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REDUCTION OF THE NEUTRALISATION TIME DURING ANTIMICROBIAL ACTIVITY TESTING OF DISINFECTANTS ACCORDING TO EUROPEAN STANDARDS

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ABSTRACT

Background. Evaluation of the biocidal activity of chemical disinfectants and antiseptics according to European Standards (EN) is based on determination of the reduction of the number of viable test microorganisms under defined conditions.
Objective. The objective of this study was to investigate whether reducing the neutralization time required following declared product contact times for the tested microorganisms yields method validations.
Material and methods. This study was conducted on 14 products containing active substances from different chemical groups: alcohols, aldehydes, biguanides, quaternary ammonium compounds, phenols, amines derivatives, oxidizing agents. These products were tested according to phase 1 tests: EN 1040:2005 and EN 1275:2005 and then according to phase 2, step 1 tests: Draft EN 13727:2005 and EN 13624:2003. Biocidal activity was evaluated using the following test organisms: *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 15442, *E. coli* NCTC 10538, *E. coli* ATCC 10536, *E. hirae* ATCC 10541, *C. albicans* ATCC 10231 and *A. brasiliensis* ATCC 16404.
Results. Validation C results for all products and tested microorganism strains were at least half of the density of the suspension for validation (Nvo) after only 10 s of neutralization. Furthermore, results from test procedures performed in parallel were also positive except 5 products toward *A. brasiliensis*.
Conclusions. The results of our study confirm that the contact time described in the European Standards phase 1: EN 1040 and EN 1275, as well as phase 2, step 1: Draft EN 13727 and EN 13624 can be precisely determined in spite of reducing the neutralization time from 5 minutes to even 10 seconds.

Key words*: antiseptics, disinfectants, neutralization time**, antimicrobial activity, European Standards*

STRESZCZENIE

Wprowadzenie. Badanie aktywności antyseptyków wg norm PN-EN polega na określeniu stopnia redukcji liczby komórek drobnoustrojów testowych, uzyskanego w deklarowanym czasie kontaktu.
Cel badań. Celem pracy było zbadanie, czy skrócenie czasu neutralizacji preparatu wynoszącego 5 minut po określonym czasie jego kontaktu z drobnoustrojami testowymi, zapewni zwalidowanie metody.
Materiał i metody. Wybrano 14 preparatów zawierających substancje czynne należące do różnych grup chemicznych: alkohole, aldehydy, biguanidyny, czwartorzędowe związki amoniowe, fenole, pochodne amin, związki utleniające. Badania przeprowadzono wg norm fazy 1: EN 1040:2005 i EN 1275:2005, a następnie wg norm fazy 2 etapu 1: Draft EN 13727:2005 and EN 13624:2003. Do badań użyto zalecane standardowe szczepy bakterii i grzybów: *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 15442, *E. coli* NCTC 10538, *E. coli* ATCC 10536, *E. hirae* ATCC 10541, *C. albicans* ATCC 10231 and *A. brasiliensis* ATCC 16404.
Wyniki. Wyniki walidacji C dla wszystkich produktów i badanych szczepów mikroorganizmów spełniały wymagania norm, czyli były równe co najmniej połowie gęstości zawiesiny do walidacji (Nvo) zaledwie po 10 s neutralizacji. Ponadto, w równolegle przeprowadzonych testach aktywności przeciwdrobnoustrojowej uzyskano pozytywne wyniki, z wyjątkiem 5 produktów wobec *A. brasiliensis*.
Wnioski. Wyniki badań potwierdzają, że czas kontaktu określany wg norm fazy 1 EN 1040:2005 i EN 1275:2005, jak również fazy 2 etapu 1: Draft EN 13727:2005 i EN 13624:2003, może być precyzyjnie określony mimo skrócenia czasu neutralizacji z 5 minut nawet do 10 sekund.

Słowa kluczowe*: antyseptyki, środki dezynfekcyjne, czas neutralizacji, aktywność przeciwbakteryjna, Normy Europejskie*

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INTRODUCTION

Human environment is plenty of microorganisms belonging to bacteria, fungi and viruses which have the ability to survive on surfaces for a very long time, even several months [5]. They can be a source of infection by transfer of pathogens in directly or indirectly way, for example through the hands of medical personnel. The use of disinfectants with proven antimicrobial activity allows preventing or reducing infections, including nosocomial infections. Test methods which take into account the parameters that influence the activity of these preparations, are developed by the European Committee for Standardization (CEN) and adopted as a standard by the Polish Committee for Standardization (PKN) [6, 7, 8]. Evaluation of the biocidal activity of chemical disinfectants and antiseptics according to European Standards (EN) is based on determination of the reduction of the number of viable test microorganisms under defined conditions. Exact measurement of the contact time between the analysed product and the microbial cells is crucial. Currently, two methods are commonly used to end product-microbe contact and stop the reaction: dilution-neutralization and membrane filtration. The dilution-neutralization method stops the reaction chemically, using an appropriate neutralizer. According to EN, neutralization time is estimated to be 5 min. +/- 10 s. In addition, the actual contact time indicated by the EN and determined during testing is extended by the time required for neutralization. Thus, reducing this neutralization time would enable more precise determination of the real preparation activity. This is particularly important when evaluating preparations, such as hygienic hand disinfection products, for which declared contact times are often very short (e.g. 30 seconds).

The objective of this study was to investigate whether reducing the neutralization time required following declared product contact times for the tested microorganisms yields method validations.

MATERIAL AND METHODS

This study was conducted on 14 products containing active substances from different chemical groups: alcohols, aldehydes, biguanides, quaternary ammonium compounds, phenols, amines derivatives, oxidizing agents. The 14 products were designed for different applications: hygienic and surgical hand disinfection; pre-operative disinfection of patient skin; disinfection of surfaces and medical devices; and for external and uterinal applications in veterinary hospitals. In the Table 1 information about each of the tested products, including area of application and composition of active substances is presented.

Table 1. Characteristics of products analyzed according to EN 1040 and EN 1275

Name of product (Manufacturer)	Use of the product	Active substances
Aldizol (Farmaceutyczno-chemiczna Spółdzielnia Pracy „Septoma”)	surfaces: walls, floors, equipment, medical devices	o-phenylphenol 7.0%, 4-chloro-3-methylphenol 4.5%, glutaraldehyde 4.0%
Biolugol (Biowet Drwalew S.A.)	skin of cattle, horses, sheep, goats	100 mL: iodine 9.0 g, potassium iodide 24.0 g
Bioseptol 70 (Bioetanol AEG Sp.z.o.o.)	skin - hygienic and surgical disinfection	100 g: ethanol 64.75 g, 2-propanol 0.72 g, phenoxyethanol 2 g
Hydrex S (Adams Healthcare Ltd., Oddział Ecolab)	human skin	100 g: chlorhexidine digluconate 3.876 g, mixture of alkyl (C12-14) dimethyl ammonium oxides 11.3 g
Incidin Active (Ecolab GmbH & Co. OHG)	inanimate surfaces	sodium percarbonate, tetraacetylethylenediamine, the active ingredient in a solution of 2%: 1000 mg/kg (ppm) of peracetic acid
Incidin Extra (Ecolab GmbH & Co. OHG)	inanimate surfaces	benzalkonium chloride 15%, polyhexamethylene - biguanide hydrochloride 2%, 2-phenylphenol 2%
Laudamonium (Ecolab Gmbh & Co. OHG)	inanimate surfaces	benzyl alkyl ammonium chloride 9.99 g/100 g
Optim 33TB (SciCan Ltd.)	inanimate surfaces	hydrogen peroxide 0.5%
Sekusept Plus (Ecolab GmbH & Co. OHG)	medical devices	glucoprotamine 25 g/100 g
Sekusept Pulver (Ecolab GmbH & Co. OHG)	medical devices	100 g: sodium perborate 20.0 g, tetraacetylethylenediamine 15.0 g
Septyl „R” (Farmaceutyczno-chemiczna Spółdzielnia Pracy „Septoma”)	inanimate surfaces	100 g: biphenyl-2-ol 13.0 g, chlorocresol 1.0 g
Solutio Iodi Spirituosa (Biowet Drwalew S.A.)	skin of cattle, horses, sheep, goats	iodine 7.5g/250 g
Spitaderm (Ecolab Deutschland GmbH)	human skin	100 g: 2-propanol 70 g, chlorhexidine digluconate 0.5 g, hydrogen peroxide 30% - 1.5 g
Sterisol Preop (Sterisol AB)	inanimate surfaces	ethanol 70 w/w %, isopropyl alcohol 10 w/w %

Table 2. Assays conditions for antibacterial (B-bacteria)) and antifungal (F-fungi) activity testing of selected products

Name of product	Conditions recommended by the manufacturers		Conditions of tests performed in NMI according to ENs	
	c	t	c	t
Aldizol	B and F - 2.5% dirty conditions 1.75% - clean conditions	B and F - 15 min.	B, *C. albicans* - 1.75% ; *A. brasilliensis* - 4%	B and F - 15 min.
Biolugol	B and F - solutions: 1:10, 1:30, 1:50, 1:400, 1:800	no information	B and F - 2%	B and F - 5 min.
Bioseptol 70	B and F - 100%	B - 1 min. G - 15 min.	B and F - 100 %	B - 1 min. ; F - 15 min.
Hydrex S	B and F - 100%	B and F - 5 min.	B and *C. albicans* - 6%	B - 1 min; *C. albicans* - 5 min.
Incidin Active	B and yeast - 1%	B and yeast - 5 min.	B and F - 1%	B and F - 5 min.
Incidin Extra	B and F - 1 %	B and F - 1 h	B - 0.5% *C. albicans* - 2%	B and *C. albicans* - 15 min.
Laudamonium	B and F - 1 %	B and F - 1 min.	B - 1% *C. albicans* - 2%	B - 1 min. *C. albicans* - 15 min.
Optim 33TB	B and *C. albicans* - 100%	B - 1 min. *C. albicans* - 15 min.	B and *C. albicans* - 100%	B - 1 min. *C. albicans* - 15 min.
Sekusept Plus	B and *C. albicans* - devices: 1.5% - 1 h 2.5% - 30 min. 4.0% - 15 min. surfaces: 1.5% - 4 h	1.5% - 1 h 2.5% - 30 min. 4.0% - 15 min. surfaces: 1.5% - 4 h	B - 0.5% *C. albicans* - 4%	B (without *P. aeruginosa*) - 1 min. *C. albicans* + *P. aeruginosa* - 5 min.
Sekusept Pulver	B and F - 2 % + 2% A	B - 15 min. F - 30 min.	B and F - 2 % + 2% A	B - 15 min.F - 30 min.
Septyl „R”	B - 2.5%	B - 15 min.	B and *C.albicans* - 1.5%	B and *C. albicans* - 15 min.
Solutio Iodi Spirituosa	B and F - 100%	no information	B and F - 100%	B and F - 5 min.
Spitaderm	B and F - 100%	B and F - 15 s - 1.5 min.	B and F - 100%	B and *C. albicans* - 1 min. *A. brasiliensis* - 15 min.
Sterisol Preop	no information	no information	B and F - 100%	B and F - 30 s

c - concentration of tested product; t - contact time; A - activator, NMI – National Medicines Institute

Table 2. contains working solution concentrations and contact times recommended for application by manufacturers as well as conditions of assays performed at the National Medicines Institute (NMI), according to appropriate European Standards.

This study was conducted according to phase 1 tests: EN 1040:2005 and EN 1275:2005 and then according to phase 2, step 1 tests: Draft EN 13727:2005 and EN 13624:2003 [1, 2, 3, 4]. The Antibiotics and Microbiology Department of National Medicines Institute possesses accreditation of Polish Center of Accreditation (PCA) and attestation of European Directorate for the Quality of Medicines (EDQM) on evaluation of bactericidal and yeasticidal activity tests performed according to European Standards. For tests conducted according to ENs phase 2, step 1, product test solutions were prepared in hard water and dirty conditions were applied: a mixture of 3 g/L bovine albumin solution with 3 mL/L sheep erythrocytes.

For each product toxicity of neutralizer was tested. In parallel studies, the procedure and validation for dilution-neutralization (C) were evaluated. A step-by-step reduction of the neutralization time from 5 minutes to 10 seconds (5 min., 4 min., 3 min., 2 min., 1 min. and 10 s) or application of only the shortest neutralization time (10 s) were evaluated. There was no possibility to use shorter time then 10 seconds for technical reasons.

The validation C consisted of three stages: (1) 8 mL of the product test solution was mixed with 1 mL of diluent and 1 mL of water or interfering substance and incubated for a time equal to the contact time used in the test procedure; (2) 1 mL of sample of this mixture was transferred into 8 mL of neutralizer for a defined neutralization time - according to EN 5 min. or according to our investigations from 5 min. to 10 s; (3) after neutralization, 1 mL of strain suspension (Nv) was added to the mixture from step 2) and incubation was performed for 30 min.

Validation C of the dilution-neutralization method was achieved if a minimum of half of the microorganism cells from the suspension added in step 3) (Nvo = Nv/10) were able to grow, indicating that the applied neutralization time was sufficient to neutralize the biocidal activity of the product: allowing the microorganisms to grow.

For products: Hydrex S and Sekusept Plus the neutralizer was composed of: lecithin 3.0 g/L, polysorbate 80 30.0 g/L, sodium thiosulphate 5.0 g/L, L-histidine 1.0 g/L, saponin 30.0 g/L in diluent (tryptone - pancreatic extract of casein 1.0 g/L, sodium chloride 8.5 g/L).

For the other evaluated preparations, the commercial Dey/Engley neutralizing broth was used (composition: pancreatic digest of casein 5.0 g/L, yeast extract 2.5 g/L, dextrose 10.0 g/L, sodium thioglycollate 1.0 g/L, sodium thiosulphate 6.0 g/L, sodium bisulphite 2.5 g/L, polysorbate 80 5.0 g/L, lecithin 7.0 g/L, bromocresol purple 0.02 g/L).

A product was considered to meet the above standards if it demonstrated at least a 5 decimal log reduction of bacteria and at least a 4 decimal log reduction of fungi.

Bactericidal activity was evaluated using the following test organisms: *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 15442, *Escherichia coli* NCTC 10538, *Escherichia coli* ATCC 10536 and *Enterococcus hirae* ATCC 10541. The density of bacterial suspensions used for the tests were adjusted to 1.5–5.0x10^8 cfu/mL (N), and for validation to 3.0x10^2–1.6x10^3 cfu/mL (Nv).

Taking into consideration the European Standards requirements, the preparations for skin and mucous membranes antisepsis (Biolugol, Bioseptol 70, Hydrex S, Solutio Iodi Spirituosa and Sterisol Preop) were analysed with the use of *E. coli* NCTC 10538, while preparations for disinfection of inanimate surfaces, like Aldizol, Incidin Extra, Laudamonium, Sekusept Plus and Septyl „R" were analysed with application of *E. coli* ATCC 10536.

Fungicidal activity was evaluated using the following test organisms: *Candida albicans* ATCC 10231 and *Aspergillus brasiliensis* ATCC 16404. The density of fungal suspensions used for the tests were adjusted to 1.5–5.0x10^7 cfu/mL (N), and for validation to 3.0x10^2–1.6x10^3 cfu/mL (Nv).

RESULTS AND DISCUSSION

In the past different non-standardized, sometimes developed by manufacturers of the disinfection preparations, test methods were used for determination of the antimicrobial activity of antiseptic and disinfection preparations. Starting from 1997, the European Com-

Table 3. Scores for bacterial strains - neutralization time - 10 s

Name of product c/t	*S. aureus* ATCC 6538			*P. aeruginosa* ATCC 15442			*E. coli* ATCC 10536* ATCC 10538			*E. hirae* ATCC 10541		
	R	Nvo	C	R	Nvo	C	R	Nvo	C	R	Nvo	C
Aldizol 1.75%/15 min.	>5.24	85	80	>5.26	98	91	>5.21*	74	72	>5.13	52	49
Biolugol 2%/5 min.	>5.24	85	82	>5.36	80	53	>5.31	78	75	>5.05	36	33
Bioseptol 70 100%/1 min.	>5.24	85	79	>5.36	80	50	>5.31	78	71	>5.05	36	35
Hydrex S 6%/1 min.	>5.24	85	83	>5.36	80	69	>5.31	78	78	>5.05	36	34
Incidin active 1%/5 min.	>5.17	56	48.5	>5.41	117	113.5	>5.21	65	60.5	>5.29	94	76
Incidin Extra 0.5%/15 min.	>5.24	85	66	>5.36	80	54	>5.28*	85	67	>5.05	36	36
Laudamonium 1%/1 min.	>5.24	85	72	>5.36	80	73	>5.28*	85	62	>5.05	36	32
Optim 33TB 100%/1 min.	>5.19	60.5	58.5	>5.33	75	44.5	>5.29	78	40.5	>5.19	63.5	62.5
Sekusept Plus 0.5%/1 min.	>5.16	83	82	>5.26	98	89	>5.26*	74	58	>5.06	52	44
Sekusept Pulver 2% + 2% A/15 min.	>5.17	48.5	60.5	>5.41	113	98	>5.21	69	65	>5.29	117.5	76
Septyl "R" 1.5%/15 min.	>5.24	85	69	>5.36	80	56	>5.28*	85	81	>5.05	36	34
Solutio Iodi Spirituosa 100%/5 min.	>5.24	85	78	>5.36	80	62	>5.31	78	66	>5.05	36	35
Spitaderm 100%/1 min.	>5.17	55.5	48.5	>5.41	113.5	108.5	>5.36	89	48	>5.29	76	75.5
Sterisol Preop 100%/30 s	>5.24	85	81	>5.36	80	56	>5.31	78	66	>5.05	36	32

c - concentration of tested solution; t - contact time; R - reduction; Nvo - number of cells per mL in validation suspension/10; C - number of cells per mL in dilution-neutralization method validation; A - activator; * - tests performed with use of *E. coli* ATCC 10536

mittee for Standardization has created several European Standards concerning evaluation of bactericidal, fungicidal and virucidal activity of chemical disinfectants and antiseptics applicable in different areas. The most important, from public health point of view, is the medical area [7]. All assays described in European Standards have to be validated. For neutralization process, the validation C is the most important.

During performed investigations, it was proved that validation C results for all products and tested microorganism strains were at least half of the density of the suspension for validation (Nvo) after only 10 s of neutralization. It means that results of antimicrobial activity testing obtained with the application of such short neutralization time are reliable. Furthermore, results from test procedures performed in parallel were also positive for most of the products. For products which showed no high activity against *A. brasiliensis* (log R< 4), the positive validation C scores observed in these cases are meaningless, since products with no proper biocidal activity do not require neutralization. No correlation was observed between neutralization time (10 s, 1, 2, 3, 4 or 5 min.) and the number of recovered microorganisms in validation C.

Particular results of evaluation of bactericidal and fungicidal activities of tested preparations and C validation are combined in Tables 3 and 4.

Performed investigations have shown, that limiting the neutralization time, allow for precise determination of the real preparation activity, what is especially important for the preparations with declared very short contact time.

Table 4. Scores for fungal strains - neutralization time - 10 s

Name of product c/t	*C. albicans* ATCC 10231			*A. brasiliensis* ATCC 16404		
	R	Nvo	C	R	Nvo	C
Aldizol 1.75%/15 min.	>4.38	92	81	>4.05	88	87
Biolugol 2%/5 min.	>4.38	92	79	>4.25	88	50
Bioseptol 70 100%/15 min.	>4.38	92	90	>4.25	69	68
Hydrex S 6%/5 min.	>4.35	76	70	<3.00	54	45
Incidin Active 1%/5 min.	>4.35	89	83	<4.09	43.5	38.5
Incidin Extra 2%/15 min.	>4.38	92	86	<3.18	69	64
Laudamonium 2%/5 min.	>4.38	92	89	<3.18	69	67
Optim 33TB 100%/15 min.	>4.32	82	76	Nt	Nt	Nt
Sekusept Plus 4%/5 min.	>4.38	92	80	<3.18	69	65
Sekusept Pulver 2% +2% A/30 min.	>4.35	89	80	<4.09	40	38.5
Septyl "R" 1.5%/15 min.	>4.38	92	82	<3.18	69	68
Solutio Iodi Spirituosa 100%/5 min.	>4.38	92	76	>4.25	69	53
Spitaderm 100%/1 min.	>4.35	90.5	89	<4.02	38.5	37
Sterisol Preop 100%/30 s	>4.38	92	90	>4.25	69	66

c - concentration of tested solution; t - contact time; R - reduction; Nvo - number of cells per mL in validation suspension/10; C - number of cells per mL in dilution-neutralization method validation; Nt - not tested; A - activator

CONCLUSIONS

The contact time of European Standards phase 1: EN 1040 and EN 1275, as well as phase 2, step 1: Draft EN 13727 and EN 13624 can be precisely determined in spite of reducing the neutralization time from 5 minutes to as short as 10 seconds. It is possible, that also in case of other European Standards, neutralization time may be reduced.

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