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Original Article

Evaluation of Neutralizing Efficacy and Possible Microbial Cell Toxicity of a Universal Neutralizer Proposed by the CTPA

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Abstract

The purpose of this research was to study the neutralization efficacy of a universal neutralizer proposed by the CTPA on a range of antimicrobial agents and its potential toxicity for microorganisms that are used for antimicrobial preservation testing. Several types of antimicrobial agents including a mixture of methylisothiazolinone and its 5-chloro derivative, dimethylol dimethyl hydantoin, Quaternium-15®, Bronopol®, benzalkonium chloride, phenoxyethanol, methyl and propyl paraben, chlorhexidine, imidazolidinyl urea, triclosan and thiomersal built-in pharmaceutical, cosmetic or health care products, as well as 6 types of these antimicrobials, i.e. a combination of methyl paraben (0.18% w/v) and propyl paraben (0.02%), imidazolidinyl urea (0.3%), chlorhexidine (0.01%), benzalkonium chloride (0.02%), Bronopol® (0.1%) and thiomersal (0.02%) in their pure and soluble form were tested. Validation of microbial recovery was carried out according to the US Pharmacopeia 27 guidelines. The universal neutralizer could inactivate all the studied antimicrobial agents against different strains tested except Staphylococcus aureus. With regards to this microorganism, only seven preservative chemicals consisting of parabens, dimethylol dimethyl hydantoin, Quaternium-15®, Bronopol®, benzalkonium chloride, Phenonip® and imidazolidinyl urea were effectively inactivated. In addition, a pure solution of 0.02% thiomersal retained its antimicrobial properties against all studied microorganisms. The neutralizer solution showed no toxicity on any of the test organisms. In conclusion, CTPA proposed neutralizing solution is not an inclusive neutralizer. Moreover, each organism to be used in the test must be included in the validation study.

Keywords: Preservative; Antimicrobial agent; Neutralization; Validation of microbial recovery.

Introduction

In various microbiological assessments of pharmaceuticals, cosmetics and health care products including sterility testing, microbial quality control of non-sterile products and antimicrobial preservative effectiveness testing, the elimination of remaining antimicrobial properties is of great importance while recovering microorganisms. The presence of inhibitory compounds in culture media will hinder the

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growth of possibly present microorganisms leading to inaccurate laboratory findings (1). There are different methods to inhibit residual antimicrobials including dilution, membrane filtration and chemical neutralization (2). Dilution is useful for those antimicrobials that have a large dilution coefficient (η) and little affinity for binding to the cell (3). A variation on dilution is filtration of the suspension to remove the antimicrobials. This technique, however, has some limitations since the antimicrobial may bind either to the membrane filter or to the cells, inhibiting recovery (4-7). Eventually, residual antimicrobials can be inhibited by chemical neutralization (2, 3, 8). The selected chemical neutralizer should not only be able to completely inactivate all of the biostatic activity of the residual antimicrobial agent likely to be carried over into recovery media, but also be inherently non-toxic to the test organisms (9).

At present, there is no single agent that has the ability to inactivate all antimicrobial substances. According to the need, researchers use different neutralizers in the recovery procedure, while evaluating different antimicrobial agents. Selecting a suitable chemical neutralizer for each preservative class, on the other hand, is a cumbersome, costly and time-consuming task. Therefore, it is desirable to develop a neutralizing solution that is capable of inactivating an extensive range of antimicrobial agents. In review of literature, there are some reports of broad-spectrum neutralizers, which have been invented by some investigators (4, 9, 10). However, Sutton et al. (1) showed that purportedly wide-spectrum neutralizing solutions such as Dey-Engley neutralizing broth are inadequate for all index organisms against all biocides tested. The Cosmetic, Toiletry and Perfumery Association (CTPA) has also introduced a formula known as the universal neutralizer (11). To the best of our knowledge, the efficacy and non-toxicity of the universal neutralizer have not been previously reported. Thus, the objective of the present study was to evaluate the neutralization efficacy on a range of antimicrobial agents of the universal neutralizer proposed by CTPA and its potential toxicity for certain index organisms.

Experimental

Materials

All culture media, raw media bases and chemicals were obtained from Merck Co. (Germany). The preservatives including methyl & propyl parabens (Merck, Germany), imidazolidinyl urea (Seppic, France), chlorhexidine digluconate (Medichem, Spain), benzalkonium chloride (Fef Chemicals, Denmark), Bronopol (Grodab Chemie, Germany) and thiomersal (Merck, Germany) were kindly donated by different pharmaceutical or cosmetic manufacturers.

Test organisms

Test organisms were those specified by US Pharmacopeia 27 for testing antimicrobial preservation effectiveness (12). These organism included *Staphylococcus aureus* (*S. aureus*) ATCC 6538, *Escherichia coli* (*E. coli*) ATCC 8739, *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 9027, *Candida albicans* (*C. albicans*) ATCC 10231 as well as *Aspergillus niger* (*A. niger*) ATCC 16404, which were maintained deeply frozen in our laboratory.

Test samples

A number of pharmaceutical, cosmetic or health care products (Table 1) containing various antimicrobial agents such as a mixture of methylisothiazolinone and its 5-chloro derivative (Kathon CG®), dimethylol dimethyl hydantoin (DMDM hydantoin), Quaternium-15®, Bronopol®, benzalkonium chloride, phenoxyethanol, methyl and propyl paraben, chlorhexidine, imidazolidinyl urea, triclosan and thiomersal were evaluated. Moreover, 6 types of these antimicrobials including a combination of methyl paraben (0.18% w/v) and propyl paraben (0.02%), imidazolidinyl urea (0.3%), chlorhexidine (0.01%), benzalkonium chloride (0.02%), Bronopol (0.1%) and thiomersal (0.02%) in their pure and soluble form were incorporated in the study. The solutions of pure preservatives were prepared in distilled water. Their pHs were adjusted at 7.0 ± 0.1, except the Bronopol solution that had a pH<5 for more stability. All the solutions were then filter sterilized.

Neutralizer evaluation procedure

Validation of microbial recovery was carried out according to the US Pharmacopeia 27 guidelines (2). The universal neutralizer was prepared with the following composition, (g/l): Lecithin, 3; Polysorbate 80, 30 ml; Sodium thiosulfate pentahydrate, 5; L-Histidine, 1; Proteose peptone, 1; Sodium chloride, 2.92; $Na_2HPO_4.12\ H_2O$, 18.16; KH_2PO_4, 3.6. The final pH was adjusted at 7.0 ± 0.1 (11).

The validation procedure required four treatment groups for comparison. One ml or g of test sample or sterile peptone solution (1 g/l; pH 7.1 ± 0.2) was added to a tube containing 9 ml of the neutralizing solution. These suspensions were then incubated for 10 min on bench top at room temperature. These tubes represented the "test" and "peptone control group", respectively. A third tube, containing 10 ml sterile saline solution, was prepared and served as the "viability control". An extra fourth tube was also included in the validation as "dilution control". The dilution control contained 9 ml saline solution plus 1 ml or g of test product. Each milliliter of these solutions was inoculated with less than 100 colony-forming units of the challenge organisms, which had been previously cultured under the conditions described in the US Pharmacopeia (12). Inoculated suspensions were incubated for an additional 10 min on the bench top at ambient temperature. The bacteria were recovered by plating 2 samples of 1 ml each into trypticase soy agar medium and incubating at 30-35°C for three days. Sabouraud dextrose agar medium supplemented with chloramphenicol was used for fungal culture and the resulting plates were incubated at 20-25°C for 3-5 days. All the experiments were repeated three times.

Analysis of data

The neutralizer toxicity (NT) was determined as follows: the number of organisms recovered in the "peptone control group" divided by the number of organisms in the "viability control". The neutralizer was considered non-toxic for test organisms if the ratio was not less than 0.70. Neutralizer efficacy (NE) was calculated as follows: the number of recovered organisms in the "test group" divided by the number of recovered organisms in the "peptone control group". A NE ratio not less than 0.70 was considered as an acceptable neutralization efficacy. The ratio of the number of organisms recovered in the "dilution control" and the number of organisms in the "viability control" was also calculated. If the ratio was not less than 0.70 then a simple dilution by itself would be sufficient to inactivate the pertinent antimicrobial agent. According to USP 27 (2), at least three independent replicates of the experiment should be performed, and each should demonstrate that the neutralizing solution is efficient but non-toxic.

Results and Discussion

NT ratios determined were found to be not less than 0.70 with respect to all the challenge organisms (Table 2), indicating that the "universal neutralizer" had no toxicity on them. Other investigators, however, have reported the toxic effect of thiosulfate component, which exists in the neutralizer, on *staphylococci* (13, 14). Adverse effect of lecithin on bacteria has also been previously demonstrated (1, 8). Other

Table 1. Antimicrobial ingredients of the products studied.

Product	Antimicrobial agent(s)
Body scrub	Dimethylol dimethylhydantoin-1,3-bis (hydroxymethyl)-5,5-dimethyl-2,4-imidazolidone-dion (DMDM Hydantoin)
Shower gel	2-Methyl-3(2H) isothiazolinone and 5-chloro derivatives (Kathon CG®)
Skin care lotion	1-(3-Chloroallyl)-3,5,7-triazo-azoniaadamantane chloride (Quaternium-15®) and propyl paraben
Power shower clean-up	Phenoxy ethanol, Methyl paraben and Propyl paraben
Antibacterial hand lotion	Imidazolidinyl urea, Methyl paraben and Propyl paraben
Antibacterial hand gel	Triclosan and ethanol
Shampoo	2-Bromo-2-nitro propane-1,3 diol (Bronopol®)
Naphazoline HCl eye drop	Benzalkonium chloride
DPT vaccine	Thiomersal
Mouthwash	Chlorhexidine digluconate

Table 2. Toxicity of the universal neutralizer.

Replicate	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*	*A. niger*
# 1	0.96[a]	1.02	0.94	0.88	0.91
# 2	1.08	0.89	1.06	1.00	1.06
# 3	1.00	1.00	1.00	0.95	0.89

[a] Numbers indicate neutralizer toxicity (NT), which is the ratio between the number of organisms recovered in the "peptone control group" to the number of organisms in the "viability control". Acceptable NT ratios are defined as ≥ 0.70.

researchers have also evaluated the microbial toxicity of other neutralizing solutions (1). They found that with the exception of Dey-Engley broth and NIH thioglycollate media that show no adverse effects, other neutralizing solutions such as AOAC diluting broth, Letheen broth, trypticase with tween and trypticase soy broth with tween 80 and lecithin were toxic against at least one index organism used.

To establish whether the neutralizer was capable of inactivating the antimicrobial agents, NE ratios of the "universal neutralizer" regarding different antimicrobials as built-in products and as their pure forms were determined (Table 3). It should be noted that the concentrations of pure antimicrobials were intentionally chosen at maximum quantities as appeared in the literature. NE ratios not less than 0.70 expressed desirable effectiveness of the neutralizing solution. Conversely, if NE ratios were less than 0.70, it would be concluded that either the neutralizer was toxic for test organisms or it was not of sufficient neutralization effectiveness. The former hypothesis is ruled out herein as the study has shown that the neutralizer has no adverse effect on the organisms. Consequently, any inactivation failure would be attributable to the latter assumption. According to our findings, the "universal neutralizer" was suitable for neutralizing most of the studied antimicrobials against all the challenge organisms except *S. aureus*. With regard to this organism, inhibitory effects of Kathon CG, triclosan, chlorhexidine, thiomersal and chlorhexidine 0.01% (pure solution) remained intact after treatment with the neutralizing solution. This is attributable to

Table 3. Neutralization efficacy of the universal neutralizer.

Antimicrobial agent	*S. aureus*			*E. coli*			*P. aeruginosa*			*C. albicans*			*A. niger*		
	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3
Kathon CG	0.00[a]	0.00	0.00	0.88	0.79	0.84	1.03	0.96	1.20	1.00	0.87	0.82	0.89	0.95	1.00
DMDM Hydantoin	0.90	0.81	1.01	0.83	0.79	0.84	1.02	0.96	0.99	0.73	0.99	1.00	0.95	0.78	1.01
Quaternium-15	1.02	0.97	1.10	0.98	0.76	1.00	1.10	1.20	0.90	1.20	0.89	0.98	1.10	0.93	1.10
Phenonip	0.78	0.80	0.76	0.83	0.76	0.98	1.20	1.07	1.10	0.88	0.87	1.10	1.10	0.82	0.99
Imidazolidinyl urea	0.78	0.78	0.80	0.80	0.90	0.81	0.87	1.10	0.90	0.96	1.00	0.87	1.09	1.10	1.10
Triclosan	0.00	0.00	0.00	0.80	0.73	0.95	0.93	0.80	0.98	0.83	0.94	0.86	1.01	0.87	1.00
Chlorhexidine	0.43	0.48	0.30	0.88	0.90	0.96	1.20	1.02	1.09	1.01	0.82	0.88	1.01	0.83	1.00
Bronopol	0.80	0.78	0.81	0.72	1.01	0.98	1.01	1.20	1.10	1.02	0.83	0.88	0.84	0.82	1.00
Benzalkonium chloride	1.02	0.87	0.98	0.71	0.74	0.91	1.20	1.00	0.99	1.10	0.78	0.99	0.83	0.74	0.79
Thiomersal	0.00	0.00	0.00	0.93	0.85	0.80	1.10	0.99	0.90	0.92	1.01	0.98	1.03	1.10	1.00
Methyl paraben 0.18% & Propyl paraben 0.02% [b]	1.14	0.83	1.00	0.85	0.97	1.08	1.10	0.87	0.83	1.20	1.10	0.90	1.20	1.10	0.93
Imidazolidinyl urea 0.3% [b]	0.93	0.78	0.90	0.95	0.90	0.99	1.20	1.10	0.90	1.10	1.20	0.96	1.10	0.90	1.03
Chlorhexidine 0.01% [b]	0.52	0.63	0.20	0.95	1.12	0.91	1.20	0.80	0.90	1.10	1.10	0.96	1.20	0.96	0.98
Bronopol 0.1% [b]	1.06	0.78	1.00	0.74	0.93	0.85	1.10	1.00	0.90	1.09	1.20	0.98	1.20	0.80	0.86
Benzalkonium chloride 0.02% [b]	1.08	0.77	1.00	0.90	1.10	0.79	1.09	0.94	0.90	1.04	0.78	0.86	1.01	0.93	0.92
Thiomersal 0.02% [b]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

[a] Numbers indicate neutralizer efficacy (NE), which is the ratio between the number of organisms recovered in the "test group" to the number of organisms in the "peptone control group". Acceptable NE ratios are defined as ≥ 0.70.

[b] The solutions of pure preservatives were prepared in distilled water. Their pH was adjusted at 7.0 ± 0.1, except the Bronopol solution that had a pH<5. All the solutions were filter sterilized.

Table 4. Evaluation of dilution effect on neutralization of various tested antimicrobial agents against index organisms.

Antimicrobial agent	*S. aureus*			*E. coli*			*P. aeruginosa*			*C. albicans*			*A. niger*		
	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3
Kathon CG	0.00 [a]	0.00	0.00	0.63	0.51	0.60	0.90	0.82	0.80	0.00	0.00	0.00	0.00	0.00	0.00
DMDM Hydantoin	0.00	0.01	0.00	0.46	0.50	0.50	0.18	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.00
Quaternium-15	1.30	1.00	1.10	0.66	0.66	0.60	0.93	0.75	0.84	0.98	0.85	1.10	1.04	0.87	1.10
Phenonip	0.00	0.00	0.00	0.62	0.56	0.60	1.02	1.01	1.05	0.79	0.89	1.10	1.09	0.80	0.80
Imidazolidinyl urea	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.80	0.93	0.86	1.09	1.10	1.00
Triclosan	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.80	0.79	0.90	0.80	0.80	1.10
Chlorhexidine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.79	0.81
Bronopol	0.00	0.00	0.00	0.65	0.65	0.60	0.77	0.72	0.96	0.90	0.80	0.79	0.00	0.00	0.00
Benzalkonium chloride	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.42	0.30	0.22	0.88	0.91	0.77
Thiomersal	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.30	0.00	0.00	0.00	0.00	0.00	0.00
Methyl paraben 0.18% & Propyl paraben 0.02% [b]	0.90	0.80	0.80	0.80	0.88	0.91	1.20	0.92	0.87	1.20	1.10	0.87	0.94	0.87	0.97
Imidazolidinyl urea 0.3% [b]	0.00	0.20	0.60	0.00	0.00	0.00	0.00	0.40	0.20	1.20	1.20	0.96	0.82	0.92	1.00
Chlorhexidine 0.01% [b]	0.00	0.00	0.00	0.50	0.00	0.20	0.00	0.00	0.00	0.56	0.60	0.54	0.79	0.88	0.80
Bronopol 0.1% [b]	0.00	0.13	0.20	0.40	0.30	0.10	0.78	0.80	0.82	1.20	0.90	0.98	0.20	0.40	0.00
Benzalkonium chloride 0.02% [b]	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.14	0.00	0.00	0.00	0.00	0.92	1.20	0.98
Thiomersal 0.02% [b]	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.42	0.40	0.00	0.00	0.00	0.00	0.00	0.00

[a] Numbers represent the ratio between the number of organisms recovered in the “dilution control” to the number of organisms in the “viability control”. Acceptable ratios are defined as ≥ 0.70.

[b] The solutions of pure preservatives were prepared in distilled water. Their pH was adjusted at 7.0 ± 0.1, except the Bronopol solution that had a pH<5. All the solutions were filter sterilized.

higher sensitivity of this gram-positive bacterium to antibacterial agents. This finding reiterates the importance of inclusion of each single organism to be recovered in the validation program as discussed by others (1). In addition, a pure solution of 0.02% thiomersal retained its properties in the presence of all studied organisms, showing that neutralization effectiveness can be dependent on the concentration of antimicrobial agent.

When using the “universal neutralizer”, two types of mechanisms, namely, dilution and chemical neutralization may contribute to inactivation of antimicrobial agents. Thus, it is essential to understand whether there is any difference between the “universal neutralizer” and a simple diluent like “saline solution”. Considering the fact, a fourth control group called as “dilution control” was also used in the validation study. For this purpose, number of organisms recovered from “dilution control” and those recovered from “viability control” were compared and reported (Table 4). The obtained ratios indicated, when using a simple dilution in saline solution, only parabens were inactivated properly against all the index organisms. This is correlated to high dilution coefficients of the agents, as mentioned in other references (2, 3). On the other hand, DMDM hydantoin was found to be the only antimicrobial agent not inactivated by simple dilution against any of test organisms. Nevertheless, it was successfully neutralized by the “universal neutralizer”. Dilution mechanism demonstrated various degrees of neutralizing effect, depending on the type of antimicrobial agent and the type of organism. For example, benzalkonium chloride, both as its pure form and as it is present in products, was inactivated by simple dilution when tested against fungi. This chemical compound has a low dilution coefficient (0.8-2.5) against bacteria and as a result is not effectively neutralized by dilution (15). Surprisingly, this coefficient increases to 9 when it is tested against fungi (15). This seems to be the reason why this compound has been suitably inactivated by dilution in our study. Some of our findings were beyond expectation and we could not find any rationale for them. For example Bronopol was astonishingly inactivated by dilution against *P. aeruginosa* and *C. albicans* in spite of its low dilution coefficient as 0.7. The other case was about Phenonip, a combination of parabens and phenoxyethanol both having high

dilution coefficients, which resisted inactivation by dilution when tested against *S. aureus* and *E. coli*. This could be related to strong effect of this preservative on bacteria compared to fungi (15). On the other hand, this preservative combination shows synergy that intensifies the antibacterial effects. Generally speaking, the “universal neutralizer” is superior to saline solution, when it comes to inactivate various antimicrobial agents against various index organisms. In other words, the presence of the chemical components in its formula and their neutralizing effects are necessary.

Briefly, CTPA proposed neutralizing solution is not an inclusive neutralizer. Moreover, each organism to be used in the validation of microbial recovery must be included in the test.

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