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Original Article

Evaluation of preservative effectiveness in ophthalmic drops by microbial challenge test

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ABSTRACT

Ophthalmic drops face the problem of microbial spoilage during their use and storage which affects consumer safety. Protection of these multiple dosage products against microbial contamination is usually achieved by addition of a suitable preservative, but sometimes some clinical hazards are observed even after addition of preservatives. The aim of present study was to evaluate and compare the effectiveness of different preservatives present in three ophthalmic drop samples collected from local market through microbial challenge test. The samples of ophthalmic drops were challenged with 3 bacterial and 2 fungal strains and results were periodically (0, 7, 14 and 28 days) investigated. The number of survive microorganisms were determined using standard microbiological dilution pour-plate method. More than 1 log reduction of microbial counts was observed in all samples at 7 day. Moreover, the log reductions in microbial count were significantly increased up to 28 day. Results showed that ophthalmic drop samples having different preservatives i.e. Benzalkonium chloride, Benzyl alcohal and Phenyl mercuric nitrate were effective against all the challenged microorganisms. However, Phenyl mercuric nitrate was found to be most effective. Hence, from this study it is concluded that preservatives present in all tested ophthalmic drop samples are effective in preventing contamination of the product during their use and storage.

Keywords: Ophthalmic drops, preservative, pharmacopoeia, microorganisms.

INTRODUCTION

Ophthalmic drops are sterile aqueous or oily solutions or suspensions of one or more active substances intended for instillation into the eye [1]. Ophthalmic drops are normally packed and supplied in suitable multidose containers that allow successive drops of the preparation to be administered [2, 3]. In their uses, microbial contamination may lead to product degradation or may give rise to serious ocular infections [2, 4]. Protection of these multiple dosage products against microbial contamination is usually achieved by addition of a suitable preservative [5-7].

The primary purpose of adding antimicrobial preservatives to pharmaceutical dosage forms is to prevent adverse effects arising from contamination by microorganisms that may be introduced inadvertently during or subsequent to the manufacturing process [8]. However, antimicrobial agents should not be used solely to reduce the viable microbial count as a substitute for good manufacturing procedures [8-10]. To prevent microbial contamination, addition of preservatives is needed according to microbial sensibility of the pharmaceutical product and its use by consumers. Preservatives are effective for control of yeast, moulds and bacterial growth.

The challenge test (antimicrobial effectiveness test) is design to measures the level of biological activity possessed by the preservative system of a pharmaceutical products. Preservative efficacy test includes artificial contamination of a formulation with a predetermined number of microorganisms followed by periodic removal of samples at fixed time intervals which, after recovery in suitable media, are used for the viable count of the microorganisms present in the formulation. The organism specified for used in the tests are intended to be representative of those that might be expected to be found in the environment in which the preparation is manufactured, stored and used [8]. Manufacturers usually test the ability of liquid preparation to maintain minimum microbial growth by deliberately inoculating the final product with a suitable microorganism such as *Escherichia coli,*

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Pseudomonas aeruginosa, *Staphylococcus aureus*, *Candida albicans*, *Aspergillus brasiliensis* and monitor the level of contamination at several time intervals [11-15]. The test then compares the level of microorganisms found on a control sample versus the test sample over a period of 28 days.

An ideal preservative should have a broad spectrum of activity against microorganisms and be compatible with different ingredients of a product and its packaging [16]. Preservative should be effective at low concentration against all possible microorganisms and nontoxic in nature [17]. Additionally, preservative must be active in the complete formulation with its lowest concentration and be effective and stable over the range of pH values [18, 19].

The aim of present study was to evaluate and compare the effectiveness of most commonly used preservatives present in ophthalmic drop samples collected from local stakeholders through microbial challenge test.

MATERIALS AND METHODS

Chemicals and Reagents: Microbiological dehydrated media, Soyabean casein digest agar and Sabouraud dextrose agar were procured from HiMedia, Mumbai. NaCl used for the harvesting of microorganisms from media slants was obtained from Merck Ltd. Milli-Q water was used to dissolve dehydrated media and to prepare 0.9 % NaCl solution. Three commercial samples of ophthalmic drops having different preservatives were randomly collected from local market. The ingredients of these products are mentioned in Table 1.

Challenged Microorganisms: The standard microbial strains procured from American Type Culture Collection (ATCC) were used in preservative effectiveness test. The Gram-negative bacteria *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027; Gram-positive bacteria *Staphylococcus aureus* ATCC 6538; yeast *Candida albicans* ATCC 10231 and mould *Aspergillus brasiliensis* ATCC 16404 were used as challenged microorganisms in preservative effectiveness test as prescribed in different Pharmacopoeias [8-10].

Preparation of Microbiological Media: Primary objective of microbiological media is to support the rapid growth of the microorganism being used in the preservative effectiveness test. Soyabean casein digest agar media was used for the recovery of bacteria having ingredients; pancreatic digest of casein (15.0 g/l), peptic digest of soyabean meal (5.0 g/l), sodium chloride (5.0 g/l) and agar (15.0 g/l). On the other hand Sabouraud dextrose agar media having ingredients; peptones (10.0 g/l), dextrose monohydrate (40.0 g/l) and agar (15.0 g/l) was used for the recovery of yeast and mould. Dehydrated media were dissolved in the Milli-Q water and pH was adjusted as per instructions on the dehydrated media container. The media were sterilized in the autoclave at 121^{o}C and 15 psi for 15 minutes. Sterilized media were tested for growth promotion test for best recovery of challenged microbial strains.

Preparation of Inoculums and its Standardization: The ATCC microbial culture strains were revived and sub-cultured on the slants of Soybean casein digest agar and Sabouraud dextrose agar for the growth of bacteria and fungi respectively. The slants of *E. coli*, *P. aeruginosa* and *S. aureus* were incubated at 30-35ºC for 24 hours. The slants of *C. albicans* were incubated at 20-25ºC for 48 hours whereas; the slants of *A. brasiliensis* were incubated at 20-25ºC for 5 days. After the incubation period sterilized saline solution (0.9% w/v NaCl) was used to harvest the bacterial and fungal cultures from agar slants through proper shaking to prepare the microbial suspension. Each microbial suspension was serially diluted with the sterile 0.9% NaCl solution to give a microbial count of 1×10^{8} CFU/ml [8]. The number of CFU was determined by dilution pour-plate method.

Test Methodology: The preservative effectiveness test was performed by challenging the samples of ophthalmic drops with microorganism *E. coli, P. aeruginosa, S. aureus, C. albicans and A. brasiliensis*. The 20 ml of each ophthalmic drop sample were taken in sterile tubes (50 ml) for each challenged microorganisms. Each sample tube was inoculated with one of the prepared and standardised inoculum in such a way that after inoculation the final concentration of microorganism remains between 1×10^{5} and 1×10^{6} CFU/ml. All the inoculated tubes were incubated at 20-25^{o}C for 28 days and viable counts were periodically determined by pour-plate method at 0, 7, 14, and 28 days subsequent to the inoculation. The preservative effectiveness test was performed by following the standard protocol described in Indian Pharmacopoeia and United States Pharmacopoeia.

RESULTS

All the three samples of Ophthalmic drops were challenged with *E. coli, P. aeruginosa, S. aureus, C. albicans, A. brasiliensis* and the level of contamination was monitored on 0, 7, 14 and 28

days by counting the colony forming units (CFU) of microorganisms by pour-plate method subsequent to the inoculation. From the calculated concentration of CFU/ml present at the start of the test (0 day), the log reduction in CFU/ml for each microorganism at the different time intervals (7, 14, and 28 days) were calculated.

Product-A: The challenge of product-A with tested microbes, heavy growth of *E. coli, P. aeruginosa, S. aureus, C. albicans* and *A. brasiliensis* were found on zero day. The counts of *E. coli, P. aeruginosa* and *S. aureus* were extensively decreased on 7 day and observed nil on and after 14 day. However, the counts of *C. albicans* and *A. brasiliensis* were decreased upto 14 day and found nil on 28 day. On 28 day the numbers of tested microorganisms had decreased more than 5 log reduction from the zero day counts. The results are mentioned in Table 2. Microbial testing of product-A (negative control) indicated no growth of any of the tested microbes.

Product-B: The microbial challenge test of product-B showed the intensive growth of *E. coli, P. aeruginosa, S. aureus, C. albicans* and *A. brasiliensis* on zero day. The counts of these microorganisms were significantly decreased on 7, 14 and 28 days. The numbers of all tested microorganism on 7 day were at least 1 log lower than initial (zero day) counts. Whereas, on 28 day their numbers had decreased more than 5 log reduction from the zero day counts. The count of all challenged microorganism were found nil on 28 day. The negative control showed no growth of all the tested microorganisms. The results are depicted in Table 3.

Product-C: Product-C showed heavy growth of *E. coli, P. aeruginosa, S. aureus, C. albicans* and *A. brasiliensis* on zero day. The counts of *E. coli, P. aeruginosa, S. aureus C. albicans and A. brasiliensis* were found more than 2 log lower than initial (zero day) counts on 7 day and became nil on and after 14 day. The negative control showed no microbial growth. The results of the challenge test for the product-C are presented in Table 4.

Normal Saline (Control): Normal Saline (0.9% w/v NaCl) was used as a control in this experimental study. The normal saline was challenged with the tested microbes. The heavy growth of all tested microbes was observed on zero day and growth was slightly declined on 7, 14 and 28 days (Figure 1).

DISCUSSION

Ophthalmic preparations like ophthalmic drops are required to be sterile. However, the accidental microbial contamination of such products while in use and home storage might adversely affect the health of the patient. So, preservative are added in them. In order to minimize the risk of spoilage of ophthalmic drops by microbial contaminants, an antimicrobial preservative is incorporated in these formulation which preferably kill low level of microbial contaminants introduced during the manufacturing process, storage or repeated use [20]. Preservative effectiveness testing is based on inoculation of sample with a determined amount of colony forming units of microbial suspension and investigation of preservative effectiveness of sample at certain interval of time by observing the number of CFU of challenged microorganisms [21].

The current study is dealing with growth of *E. coli, P. aeruginosa, S. aureus, C. albicans* and *A. brasiliensis* inoculated into different ophthalmic drops manufactured by different pharmaceutical companies and which consist of different preservative system i.e. Benzalkonium chloride (Ophthalmic drop-A), Benzyl alcohal (Ophthalmic drop-B), Phenyl mercuric nitrate (Ophthalmic drop-C). The antimicrobial effectiveness testing of Ophthalmic drop-A having Benzalkonium chloride as a preservative showed more than 2 log reduction of all bacterial strains on 7 day and on the same day among all the challenged bacterial strains maximum 3.6 log reduction of *P. aeruginosa* was observed from the initial day (0 day) count. The CFU of bacterial strains were became nil on 14 and 28 days. Whereas, in case of fungal strains more than 1 log reduction were observed on 7 day from initial count and *A. brasiliensis* showed maximum 2 log reduction. The CFU of fungal strains were continuously reduced up to14 day and became nil on 28 day.

In case of Opthalmic drop-B having preservative Benzyl alcohal, the reducion of more than 1 log and 3 log of bacterial strains were observed on 7 and 14 days respectively from the initial day count. On 7 day *E. coli* showed maximum log reduction i.e. 2.2 from the initial count. On the other hand the count of fungal strains showed more than 1 log reduction on 7 day from the initial count and *A. brasiliensis* showed maximum 1.8 log reduction. All fungal count became nil on 28 day.

The Ophthalmic drop-C containing Phenyl mercuric nitrate as a preservative showed more than 2 log reductions of bacterial strains on 7 day from the initial count and maximum 4 log reduction of *P. aeruginosa* was found on the same day. The bacterial growth became nil on and after 14 day. However; the fungal count showed more than 2 log reduction on 7 day and *C. albicans*

showed maximum log reduction 3 on 7 day from the initial count. Like bacterial strains all fungal strains also became nil on and after 14 day. The results of this study showed that all challenged ophthalmic drops met the preservative effectiveness criteria of different pharmacopoeias and Phenyl mercuric nitrate in Ophthalmic drop-C was found as most effective preservative.

CONCLUSION

Microbial contamination of ophthalmic drops packaged in multi dose containers is a serious risk factor for ocular infections. So, adequate preservation of such preparations is very essential to minimize the risk of infection associated with inadvertent microbial contamination. Such preparations are protected by the addition of antimicrobial preservatives that prevent the alterations and degradation of the product formulations. An ideal preservative should have a broad spectrum of activity against microorganisms. The significant of present study was to evaluate and compare the effectiveness of different preservatives system i.e. Benzalkonium chloride (Ophthalmic drop-A), Benzyl alcohal (Ophthalmic drop-B), Phenyl mercuric nitrate (Ophthalmic drop-C) present in three different ophthalmic drops through microbial challenge test. The numbers (CFU) of challenge microorganisms were found continuously decreasing from initial day (zero day) to 28 day in tested ophthalmic drop samples. All the tested ophthalmic drops having different preservatives i.e. Benzalkonium chloride, Benzyl alcohal and Phenyl mercuric nitrate were capable of resisting the growth of microorganisms studied over 28 days. However, Phenyl mercuric nitrate was found to be most effective. From this study it is concluded that preservatives present in tested ophthalmic drops have considerable antimicrobial activity against microorganisms and effective in preventing contamination of ophthalmic drops during their storage and use.

Table 1: Composition and nature of packaging of tested ophthalmic drops.

Product (Ophthalmic Drops)	Composition	Packaging Size	Nature of packaging
A	Ketorolac Tromethamine 0.5% w/v; Benzalkonium chloride 0.02 % v/v	5 ml	Plastic
B	Indomethacin 1% w/v; Benzyl alcohal 1% v/v	5 ml	Plastic
C	Dexamethasone sodium phosphate 0.5% w/v; Chloramphenicol 0.5% w/v; Phenyl mercuric nitrate 0.001% w/v	10 ml	Plastic

Table 2: Antimicrobial effectiveness testing of Ophthalmic drop-A.

Name of Organisms	Inoculum Concentration	Microbial Count (CFU/ml)				Log Reduction (from 0 day count)		
		0 day	7^{th} days	14^{th} days	28^{th} days	7^{th} days	14^{th} days	28^{th} days
Escherichia coli ATCC 8739	7×10^{8}	6×10^{6}	8×10^{3}	Nil	Nil	2.9	6.8	6.8
Pseudomonas aeruginosa ATCC 9027	5×10^{8}	8×10^{6}	2×10^{3}	Nil	Nil	3.6	6.9	6.9
Staphylococcus aureus ATCC 6538	8×10^{8}	3×10^{6}	6×10^{3}	Nil	Nil	2.7	6.5	6.5
Candida albicans ATCC 10231	7×10^{7}	2×10^{6}	4×10^{4}	3×10^{2}	Nil	1.7	3.8	6.3
Aspergillus brasiliensis ATCC 16404	5×10^{7}	4×10^{5}	4×10^{3}	1×10^{1}	Nil	2.0	4.6	5.6

Table 3: Antimicrobial effectiveness testing of Ophthalmic drop-B.

Name of Organisms	Inoculum Concentration	Microbial Count (CFU/ml)				Log Reduction (from 0 day count)		
		0 day	7^{th} days	14^{th} days	28^{th} days	7^{th} days	14^{th} days	28^{th} days
Escherichia coli ATCC 8739	7×10^{8}	3×10^{6}	2×10^{4}	2×10^{1}	Nil	2.2	5.2	6.5
Pseudomonas aeruginosa ATCC 9027	5×10^{8}	5×10^{6}	4×10^{4}	9×10^{1}	Nil	2.1	4.7	6.7
Staphylococcus aureus ATCC 6538	8×10^{8}	7×10^{6}	8×10^{4}	3×10^{3}	Nil	1.9	3.3	6.8
Candida albicans ATCC 10231	7×10^{7}	4×10^{5}	3×10^{4}	5×10^{2}	Nil	1.1	2.9	5.6
Aspergillus brasiliensis ATCC 16404	5×10^{7}	5×10^{5}	8×10^{3}	3×10^{2}	Nil	1.8	3.2	5.7

Table 4: Antimicrobial effectiveness testing of Ophthalmic drop-C.

Name of Organisms	Inoculum Concentration	Microbial Count (CFU/ml)				Log Reduction (from 0 day count)		
		0 day	7^{th} days	14^{th} days	28^{th} days	7^{th} days	14^{th} days	28^{th} days
Escherichia coli ATCC 8739	7×10^{8}	2×10^{6}	2×10^{3}	Nil	Nil	3.0	6.3	6.3
Pseudomonas aeruginosa ATCC 9027	5×10^{8}	6×10^{6}	7×10^{2}	Nil	Nil	4.0	6.8	6.8
Staphylococcus aureus ATCC 6538	8×10^{8}	4×10^{6}	5×10^{3}	Nil	Nil	2.9	6.6	6.6
Candida albicans ATCC 10231	7×10^{7}	6×10^{5}	7×10^{2}	Nil	Nil	3.0	5.8	5.8
Aspergillus brasiliensis ATCC 16404	5×10^{7}	8×10^{5}	6×10^{3}	Nil	Nil	2.1	5.9	5.9

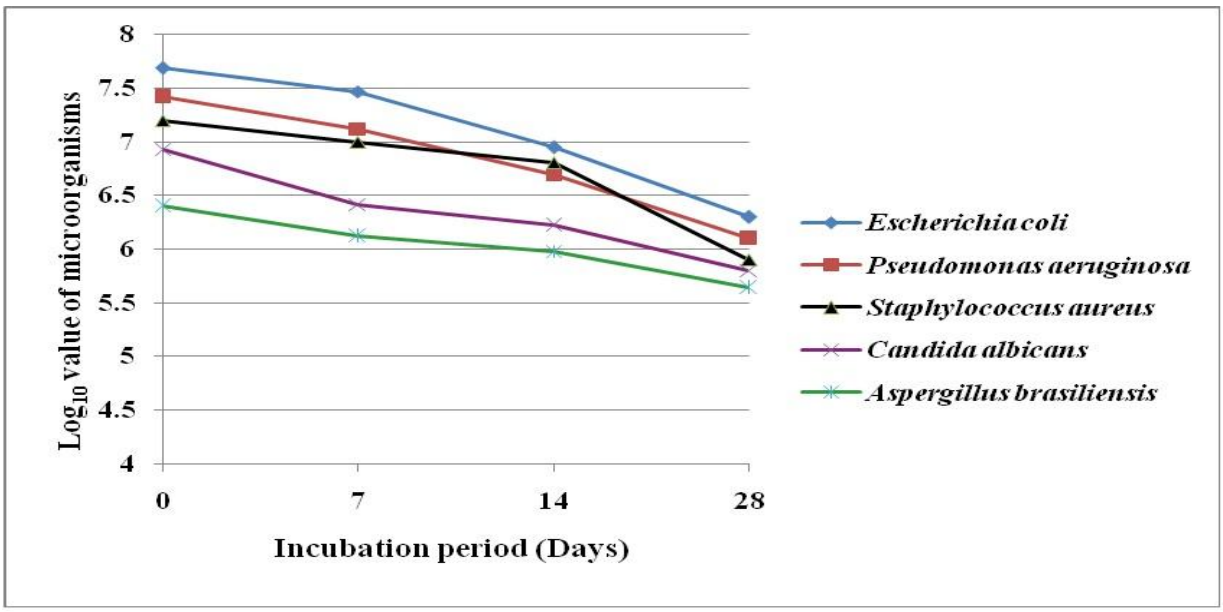

Figure1: Graph showing the numbers (Log_{10} value) of challenged microorganisms periodically (0, 7, 14 and 28 days) investigated after inoculation into normal saline.

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