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SEVEN DAY VIABLE ANALYSIS OF SOME OPHTHALMIC PREPARATIONS WITH DIFFERENT CONCENTRATIONS OF BENZALKONIUM CHLORIDE

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ABSTRACT

The efficacy of zinc sulphate and boric acid ophthalmic preparations, containing benzalkonium chloride as preservative have been investigated against Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* and Gram-positive *Staphylococcus aureus* bacteria. The viability of bacteria was checked after 7 days though *they* were completely suppressed after 24 hours of inoculation. Zinc sulphate increased the antimicrobial activity of benzalkonium chloride, whereas the boric acid or its combination with zinc sulphate reduced the same. The *Pseudomonas aentginosa* was found to be viable against zinc sulphate, boric acid or its combination at a concentration of 0.01% to 0.005% of the preservative. *Eschenchia coli* and *Staphylococcus aureus* also exhibited resistance but to a lesser extent than *Pseudomonas aeruginosa.* This viability may be dangerous in case of multidose ophthalmic preparations.

Introduction

There is a general agreement that microbial challenges are the only means of evaluating preservative efficacy in ophthalmic products. For such evaluation a number of procedures have been recommended by Rdzok et al. (1955), Anderson and Crompton (1967), Erikson (1970), Bernard (1973) and Norton et al. (1974). Antimicrobial agent effectiveness test, applies to ophthalmic products in the original unopened container. The USP. XIX and BP test provides a basis for evaluating the effectiveness of preservative (Allen, 1959). The preservative may not be effective at different stages of an organism's life (Leitz, 1972) as they may loose its activity or interact with the walls of the containers (Raiman, 1979; Brown and Richard, 1965).

The viability of *Pseudomonas aeruginosa* over 7 days suggests that chances 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 (Berry and Bean, 1954), and provides information on preservative availability (Mitchel, 1964).

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

Materials and Method

Test organism used in this study were Gram-negative (*Pseudomonas aeruginosa* ATCC9027, *Eschenchia coli* ATCC10231) and Gram- positive *(Staphylococcus au*reus ATCC6536) bacteria. Subcultures were made in tryptic soya agar what was diluted at 10^7 viable cells per ml. Ophthalmic preparations, boric acid 1.6% and zinc sulphate, 0.25%, and 0.46%, (Merck) were tested against the above listed organisms. The various ophthalmic preparations formulated in the lab were designated as Z, Z1, B, Z1B and contained the following: Z (0.25% zinc sulphate), Z1 (0.46% zinc sulphate), B (1.6% boric acid). Z1B (Zinc sulphate 0.46% + boric acid 1.6%). They were prepared 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 all the four formulations (Z2, Z1, B, Z1B) separately.

Bacterial cells were added to prewarmed ophthalmic solutions held at 20°C in a conical flask to a final concentration of 10^6 cells per ml. 1 ml of the sample was withdrawn at intervals of 01 hour, 24 hours, 7 and 14 days. The samples were plated on tryptic soya agar (oxide), incubated at 37°C for twenty four (24) hours and then the colonies which appeared were counted.

Results

The formulation Z (zinc sulphate 0.25%) with 0.1% to 0.05% concentration of benzalkonium chloride was found quite effective and no colonial growth was observed after one hour, twenty four hours, and seven and fourteen days against *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* (Table 1). This effectiveness was same for preparation Z1 (zinc sulphate, 0.46%) (Table 2) and preparation B (boric acid, 1.6%) (Table 3).

ZIB (zinc sulphate 0.46% + boric acid 1.6%) was also generally effective with 0.1% to 0.05% concentration of benzalkonium chloride against *Pseudomonas aeruginosa,*

*Staphylococcus au*reu*s* except *Escherichia coli* whereas numerous and 02 colonies were counted after 01 and 24 hour respectively (Table 4). Zinc sulphate (0.25%) with 0.01% benzalkonium chloride was also effective, 4 and 3 colonies appeared after one hour and 24 hours respectively against *Pseudomonas aeruginosa* and 39, 4 and 6 colonies appeared after one hour, 24 hours and 14 days, respectively against *Staphylococcus au*reu*s*. No viability against *Escherichia coli* was noted (Table 1). Similar results were obtained with 0.005% - 0.001% concentration of the preservative that has been responded fluctually *by Pseudomonas aeruginosa,* to entire contact times, extensively *by St*a*phylococcus* mucus after one hour (115, 300 colonies per 0.0005% and 0.001% respectively) and highly *by Escherichia coli* (Table 1). Preservative may act as bacteriostatic or bactericidal and lower concentrations of preservative may be more effective as compared to higher concentrations (Anderson and Crompton, 1967). The colonial response observed against (Zl) zinc sulphate 0.46% + benzalkonium chloride 0.01% and 0.005% concentration after 7 days. No bacterial growth was observed after 24 hours of inoculation and extensive growth was noted at 0.01% as compared to lower concentration (0.005%) by *Pseudomonas aeruginosa,* (25/0.01% and 05/0.005%. Table 2). Zl was found more effective against *Staphylococcus aureus* and *Escherichia coli.*

The preparation B (boric acid 1.6%) with 0.01% - 0.001% benzalkonium chloride was found completely ineffective against both Gram negative and Gram positive bacteria after 01 hour, and 14 days (Table 3). This ineffectiveness was also observed with ZIB (zinc sulphate 0.46% + boric acid 1.6%) for the same range of preservative (Table 4).

Discussion

A chemical assay of preservative concentration may be insufficient since it is wholly unrelated to the bioactivity present (Raiman, 1970; Crompton, 1962). The concentration of preservative becomes unrelated when we take into consideration the mode of action of preservative (bactericidal/bacteriostatic). The minimum inhibitory concentration (MIC) does not distinguish between death of bacteria and the suppression of their growth. If a compound is bactericidal there will be no growth in the tubes containing concentrations greater than MIC (Calaghan, 1984).

In the present study, the viability of *Pseudomonas aeruginosa,* especially, and *Staphylococcus aureus* and *Escherichia coli* generally reappeared after seven days even it was completely suppressed after 24 hours of inoculation against preparations Z, Zl, B, ZIB (Table 5). Vegetative bacteria are killed fairly rapidly by most preservatives. In general, Gram negative bacilli, especially *Pseudomonas aeruginosa* are less sensitive than Gram positive organism (Lynn and Hugo, 1984).

Pseudomonas aeruginosa showed growth with 0.005% to 0.001% benzalkonium chloride. The growth of *Pseudomonas aeruginosa* after 7 days was increased upto 500-3500% o the number of colonies observed after 24 hours of inoculation against Z1 (zinc sulphate 0.46%) with 0.01% of benzalkonium chloride. The number of colonies were increased upto 400% after 7 days as compared to 24 hours of inoculation, against same (Zl: zinc sulphate 0.46%) with 0.005% of benzalkonium chloride (Table 5). The pronounced effect of lesser concentration (0.005% < 0.01%) of preservative, maybe due to the fact that 0.005% should exert prelytic effect whereas 0.01% preservative was unable to produce bactericidal action (Scharaff and Manpin, 1960). Benzalkonium chloride exhibits two zones of activity depending on the concentration in which the membrance permeability is not altered and IInd zone in which membrane permeability is altered (bactericidal) due to loss of potassium.

The innumerable number of colonies of *Pseudomonas aeruginosa* appeared after seven days though completely suppressed after 24 hours of inoculation against boric acid 1.6% (with 0.005% benzalkonium chloride) and boric acid 1.6% + zinc sulphate 0.46% (with 0.01% to 0.005% benzalkonium chloride). The boric acid 1.6% (with 0.1% benzalkonium chloride) generally showed the growth of *Staphylococcus aureus* and *Escherichia coli* after 01 hour, 24 hours, and 7 days. Lower concentrations of pre-servative (0.005% to 0.001% benzalkonium chloride) with Z1 (zinc sulphate 0.46%) and Z1B (zinc sulphate 0.46% + boric acid 1.6%) responded extensive reviability (growth after 7 days.

Though it was completely suppressed, after 24 hours of inoculations) of *Escherichia coli*

In choosing a suitable preservative, the problem is that of maintaining an adequate preservative concentration in the product to ensure satisfactory preservation (Bloomfield, 1988). The seal of rubber container is broken at the first use of dropper assembly, and ophthalmic preparation is exposed to thrust of contaminated air entering through opening, since preservatives are removed from solution and used up during their inactivation of micro-organism (Beveridge, 1984). Most preservatives are metabolized by many bacteria and fungi. Various components of formulations may in-crease the resistance and longevity of contaminated micro-organism. Benzalkonium chloride has been reported to be utilized at concentrations well below the use level. Contaminants may also be introduced via raw material or from residual microbial contaminants within manufacturing equipment (Beans, 1972).

Multiple dose container should be utilized by patient up to seven days. A hapazard danger of contamination may appear immediately within seven days of first use of dropper assembly, and same will be undetectable after 24 hours.

Table 1
Antimicrobial Effectiveness Test
Viable count of bacteria (Gram + ve, Gram -ve)
against preparation "Z" with different concentrations of benzalkonium chloride

Contact Time	Number of Bacterial Colonies: Conc. of Benzalkonium chloride 0.1%	0.05%	0.01%	0.005%	0.001%
	Z investigated against *P. aeruginosa*				
01 hour	04	03	04	13	10
24 hour	-	-	03	06	02
07 days	-	-	-	30	04
14 days	-	-	-	25	60
	Z investigated against *S. aureus*				
01 hour	-	-	39	115	300
24 hours	-	-	04	006	008
07 days	-	-	-	-	-
14 days	-	-	06	002	60
	Z investigated against *E. coli*				
01 hour	-	-	-	02	18
24 hours	-	-	-	04	15
07 days	-	-	-	-	-
14 days	-	-	-	-	-

Z: Zinc sulphate 0.25%
Gram -v, *pseudomonas aeruginosa, Escherichia coli*
Gram + ve, *Staphylococcus aureus*
No colony (-)

Table 2
Antimicrobial Effectiveness Test
Viable count of bacteria (Gram + ve, Gram -ve)
against preparation "Z1" with different concentrations of benzalkonium chloride

Contact Time	Number of Bacterial Colonies: Conc. of Benzalkonium chloride 0.1%	0.05%	0.01%	0.005%	0.001%
	Z1 investigated against *P. aeruginosa*				
01 hour	-	-	-	250	N
24 hours	-	-	-	-	03
07 days	-	-	25	004	-
14 days	-	-	-	-	-
	Z1 investigated against *S. aureus*				
01 hour	-	-	-	03	N
24 hours	-	-	-	15	05
07 days	-	-	-	-	-
14 days	-	-	-	-	-
	Z1 investigated against *E. coli*				
01 hour	-	-	04	60	50
24 hours	-	-	-	-	20
07 days	-	-	-	N	N
14 days	-	-	-	-	18

Z1: Zinc sulphate 0.46%
Gram -v, *Pseudomonas aeruginosa, Escherichia coli*
Gram + ve, *Staphylococcus aureus*
No colony (-). Numerous colonies (N)

Table 3
Antimicrobial Effectiveness Test
Viable count of bacteria (Gram + ve, Gram -ve)
against preparation "Z" with different concentrations of benzalkonium chloride

Contact Time	Number of Bacterial Colonies: Conc. of Benzalkonium chloride 0.1%	0.05%	0.01%	0.005%	0.001%
	B investigated against *P. aeruginosa*				
01 hour	-	-	150	N	N
24 hours	-	-	051	32	N
07 days	-	-	051	N	N
14 days	-	-	-	N	N
	B investigated against *S. aureus*				
01 hour	-	-	N	N	N
24 hours	-	-	30	N	N
07 days	-	-	N	N	N
14 days	-	-	50	N	N
	B investigated against *E. coli*				
01 hour	-	-	N	N	N
24 hours	-	-	25	N	N
07 days	-	-	N	N	N
14 days	-	-	N	N	N

B: Boric acid 1.6%
Gram -v, *Pseudomonas aeruginosa, Escherichia coli*
Gram + ve, *Staphylococcus aureus*
No colony (-). Numerous colonies (N)

Table 4
Antimicrobial Effectiveness Test
Viable count of bacteria (Gram + ve, Gram -ve)
against preparation "Z" with different concentrations of benzalkonium chloride

Contact Time	Number of Bacterial Colonies: Conc. of Benzalkonium chloride 0.1%	0.05%	0.01%	0.005%	0.001%
	Z1B investigated against *P. aeruginosa*				
01 hour	-	-	160	160	200
24 hours	-	-	004	003	008
07 days	-	-	N	N	N
14 days	-	-	N	N	N
	Z1B investigated against *S. aureus*				
01 hour	-	-	N	N	N
24 hours	-	-	60	40	N
07 days	-	-	18	40	N
14 days	-	-	-	N	N
	Z1B investigated against *E. coli*				
01 hour	-	N	N	100	N
24 hours	-	02	08	-	-
07 days	-	-	06	032	N
14 days	-	-	06	025	N

Z1B: Zinc sulphate 0.46% + boric acid 1.6%
Gram -v, *Pseudomonas aeruginosa, Escherichia coli*
Gram + ve, *Staphylococcus aureus*
No colony (-). Numerous colonies (N)

Table 5
Comparative increased viability after seven days to 24 hours of inoculation of bacteria against Z, Z1, B, Z1B

Contact Time	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.

References

Allen H.F. (1959). *Trans. Am. Ophthabnol. Soc.* **57***:* 377.
Anderson K. and Crompton D.R (1976). *Lancet* **ii**: 968.
Beans H.S. (1972). *J. Soc. Cosmet. Chem.* **23**: 703.
Bernard I. (1973). *Labo. Phanna problems et technique.* **21**: *55.*
Berry H. and Beans H.S. (1954). *J. Pharm. Phamacol.* **6**: 649.
Beveridge E.G. (1984). Microbial spoilage and preservation of pharmaceutical products. In pharmaceutical Microbiology (Ed: W.B. Hugo and A.D. Russel). Blackwill Scientific Publication, pp.334.
Bloomfield S.F. (1988). Control of microbiological contamination in non sterile pharmaceuticals, cosmetics and toiletries, (Eds. S.F. Bloomfield, R. Baired, R.E. leak and R. Leech) Ellis Horw000d Limited. Chichesteer, West Sussex P0191EB, England, pp.9-34.
British, Pharmacopoeia (1973). London: HMSO.
Brown R.W.M. and Richard R.M.E. (1965). *Nature.* **207**: 391.
Callaghan C.H.O. (1984). Assessment of a new antibiotic in pharmaceutical Microbiology (Eds W.B. Hugo and Russel). Blackwill Scientific Publications. pp.122.
Crompton D.O. (1962). *J. Pliant:*. **43**: 1020.
Erikson S.P. (1970). *Drug. Cosmet. Ind.* **107**: 36, 147.
Leitz M. (1972). *Bullet. Parent. Drug. Assoc.* **26**: 212.
Lynn B. and Hugo W.B. (1984). Chemical disinfectants antiseptics and preservatives. In Pharmaceutical Microbiology (Eds: W.B. Hugo and A.D. Russel). Blackwill Scientific Publications. pp.201.
Mitchel A.G. (1964). *J. Pharm. Pharmacol.* **16**: 533.
Norton D.A, Davies D.J.G., Richardson N.E., Meakin B.I. Kealle A. (1974). *J. Pharm. Pharmacol.* **26**: 841.
Raiman H.L. (1970). Problems relating to Pharmaceutical preservative including criteria for stability, efficacy and testing method. In Dating of Pharmaceuticals (Eds: Windheuser, lJ. and W.L. Blackstein) University of Wisconsin Press. pp.77.
Rdzok E.J., Grundy W.E., Kirchmeyer F.J., Sylvester J.C. (1955). *J. Am. Pharm. Assoc.* (Sci. Ed.) **44**: 631.
Scharff T.G. and Manpin W.C. (1960). *Biochem. Pharmac.* **5**:79.
United States Pharmacopoeia. XVII. (1970). Revision Easton Pennsylvania. USA. Mack Publishing Company.