CHAPTER 8

Preservative Efficacy Study

8.1 Introduction

Nasal spray products have been widely used to assist the delivery of both locally acting and systemic drugs. As nasal sprays formulations are mainly liquid they are prone to microbial contamination during venting air or through the orifice due to contact of spray tip with microbial flora of nasal cavity. The standard container closure system cannot protect the product from microbial contamination. Although the formulations were devoid of any growth supporting ingredients like sugars, celluloses, protein digests etc. the high water activity (0.9), usually associated with the aqueous based formulations has been used as an alarm for signifying the susceptibility for supporting microbial growth. Therefore, preservatives need to be used if the microbial integrity of the product has to be maintained since it is simple and cost effective method.

However, the choice and concentration of preservatives are limited by their potential adverse effects on the nasal mucosa. There remains a controversy in use of preservative, particularly in Europe, and if used the regulatory authorities require proper justification (1). For nasal products the available preservatives include Potassium Sorbate, Methylparaben, Propylparaben and Benzalkonium chloride, is one of the preservative frequently used in formulation. However use of higher concentration of BKC can cause nasal toxicity (2). Therefore it is prudent to make a rationale choice of preservative and keep the concentration of preservative to the minimum.

Preservative concentration used in formulations final container the can be justified by performing preservative efficacy test. Challenge the preparation with approved inoculums of suitable micro-organisms and store the inoculated preparation at a said temperature. Remove samples from the container at specified intervals and count the organisms in the samples. Preservative properties of the preparation are adequate if, in the conditions of the test, there is a significant fall or stagnant count in the number of micro-organisms in the inoculated preparation after prescribed times and temperatures.

Single-strain microbial challenges are made and the elected micro-organisms are supplemented, by other strains where ever required or species that may represent likely contaminants to the preparation (3).

Test micro-organisms

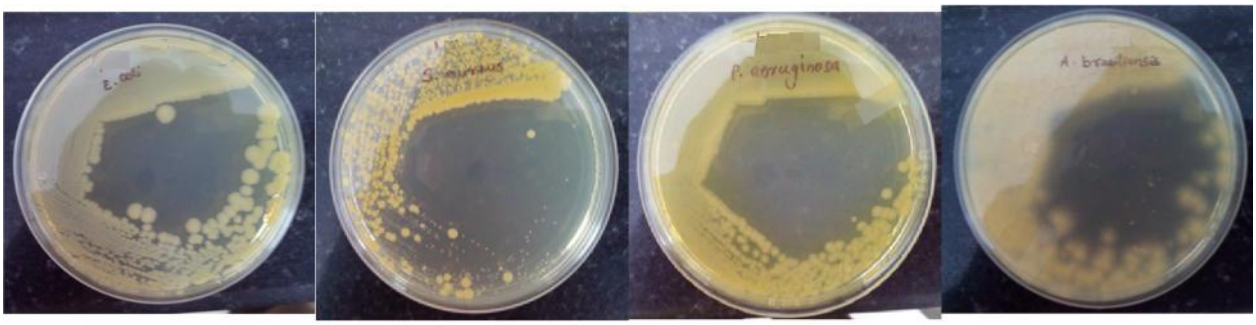
1. Pseudomonas aeruginosa ATCC 9027.
2. Staphylococcus aureus ATCC 6538.
3. Candida albicans ATCC 10231.
4. Aspergillus Niger ATCC 16404.
5. Escherichia coli ATCC 8739 and
6. Zygosaccharomycesrouxii (NCYC 381) for oral preparations containing a high concentration of sugar.

8.2 Preparation of inoculums

Bacteria were inoculated on Tryptone Soya Agarmedium or fungi were inoculated on Sabaourad Dextrose Agar medium. Incubate the bacterial cultures at 30-35 °C for 18-24 h, the culture of C. albicans at 20-25 °C for 48 h, and the culture of A. niger at 20-25 °C for 1 week or until good sporulation was obtained. Sub culturing was carried out whenever required before the micro-organism achive the optimal growth state, but it is suggested that their count be kept to a minimum.

To harvest the bacterial and C. albicans cultures, a sterile suspending fluid was used containing 9 g/l of sodium chloride R, for dispersal and bacteria grown on the surface were transferred into a suitable vessel. The culture was diluted to 10^8micro-organismsper milliliter by adding sufficient suspending fluid. To harvest the A. niger culture, a sterile suspending fluid containing 9 g/l of sodium chloride R and0.5 g/l of polysorbate 80 R was used and dilution was done to adjust the spore count to about 10^8 per milliliter. From these stock serial dilutions were prepared in saline and to determine the number of colony-forming units per mL in each suspension by plate count. The number of colonies per ml will help in determining the baseline to use in the test. The bacterial stock and dilutions were stored at 2-8^{o}C until the results of CFU were obtained (2-3 days) and used as and when required.

Figure 8.1 Sub-culture of test microorganism



8.3 Method

Five containers containing 10 ml of the product to be examined were inoculated, with harvested cultures of *Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans, Aspergillus niger*, *Escherichia coli* suspension to give an inoculums of $10^5$to 10^6 micro-organisms per mL of the preparation. The volume of the suspension of inoculums was kept to less than 1 percent of the volume of the product and thoroughly mixed to ensure homogeneous distribution. The inoculated products were kept at 20-25 °C and protected from light. Suitable sample were removed from each container, typically 0.1 ml, at zero hour and 7, 14, 28 day and number of viable micro-organisms were determined by plate count method. The residual antimicrobial activity of the product was eliminated by dilution. The ability of the system to support the growth of the test organisms was confirmed by the use of appropriate controls. The procedure was validated to verify its ability to demonstrate the required reduction in count of viable micro-organisms.

8.3.1 Criteria of Acceptance

The criteria for evaluation of antimicrobial activity, as provided by USP NF (30) for sterile nasal products (Category I), are given in Table 1 in terms of the log reduction in the number of viable micro-organisms against the value obtained for the inoculums. The no increase (NI) is defined as not more than 0.5 log unit higher than the previous value measured.

Table 8.1 Acceptance criteria for preservative effectiveness testing as per USP

	For category 1 Products
Bacteria	Not less than 1.0 log reduction from the initial calculated count at 7 days, not less than 3.0 log reduction from the initial cunt at 14 days, and no increase from the 14 days count at 28 days
Yeast and Molds	No increase from the initial calculated count at 7, 14 and 28 days

8.3.2 Formulations Tested

Table 8.2 Test formulations

Sr. no.		Benzalkonium Chloride Concentration
1.	Nasal Spray 1 (NS 1)	0
2.	Nasal Spray 2 (NS 2)	0.025% w/v
3.	Nasal Spray 3 (NS 3)	0.05% w/v
4.	Nasal Spray 4 (NS 4)	0.1% w/v

8.4 Results of Microbial challenge Test

Formulation 1:

Table 8.3 Results of microbial challenge to Formulation 1 without preservative

Microorganism	Colony Forming Units /ml			
	0 Days	7 Days	14 Days	28 Days
E. coli	3.4×10^5	<10	<10	<10
S. aureus	2.0×10^5	<10	<10	<10
P. aeruginosa	1.8×10^5	<10	<10	<10
A. brasiliensis	3.4×10^5	2.5×10^5	2.3×10^5	2×10^4
C. albicans	4.1×10^5	6×10^3	<10	<10

Table 8.4 Log reduction in microbes after microbial challenge to NS 1 without preservative

Microorganism	Log Reduction		
	7 Days	14 Days	28 Days
E. coli	4.53	4.53	4.53
S. aureus	4.30	4.30	4.30
P. aeruginosa	4.26	4.26	4.26
A. brasiliensis	0.13	0.17	1.23
C. albicans	1.83	4.61	4.61

Formulation 2:

Table 8.5 Results of microbial challenge to NS 2 with 0.025% w/v preservative

Microorganism	Colony Forming Units /ml			
	0 Days	7 Days	14 Days	28 Days
E. coli	3.0×10^5	<10	<10	<10
S. aureus	2.5×10^5	<10	<10	<10
P. aeruginosa	1.0×10^5	<10	<10	<10
A. brasiliensis	3.2×10^5	1.5×10^5	1.1×10^4	2000
C. albicans	3.8×10^5	30	<10	<10

Table 8.6 Log reduction in microbes after microbial challenge to NS 2 with 0.025% w/v preservative

Microorganism	Log reduction for formulation 2		
	7 Days	14 Days	28 Days
E. coli	4.48	4.48	4.48
S. aureus	4.40	4.40	4.40
P. aeruginosa	4.00	4.00	4.00
A. brasiliensis	0.33	1.46	2.20
C. albicans	4.10	4.58	4.58

Formulation 3:

Table 8.7 Results of microbial challenge to NS 3 with 0.05% w/v preservative

Microorganism	Colony Forming Units /ml			
	0 Days	**7 Days**	**14 Days**	**28 Days**
E. coli	1.5 X 10^5	<10	<10	<10
S. aureus	1.9 X 10^5	<10	<10	<10
P. aeruginosa	3.2 X 10^5	<10	<10	<10
A. brasiliensis	4.1 X 10^5	<10	<10	<10
C. albicans	2.8 X 10^5	<10	<10	<10

Table 8.8 Log reduction in microbes after microbial challenge to NS 3 with 0.05% w/v preservative

Microorganism	Log reduction for formulation 3		
	7 Days	**14 Days**	**28 Days**
E. coli	4.18	4.18	4.18
S. aureus	4.28	4.28	4.28
P. aeruginosa	4.51	4.51	4.51
A. brasiliensis	4.61	4.61	4.61
C. albicans	4.45	4.45	4.45

Formulation 4:

Table 8.9 Results of microbial challenge to NS 4 with 0.1% w/v preservative

Microorganism	Colony Forming Units /ml			
	0 Days	**7 Days**	**14 Days**	**28 Days**
E. coli	2.4 X 10^5	<10	<10	<10
S. aureus	1.5 X 10^5	<10	<10	<10
P. aeruginosa	3.0 X 10^5	<10	<10	<10
A. brasiliensis	3.4 X 10^5	<10	<10	<10
C. albicans	1.2 X 10^5	<10	<10	<10

Table 8.10 Log reduction in microbes after microbial challenge to Formulation 4 with 0.1% w/v preservative

Microorganism	Log reduction for formulation 4		
	7 Days	**14 Days**	**28 Days**
E. coli	4.38	4.38	4.38
S. aureus	4.18	4.18	4.18
P. aeruginosa	4.48	4.48	4.48
A. brasiliensis	4.53	4.53	4.53
C. albicans	4.08	4.08	4.08

Figure 8.2 Colony count of control sample vs preservative at T – 0 time point

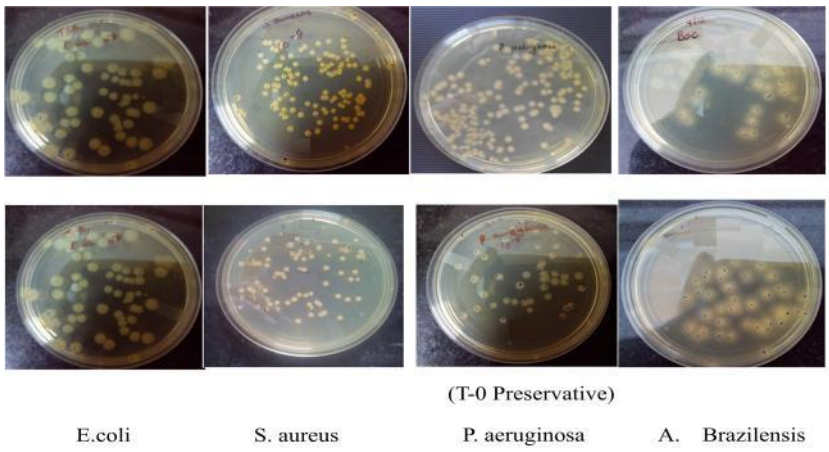


Figure 8.3 Comparison of control sample vs preservative at T – 7 time point

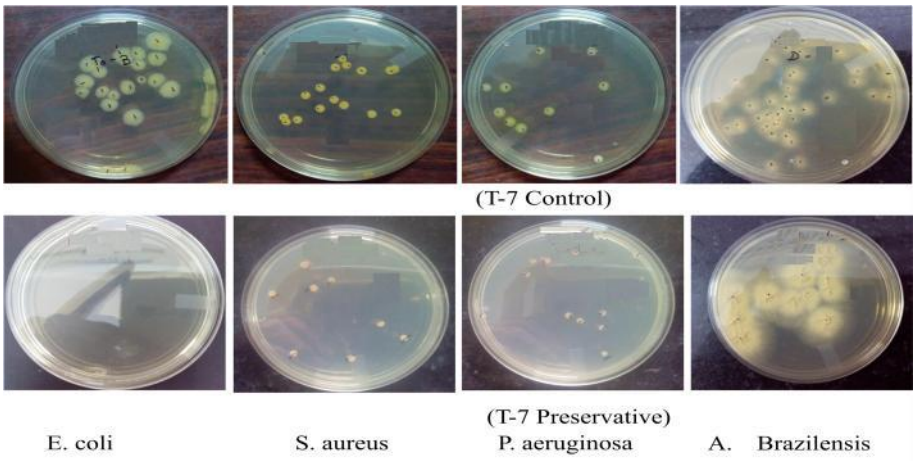


Figure 8.4 Comparison of control sample Vs preservative at T – 14 time point

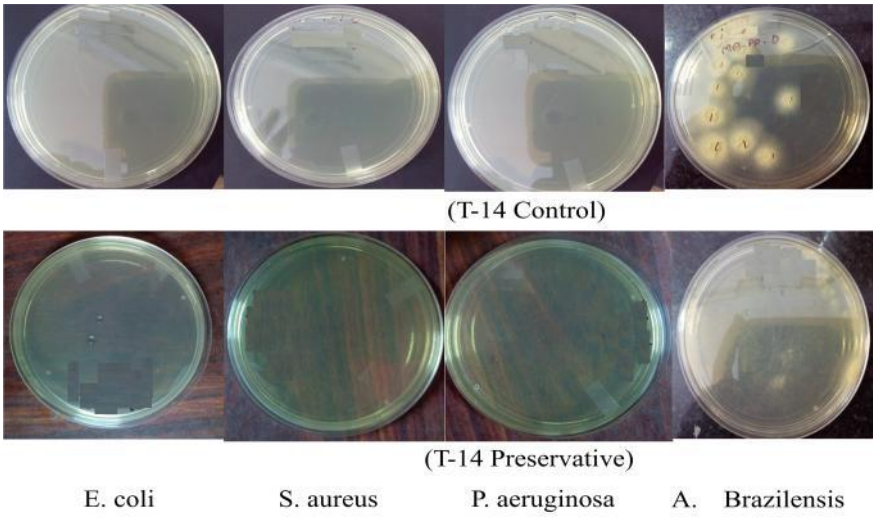
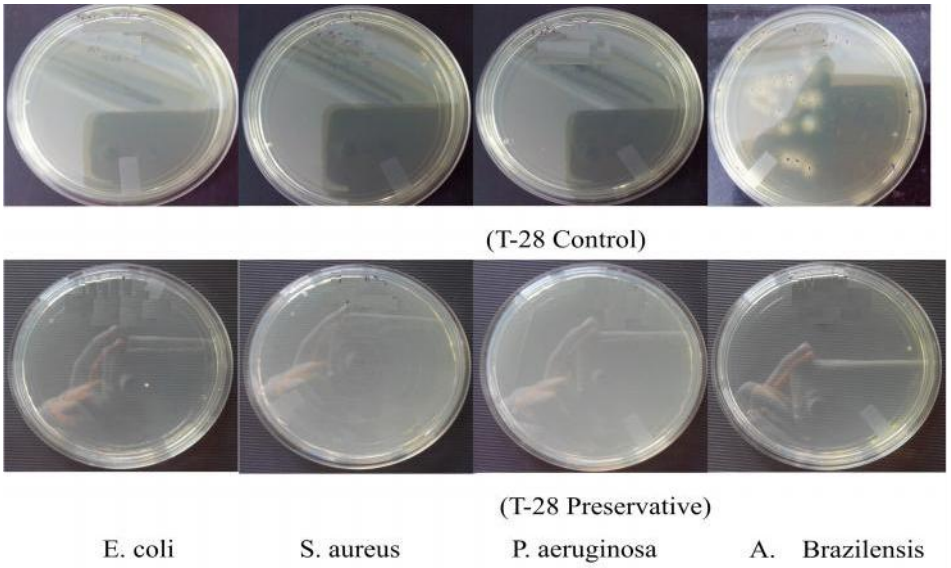


Figure 8.5 Comparison of control sample Vs preservative at T – 28 time point



8.5 Result and discussion

The growth of microorganisms in zero day count confirms that the challenge level of the test indicating the contamination level of 10^5-10^6 organisms/ml of formulation. A significant microbial count was observed on the day 7 and 14, however; there was reduction in microbial count in preservative containing formulation. The day 14 and 28 showed very low microbial counts in all the cases except the control formulations, which proves the susceptibility of the preservative free formulations to support growth of microorganisms, specifically when used in

contact with the external body cavities like nostrils. Although the formulations are devoid of any microbial growth supporting ingredients the high water activity values for aqueous formulations (> 0.9) warrant the chances of contamination from the both bacteria and fungus. Typically the fungus have the ability to grow at ambient temperature conditions (25^{o}C) and lower water activity of upto 0.87, which necessitates measures for protection against contamination by pathogenic microorganisms.

Formulations NS 1, NS 2, NS 3 and NS 4 contain different concentrations of Benzalkonium chloride 0%, 0.025%, 0.05% and 0.1% respectively. NS 1 and NS 2 formulation failed in microbial challenge test while F2 and F3 passed the test based on the acceptance criteria given by EP. It was also observed that all the organisms except Aspergillus were reduced significantly in all the formulation with and without preservatives. This can be attributed to the fact that the Aspergilus is a spore form of the fungus while the Candida is a yeast form. The sporulating forms of organisms are more resistant to the action of the antimicrobial agents. Therefore ability of the preservative to kill Aspergillus will be more important in deciding the results of preservative efficacy test. Since, it is prudent to keep the preservative concentration to the minimum, the F2 formulation containing 0.1 % of BKC can be considered to be acceptable.

8.6 References

1. Edition JPF. English Version. PMI Institute. 2002.

2. Hallen H, Graf P. Benzalkonium chloride in nasal decongestive sprays has a long-lasting adverse effect on the nasal mucosa of healthy volunteers. Clinical & Experimental Allergy. 1995;25(5):401-5.

3. Sutton SV, Porter D. Development of the antimicrobial effectiveness test as USP chapter< 51>. PDA Journal of Pharmaceutical Science and Technology. 2002;56(6):300-11.