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MICROBIOLOGICAL EVALUATION OF SOME OPHTHALMIC PREPARATIONS CONTAINING DIFFERENT CONCENTRATIONS OF BENZALKONIUM CHLORIDE

HASAN ADIL

From the Department of Pharmaceutics, Faculty of Pharmacy, University of Karachi, Karachi-75270, Pakistan.

ABSTRACT

Zinc sulphate and boric acid ophthalmic preparations, containing benzalkonium chloride as preservative, have been investigated against gram-negative and positive bacteria. Viability of bacteria was found after 7 days, although it was completely suppressed after 24 hours of inoculation. Zinc sulphate generally increases the antimicrobial activity of benzalkonium chloride, whereas boric acid or its combination with zinc sulphate reduce it. *Pseudomonas aeruginosa* was shown to be viable against zinc sulphate, boric acid, and their combination at a concentration of 0.01% to 0.005% of the preservative. *Escherichia coli* and *Staphylococcus aureus* were also found to be resistant but to a lesser extent than *Pseudomonas aeruginosa*. This viability may be dangerous in case of multidose ophthalmic preparations.

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INTRODUCTION

There is a general agreement that microbial challenges are the only means of evaluating preservative efficacy in ophthalmic products. There are many procedures recommended by Rdzok et al.[18] Anderson and Crompton, [2,11] Erikson,[12] Bernard,[4] and Norton et al.[16]

Antimicrobial agent effectiveness test applies to ophthalmic products in the original unopened container. The USP. XIX test provides a basis for evaluating preservative effectiveness. The preservative may not be equally effective at different stages of an organism's life.[13] The preservative may also lose its activity or interact with the walls of the containers.[17]

The viability of *Pseudomonas aeruginosa* over 7 days suggests that risks of hazards are present even though the preparation complies with the criteria defined in the pharmacopoeia. Therefore, in evaluating preservative efficacy, viability of bacteria after seven days is considered as an important factor,[3,5] and provides information on preservative availability.[5]

In the present work we wish to report the evaluation of a number of laboratory prepared and commercially available ophthalmic preparations containing benzalkonium chloride as a preservative and its efficacy as an antimicrobial agent.

MATERIAL AND METHODS

Test organisms used in this study were gram-negative (*Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 10231) and gram-positive (*Staphylococcus aureus* ATCC 6536) bacteria.

soya agar that was diluted to 10^7 viable cells per ml. Ophthalmic preparations (boric acid 1.6% and zinc sulphate, 0.25% and 0.46%, Merck Chemical Co.), were tested against the above-listed organisms. The ophthalmic preparations formulated in the lab were designated as Z, Z1, Z1B, and B and contained the following: Z (0.25% zinc sulphate), Z1 (0.46% zinc sulphate), B (1.6% boric acid), Z1B (zinc

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Table I. Antimicrobial effectiveness test. Viable count of bacteria (Gram+, Gram-)* against preparation Z with different concentrations of benzalkonium chloride.

contact time	conc. of benzalkonium chloride 0.1%	0.05%	0.01%	0.005%	0.001%
	Z investigated against *P. aeruginosa*				
1 hour	04	03	04	13	10
24 hours	-	-	03	06	02
day 07	-	-	-	30	04
day 14	-	-	-	25	60
	Z investigated against *S. aureus*				
1 hour	-	-	39	115	300
24 hours	-	-	04	006	008
day 07	-	-	-	-	-
day 14	-	-	06	002	60
	Z investigated against *E. Coli*				
1 hour	-	-	-	02	18
24 hours	-	-	-	04	15
day 07	-	-	-	-	-
day 14	-	-	-	-	-

Z: Zinc sulphate 0.25%

* Gram-: *Pseudomonas aeruginosa, Escherichia coli.*

Gram+: *Staphylococcus aureus.*

sulphate 0.46% + boric acid 1.6%). These were produced by dissolving the required amounts of zinc sulphate and boric acid in appropriate control solutions. The control solution was 0.9% W/v NaCl (Merck) in distilled water. Benzalkonium chloride (preservative) was added in five different concentrations (0.1%, 0.05%, 0.01%, 0.005%, 0.001%) to each of the formulations (Z, Z1, Z1B,B) separately.

Cells were added to prewarmed assayed ophthalmic solutions held at 25°C in a conical flask at a concentration of 10^6 cells per ml. 1 ml of the sample was withdrawn at intervals of 1 hour, 7 and 14 days. The samples were placed on tryptic soya agar (oxoide), incubated at 37°C for twenty four hours and then the colonies which appeared were counted.

RESULTS

Zinc sulphate (0.25%) with a 0.1% to 0.05% concentration of benzalkonium chloride was found to be quite effective against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* and no microbial growth was observed after one hour, twenty four hours, and seven and fourteen days (Table I). Zinc sulphate, 0.46% (Table II) and boric acid, 1.6% (Table III) gave the same results.

ZB (zinc sulphate 0.46%+boric acid 1.6%) with a 0.1% to 0.05% concentration of benzalkonium chloride was also effective against *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, but numerous and 02 colonies of *Escherichia coli* were present after 01 and 24 hours respectively (Table IV). Zinc sulphate (0.25%) with 0.01% benzalkonium chloride was also effective against *Pseudomonas aeruginosa*, with 4 and 3 colonies appearing after one hour and 24 hours respectively, and *Staphylococcus aureus*, with 39, 4, and 6 colonies observed after one hour, 24 hours and 14 days, respectively. No colonies of *Escherichia coli* were noted. The same results were obtained with 0.005%-0.001% concentrations of the preservative, with a fluctuant response by *Pseudomonas aeruginosa* during the entire contact period, an extensive response by *Staphylococcus aureus* after one hour (115 and 300 per 0.005% and 0.001% respectively) and a slight response by

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Table II. Antimicrobial effectiveness test. Viable count of bacteria (Gram -, Gram +)* against preparation Z1 with different concentrations of benzalkonium chloride.

contact time	conc. of benzalkonium chloride 0.1%	0.05%	0.01%	0.005%	0.001%
	Z1 investigated against *P. aeruginosa*				
1 hour	-	-	-	250	N
24 hours	-	-	-	-	03
day 07	-	-	25	004	-
day 14	-	-	-	-	-
	Z1 investigated against *S. aureus*				
1 hour	-	-	-	03	N
24 hours	-	-	-	15	05
day 07	-	-	-	-	-
day 14	-	-	-	-	-
	Z 1 investigated against *E. coli*				
1 hour	-	-	04	60	50
24 hours	-	-	-	-	02
day 07	-	-	-	N	N
day 14	-	-	-	-	18

Z1: Zinc sulphate 0.46%
* Gram -: *Pseudomonas aeruginosa, Escherichia coli.*
Gram +: *Staphylococcus aureus.*
(-) no colony (N) numerous colonies.

Table III. Antimicrobial effectiveness test. Viable count of bacteria (Gram -, Gram +)* against preparation B with different concentrations of benzalkonium

contact time	conc. of benzalkonium chloride 0.1%	0.05%	0.01%	0.005%	0.001%
	B investigated against *P. aeruginosa*				
1 hour	-	-	150	N	N
24 hours	-	-	051	32	N
day 07	-	-	051	N	N
day 14	-	-	-	N	N
	B investigated against *S. aureus*				
1 hour	-	-	N	N	N
24 hours	-	-	30	N	N
day 07	-	-	N	N	N
day 14	-	-	50	N	N
	B investigated against *E. Coli*				
1 hour	-	-	N	N	N
24 hours	-	-	25	N	N
day 07	-	-	N	N	N
day 14	-	-	N	N	N

B: Boric acid 1.6%
*Gram -: *Pseudomonas aeruginosa, Escherichia coli.*
Gram +: *Staphylococcus aureus.*
(-) no colony (N) numerous colonies.

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Table IV. Antimicrobial effectiveness test. Viable count of bacteria (Gram -, Gram +)* against preparation Z1B with different concentrations of benzalkonium chloride.

contact time	conc. of benzalkonium chloride 0.1%	0.05%	0.01%	0.005%	0.001%
	Z1B investigated against *P. aeruginosa*				
1 hour	-	-	160	160	200
24 hours	-	-	004	003	008
day 07	-	-	N	N	N
day 14	-	-	N	N	N
	Z1B investigated against *S. aureus*				
1 hour	-	-	N	N	N
24 hours	-	-	60	40	N
day 07	-	-	18	40	N
day 14	-	-	-	N	N
	Z1B investigated against *E. Coli*				
1 hour	-	N	N	100	N
24 hours	-	02	08	-	-
day 07	-	-	06	032	N
day 14	-	-	06	025	N

Z1B: Zinc sulphate 0.46 + boric acid 1.6%

*Gram -: *Pseudomonas aeruginosa, Escherichia coli.*

Gram +: *Staphylococcus aureus.*

(-) no colony (N) numerous colonies.

Escherichia coli (Table I).

Preservative activity should be designated as bacteriostatic or bactericidal,[3,11] and lower concentrations of preservative may be more effective as compared to higher concentrations. Colonies of *Pseudomonas aeruginosa* appeared in preparation Z1 (zinc sulphate 0.46%+ benzalkonium chloride 0.01% and 0.005%) after 7 days, with higher colony counts in the 0.01% concentration, although no growth was seen at 24 hours of inoculation (25/0.01% and 04/0.005%- Table II). Z1 was found to be more effective against *Staphylococcus aureus* and *Escherichia coli*.

Boric acid 1.6% (B) with 0.01%-0.001% benzalkonium chloride was found to be completely ineffective against both gram negative and gram positive bacteria after 1 hour and at 14 days following inoculation (Table III). This ineffectiveness was noticed with ZB (zinc sulphate 0.46%+boric acid 1.6%) at the same concentrations (Table IV).

DISUSSION

A chemical assay of preservative concentrations may be insufficient since the concentrations studied may be wholly unrelated to the bioactivity of the agent.[17] The concentration of the preservative loses its correlation when we take into consideration its mode of action (bactericidal/bacteriostatic). The minimum inhibitory concentration (MIC) does not distinguish between death of bacteria and suppression of their growth. If a compound is bactericidal there will be no growth in tubes containing concentrations greater than the agent's MIC.[10]

The viablity of *Pseudomonas aeruginosa* especially and *Staphylococcus aureus* and *Escherichia coli* reappeared after seven days even though it was completely suppressed after 24 hours of inoculation against preparations Z, Z1, B, and Z1B (Table V). Vegetative bacteria are killed fairly rapidly by most preservatives, although gram negative bacilli including *Pseudomonas aeruginosa* are less sensitive than gram positive organisms.[14]

Pseudomonas aeruginosa exhibited abundant growth with 0.005% to 0.001% benzalkonium chloride. This phenomenon was very extensive (500-3500% increase in number of colonies) in Z1 (zinc sulphate 0.46%) with benzalkonium chloride 0.01%, while a 400% increase was observed in the 0.005% preparation (Table V). The pronounced effect of the 0.005% concentration of preservative compared to the 0.01% may be due to the fact that the former exerts a prelytic effect, whereas the latter is

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Table V. Comparative increased viability of bacteria 7 days and 24 hrs following inoculation with Z, Z1, B, Z1B.

Preparation	Conc. of benzalkonium chloride 0.1%	0.05%	0.01%	0.005%	0.001%
		(*P. aeruginosa*)			
Z	-	-	-	*	*
Z1	-	-	**	*	-
B	-	-	-	N%	-
Z1B	-	-	N%	N%	N%
		(*S. aureus*)			
Z	-	-	-	-	-
Z1	-	-	-	-	-
B	-	-	N%	-	-
Z1B	-	-	-	-	-
		(*E. Coli*)			
Z	-	-	-	-	-
Z1	-	-	-	N%	N%
B	-	-	N%	-	-
Z1B	-	-	-	**	N%

Z: zinc sulphate 0.25%, Z1: zinc sulphate 0.46%, B: boric acid 1.6%, Z1B: zinc sulphate 0.46% + boric acid 1.6%.

* 200-500% increased number of colonies as compared to 24 hours of inoculation.

** 500-3500% increased number of colonies as compared to 24 hours of inoculation.

N% incomparable increased number of colonies as compared to 24 hours of inoculation.

unable to exert bactericidal activity.[19] Benzalkonium chloride exhibits two zones of activity depending on its concentration, the first in which membrane permeability is not altered, and the second in which membrane permeability is altered (bactericidal) due to loss of potassium ions.

Uncomparable reviability of *Pseudomonas aeruginosa* was noticed after 7 days in boric acid 1.6% (+0.005% BAC) and zinc sulphate 0.46% + boric acid 1.6% (+0.01% - 0.005% BAC). Boric acid 1.6% (+0.01% BAC) was generally ineffective against *Staphylococcus aureus* and *Escherichia coli*. Lower concentrations of preservative (0.005%-0.001%) with Z1 (zinc sulphate 0.46%) and Z1B (zinc sulphate 0.46% + boric acid 1.6%) showed extensive reviability of *Escherichia coli* after 7 days (Table V).

In addition to choosing a suitable preservative, we also have the problem of maintaining an adequate preservative concentration in the product to ensure satisfactory preservation.[7] The rubber seal of the container breaks when the medication is first used, and the ophthalmic preparation is exposed to a flow of contaminated air which enters through the opening. Preservatives are removed from the solution and used up during inactivation of micro-organisims.[6] Most preservatives are metabolized by bacteria and fungi. Various components of formulations may increase the resistance and longevity of contaminating microorganisms. Benzalkonium chloride has been reported to be utilized at concentrations well below commonly used levels. Contaminants may be introduced via raw materials or by residual microbial contaminants within manufacturing equipment.[3] These contaminants are considered to be important, because they are usually resistant pathogens. Multiple dose containers may be utilized by patients for up to seven days or more. In such cases, contamination may be undetectable after 24 hours, but is usually present after seven days.

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