabre Dermatologie, batches G01050 and G01076),

2) Chlorhexidine digluconate 0.25% (w/v) benzalkonium chloride 0.025% (w/v) and benzylic alcohol 4% (v/v) ready to use solution for cutaneous application (Biseptine Spraid® 0.25%, Laboratoire Bayer Healthcare SAS, batches KP0B1Z1 and KP0CX9W).

The tested antiseptics were diluted in water for injectable preparations (80%, 40% and 1% v/v) as recommended for ready to use products.

2.2. Strains

The bacterial strains recommended by the NF EN 13727 [17] standard were used: *Staphylococcus aureus* CIP 4.83 (ATCC 6538), *Pseudomonas aeruginosa* CIP 103467 (ATCC 15442), *Escherichia coli* CIP 54117 (NCTC 10538), *Enterococcus hirae* CIP 58.55 (ATCC 10541). These strains were provided by the Pasteur Institute Collection (Paris, France) and stored in compliance with the NF EN 12353 standard [24]. *C. albicans* DSM 1386 strain, provided by the Deutsch Collection, was used to evaluate the yeasticidal activity regarding the NF EN 13624 [18].

The maintaining and CFU numerations were performed according to the standards.

2.3. Trial Protocols

Evaluation of the bactericidal activity

Bacterial suspensions were prepared in tryptone salt at a concentration ranging from 1.5×10^8 and 5.0×10^8 CFU (colony forming units)/mL, with a final test concentration ranging between 1.5×10^7 and 5.0×10^7 CFU/mL.

The experimental conditions specified in the NF EN 13727 standard [17] for hygienic and surgical hand rubbing and washing were selected: 1 min ±5 s contact duration at a temperature of 20˚C ± 1˚C. The inactive concentration tested was 1%.

The most restrictive assay condition regarding interfering substances was selected, *i.e.* in dirty conditions (recommended for application without previous cleaning). For dirty conditions, interfering substances were a mixture of 3 g/L

bovine serum albumin (Sigma Aldrich, Saint-Quentin Fallavier, France) and 3 mL of erythrocytes (BioMérieux, Craponne, France) as indicated in the two standards.

Contact between microorganisms and product dilutions was stopped by dilution-neutralization as indicated in the standard. The absence of toxic effect of the experimental conditions and the neutralizer were checked as specified in the NF EN 13727 [17] standard, as well as the dilution-neutralization validation.

After neutralization (neutralizant composition: Tween 80 (10%), Saponin (2%), Lecithin (2%), Sodium thiosulfate (0.5%) (Sigma Aldrich), Trypcase Soja broth (Biomérieux)), residual viable bacteria were counted after incubation in corresponding agar for the control and the tested suspensions. The values of counts after trial expressed in decimal logarithms (log) were subtracted from the basal values prior antiseptic exposure in order to calculate the log reductions. The upper limit of count defined by the standard induces a lower limit in terms of log reduction (<in **Tables 1-4**). The lower limit of count defined by the standard induces an upper limit in terms of log reduction (>in the results tables). According to NF EN 13727 [17], the tested antiseptic solutions were considered bactericidal for hygienic and surgical handrub if, when used undiluted, they produced a ≥5 log reduction in colony counts; and for hygienic handwash or for surgical handwash if, when used diluted (≤50%), they produced a ≥3 log or ≥5 log reduction respectively. The bactericidal activity was considered to be maintained on the microorganisms for which a ≥5 log reduction was observed.

Assays were performed in duplicate.

Evaluation of the yeasticidal activity

Yeast suspensions were prepared in tryptone salt at a concentration ranging from 1.5×10^7 and 5.0×10^7 CFU (colony forming units)/mL, with a final test concentration ranging between 1.5×10^6 and 5.0×10^6 CFU/mL.

The experimental conditions were those specified for the EN 13727 standard [17] assays: 1 min ±5 s contact duration at a temperature of 20˚C ± 1˚C, in dirty conditions.

Results were expressed as described by EN 13727 [17] taking into account differences in the log reduction to be reached. According to NF EN 13624 [18], the tested antiseptic solutions were considered yeasticidal for hygienic and surgical handrub if, when used undiluted, they produced a ≥4 log reduction in colony counts; and for hygienic handwash or for surgical handwash if, when used diluted (≤50%), they produced a ≥2 log or ≥4 log reduction respectively. The yeasticidal activity was considered to be maintained on the microorganisms for which a ≥4 log reduction was observed.

Assays were performed in duplicate.

3. Results

3.1. Bactericidal Activity

The number of viable cells was not reduced by a factor greater than two-fold

when experimental conditions were applied, including neutralization validation (Table 1 and Table 2). Thus, it was concluded that the bactericidal assay used in this study was appropriate for determining the *in vitro* bactericidal activity of the two products.

As shown in Table 1, both products were bactericidal (reduction ≥ 5 log) on the 4 defined strains after 1 minute of contact and in dirty conditions. Under these experimental conditions, the bactericidal activity of the products was sustained even when diluted at 40% (v/v). *E. coli* CIP 54117 was the most sensitive strain with defined log reduction at the lowest concentration 1% (v/v) with solution A.

Table 1. Evaluation of bactericidal activity of solution A according to the NF EN 13727 [17].

Test organism	Mean bacterial counts (CFU/ml) at 10^{-6} dilution[a]				Test suspension[a] (log CFU/ml)	Log reduction in bacterial counts[a]		
	Suspension for validation	Experimental conditions	Neutralizing solution	+Neutralized test product		80% (v/v)	40% (v/v)	1% (v/v)
S. aureus CIP 4.83								
Assay 1	57	71	64	74	7.23	**>5.09**	**>5.09**	<2.71
Assay 2	97	106	106	115	7.52	**>5.38**	**>5.38**	<3.00
P. aeruginosa CIP 103467								
Assay 1	45	46	50	43	7.21	**>5.06**	**>5.06**	<2.69
Assay 2	121	107	112	101	7.58	**>5.43**	**>5.43**	3.24
E. coli CIP 54117								
Assay 1	61	69	58	65	7.31	**>5.16**	**>5.16**	**>5.16**
Assay 2	104	94	89	85	7.55	**>5.40**	**>5.40**	4.49
E. hirae CIP 58.55								
Assay 1	88	84	91	90	7.46	**>5.32**	**>5.32**	<2.95
Assay 2	52	50	65	48	7.27	**>5.13**	**>5.13**	<2.76

[a]Values represent the mean of duplicate counts.

Table 2. Evaluation of bactericidal activity of solution B according to the NF EN 13727 [17].

Test organism	Mean bacterial counts (CFU/ml) at 10^{-6} dilution[a]				Test suspension[a] (log CFU/ml)	Log reduction in bacterial counts[a]		
	Suspension for validation	Experimental conditions	Neutralizing solution	+Neutralized test product		80% (v/v)	40% (v/v)	1% (v/v)
S. aureus CIP 4.83								
Assay 1	57	71	64	65	7.23	**>5.09**	**>5.09**	<2.71
Assay 2	97	106	106	105	7.52	**>5.38**	**>5.38**	<3.00
P. aeruginosa CIP 103467								
Assay 1	45	46	50	57	7.21	**>5.06**	**>5.06**	<2.69
Assay 2	121	107	112	107	7.58	**>5.43**	**>5.43**	<3.06
E. coli CIP 54117								
Assay 1	61	69	58	62	7.31	**>5.16**	**>5.16**	2.98
Assay 2	104	94	89	97	7.55	**>5.40**	**>5.40**	<3.03
E. hirae CIP 58.55								
Assay 1	88	84	91	72	7.46	**>5.32**	**>5.32**	<2.95
Assay 2	52	50	65	53	7.27	**>5.13**	**>5.13**	<2.76

[a]Values represent the mean of duplicate counts.

3.2. Yeasticidal Activity

According to NF EN 13624 [18], both products were yeasticidal after 1 mn of contact at 20˚C in dirty conditions for the concentrations 80% (v/v) and 40% (v/v) (Table 3 and Table 4).

Table 3. Evaluation of yeasticidal activity of solution A according to the NF EN 13624 [18].

Test organism	Mean bacterial counts (CFU/ml) at 10^{-6} dilution[a]				Test suspension[a] (log CFU/ml)	Log reduction in bacterial counts[a]		
	Suspension for validation	Experimental conditions	Neutralizing solution	+Neutralized test product		80% (v/v)	40% (v/v)	1% (v/v)
C. albicans DSM 1386								
Assay 1	81	68	72	85	6.38	**>4.24**	**>4.24**	<1.86
Assay 2	75	60	67	69	6.35	**>4.20**	**>4.20**	<1.83

[a]Values represent the mean of duplicate counts.

Table 4. Evaluation of yeasticidal activity of solution B according to the NF EN 13624 [18].

Test organism	Mean bacterial counts (CFU/ml) at 10^{-6} dilution[a]				Test suspension[a] (log CFU/ml)	Log reduction in bacterial counts[a]		
	Suspension for validation	Experimental conditions	Neutralizing solution	+Neutralized test product		80% (v/v)	40% (v/v)	1% (v/v)
C. albicans DSM 1386								
Assay 1	81	68	72	94	6.38	**>4.24**	**>4.24**	<1.86
Assay 2	75	60	67	67	6.35	**>4.20**	**>4.20**	<1.83

[a]Values represent the mean of duplicate counts.

4. Discussion

To establish that a product has bactericidal activity under practical conditions representative of its intended use in the medical area, phase 2, step 1 quantitative suspension tests are required, such as the NF EN 13727 [17] and NF EN 13624 [18] standards respectively for the claim of bactericidal and yeasticidal activities.

The present study demonstrated that a logarithmic reduction of 5 can be achieved in dirty conditions, for a one minute contact time with the two products under assay, containing either chlorhexidine alone (0.5% w/v) or combined with benzalkonium chloride (0.25% and 0.5% respectively), regarding the mandatory Gram+ and Gram− strains for antiseptic evaluation. In the same time, we also noticed a yeasticidal activity on *C. albicans* DSM 1386 with a 4 log reduction in similar assay conditions. These reductions were observed at the higher tested concentration (80% v/v) and also at 40% (v/v). The activity was preserved for both tested products in test conditions, *i.e.* in the presence of high protein load.

Chlorhexidine digluconate is well known for its broad spectrum antiseptic activity (bactericidal against Gram+ and Gram− bacteria and yeasticidal) leading to a large use to prevent infections even regarding antibiotic resistant bacteria [25]. Benzalkonium chloride is a surfactant with detergent activity of the quaternary ammonium family (QACs) that is primarily bactericidal against Gram+ and is aimed to complete the action of chlorhexidine. Both active substances are

cationic agents and express bactericidal activity by similar mechanisms of action: lesion of the cell wall and cytoplasmic membrane, and intracellular precipitation of proteins [26] [27] [28] [29]. Combination of chlorhexidine and QACs as benzalkonium chloride was frequently considered positive. In 2004, Cabotin *et al.* [16] demonstrated that the bactericidal activity of chlorhexidine (0.2%) was impaired by albumin (0.3%) but maintained when combined with benzalkonium chloride (0.5%). We recently confirmed this positive interaction when both active substances were combined in the same concentrations and ratio than those tested by Cabotin *et al.* [30].

Here we demonstrated that chlorhexidine alone at 0.5% (w/v) presented the same or slightly higher level of antiseptic activity than a solution of chlorhexidine at a concentration 2 times lower but in combination with a very low concentration of benzalkonium chloride 0.025% (w/v) and benzylic alcohol (4%). Those results underlined the importance of the formulation and its final validation regarding interactions expected between active substances, objectives and uses. Currently, many questions are under light regarding benzalkonium chloride, a detergent and preservative found frequently in health care and household products, as an irritant and sensitizer [31] [32]. Wentworth [31] noted a progressive increase from 2001 through 2005 and 2006 through 2010 in the rate of allergic patch test results to benzalkonium chloride at the Mayo Clinic which classes this molecule among the top 10 most frequent allergens in their standard. In the same time, Isaac [32] evaluated benzalkonium chloride as an allergen (patch test) on patients with suspected allergic contact dermatitis. The prevalence of positive reaction in the tested population led the authors to recommend precautions for patients with compromised skin barriers. Most of all these studies underlined the need of a discriminate and proportionate use of molecules with antimicrobial properties as benzalkonium chloride and other AQCs to avoid potential cross-reactions.

5. Conclusion

In conclusion, this study demonstrated the efficient bactericidal and yeasticidal activity of aqueous solutions containing chlorhexidine digluconate either alone at a concentration of 0.5% (w/v) or at a concentration of 0.25% (w/v) when combined with benzalkonium chloride at 0.025% (w/v) and benzylic alcohol 4%. Regarding the respective formulations, indications and uses have to be considered carefully taking into account the interests and risks for each active substance.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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