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Evaluation of preservative effectiveness in an official antacid preparation

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Abstract

The effective antimicrobial anilide derivatives from our earlier study were subjected to preservative efficacy testing in an official antacid preparation, (Aluminium Hydroxide Gel-USP) against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Candida albicans* and *Aspergillus niger* as representative challenging microorganisms as per USP guidelines. The selected anilide derivatives were found to be effective against *C. albicans, E. coli* and *S. aureus* and showed preservative efficacy comparable to that of standard and even better in case *A. niger*. This study showed the potential of anilides to be used as a preservative in pharmaceutical products.

Key words: Anilides, preservative, Log CFU/ml.

Introduction

The presence of high degree of water in pharmaceutical product makes it to be easily contaminated with microorganisms that results in spoilage of product with loss of its therapeutic properties and if they are pathogenic, serious infection can arise. In order to minimize the risk of spoilage of pharmaceutical product by contaminants, an antimicrobial preservative is included in a formulation which preferably kill low level of contaminants introduced during the manufacturing process, storage or repeated use of multiple dose containers. Preservatives must, therefore, be stable within the formulation for the shelf life of the product and be capable of dealing with all the abuses made to it by the consumer and user (i.e. contamination during use, incorrect storage etc.) (Aker et al. 1990, Zani et al. 1997).

Different pharmacopoeias describe official methods for evaluating the effectiveness of preservative system (U.S.P. 2004, B.P. 1993). It was early recognized that evaluation of antimicrobial activity of a preservative in a finished product can only be made by testing the compound claiming preservation in the finished product.

Preservative efficacy (challenge test) involves the artificial introduction of representative microorganisms including Gram positive and Gram negative bacteria, mould and yeast into the product under study, in sufficient amounts followed by the collection of kinetic information regarding the loss of their viability.

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Lack of standardization in the performance (i.e. microorganism used as inoculum) and the criteria of the challenge test often lead to irreproducible results or results which cannot be compared with those from different laboratories. Therefore, the establishment of certain standards regarding preservation efficacy, mainly proposed by Pharmacopoeias, necessitates harmonization between the different scientific units in the industry, and between the authorities responsible for evaluation and selection of suitable preservatives (Manou et al. 1998).

In view of above, in the present study we hereby report the preservative efficacy of most effective anilide derivatives reported from our previous study (Narasimhan et al. 2007) against Gram positive *Staphylococcus aureus MTCC 2901*, *Bacillus subtilis MTCC 2063*, Gram negative *Escherichia coli MTCC 1652*, fungal strains *Aspergillus niger MTCC 8189* and *Candida albicans MTCC 227* and compared it with the standard preservatives methyl and propyl paraben, in Aluminium hydroxide gel -USP (Lachman et al. 1987).

Materials and Methods

Materials

Nutrient agar, nutrient broth, sabouraud dextrose agar and sabouraud dextrose broth were obtained from Himedia, Mumbai. Mannitol, methyl and propyl paraben were obtained from CDH, Mumbai.

Methods

Aluminium Hydroxide Gel USP was used as the pharmaceutical product for evaluation of preservative efficacy testing.

Formula: Aluminium hydroxide gel, 36 g; Mannitol, 7 g; Methyl paraben, 0.2 g; Propyl paraben, 0.02 g; Saccharin, 0.05 g; Peppermint oil, 0.005 ml; Alcohol, 1 ml; Purified water q.s., 100 ml.

The weighed quantity of aluminum hydroxide gel and mannitol were triturated with 50 ml of water in a mortar. Methyl paraben, propyl paraben, saccharin and peppermint oil were dissolved in alcohol and added to above mixture and triturated well. The volume was made up to 100 ml with purified water followed by its sterilization by autoclaving.

For preservative efficacy testing, the aluminium hydroxide gel was prepared using the preservatives mentioned in Table 1 by replacing methyl paraben and propyl paraben from the above formula. The equimolar amount of selected preservatives were calculated with reference to the amount of methyl paraben (0.0013 mol) and added into aluminum hydroxide gel.

Table 1. Amount of selected preservatives added in Aluminum Hydroxide Gel – USP.

S. No.	Preservative	Amount (g)
1.	Lauric-p-nitro anilide	0.416
2.	Stearic-p-chloro anilide	0.510
3.	Lauric acid	0.369
4.	Stearic acid	0.260

Strains: Staphylococcus aureus MTCC 2901, Bacillus subtilis MTCC 2063, Escherichia coli MTCC 1652, Candida albicans MTCC 227 and *Aspergillus niger MTCC 8189* were used in this study was originally identified as a common contaminants and prescribed in USP for preservative efficacy testing in pharmaceutical preparations.

Preservative efficacy testing in Aluminium hydroxide gel USP 2004

The preservative efficacy test was performed essentially following the standard protocol described in USP-2004. In all cases the preservative efficacy test was done in Aluminium hydroxide gel-USP with and without the preservative system. The unpreserved product was used as a control to evaluate the viability of the inoculated cells and their ability to grow in the product.

Preparation of inoculum

The representative microorganisms were inoculated in nutrient agar I.P. (*S. aureus, B. subtilis, E. coli*) and sabouraud agar I.P. (*C. albicans, A. niger*). The seeded plates were incubated at 37°C for 24 h (*S. aureus, B. subtilis, E. coli*), 37°C for 48 h (*C. albicans*) and 25°C for 7 d (*A. niger*). After the inoculation period, suspensions of microorganisms were prepared in sterile saline solution (0.9% w/v NaCl) to give a microbial count of 1x 10^4 CFU/ml.

Test Procedure

Aluminium hydroxide gel-USP in their final container was used in the challenge test. The preparation was inoculated with the microbial cell suspension with a cell count of 1 x 10^4 CFU/ml. The inoculum never exceeded 1% of the volume of the product sample. Inoculated samples were mixed thoroughly to ensure homogeneous microorganism distribution and incubated. The CFU/ml of the product was determined at an interval of 0, 7, 14, 21 and 28 days on agar plate. The log values of number of CFU/ml (Table 2, Table 6) of aluminium hydroxide gel was calculated and compared as per the guidelines of USP 2004.

Criteria of acceptance for preservative system

As per USP NF 2004 the requirement for antacid made with an aqueous base, preservative effectiveness are met if there is no increase from initial calculated count at 14th and 28th days in case of bacteria, yeast and moulds. Where, no increase is defined as not more than 0.5 $\log_{10}$ higher than previous value measured (USP 2004).

Results and Discussion

In case of *B. subtilis,* selected anilides (Lauric-p-nitro anilide, stearic-p-chloro anilide) showed less than 0.5 $\log_{10}$ values of increment of CFU/ml at 14th and no increment at 28th day from its previous $\log_{10}$ values of CFU/ml. So they pass preservative efficacy testing. The parent lauric acid and standard preservative were active on 14th day but fails to meet the required limit on 28th day (Table 2).

Table 2. Bacterial count of *B. subtilis* in aluminium hydroxide Gel-USP supplemented with preservatives.

Preservative added	Log CFU/ml (Time in days)				
	0	7	14	21	28
Lauric acid	0.000	0.602	0.301	0.000	0.778
Lauric-p-nitroanilide	0.301	0.602	0.698	0.000	0.000
Stearic acid	0.602	1.204	0.903	0.000	0.477
Stearic-p-chloroanilide	0.602	0.477	0.301	0.000	0.000
Methyl and Propyl paraben	0.602	0.477	0.000	0.000	0.778
Control	0.698	0.602	1.113	0.301	0.845

In case of *S. aureus* both selected anilide derivatives, parent acids and standard meets USP 2004 guidelines for preservative effectiveness testing and results are comparable to that of standard (Table 3).

Table 3. Bacterial count of *S. aureus* in aluminium hydroxide Gel-USP supplemented with preservatives.

Preservative added	Log CFU/ml (Time in days)				
	0	7	14	21	28
Lauric acid	0.778	0.000	0.000	0.698	0.477
Lauric-p-nitroanilide	0.477	0.301	0.301	0.000	0.000
Stearic acid	1.000	0.477	0.301	0.301	0.602
Stearic-p-chloroanilide	0.698	0.301	0.301	0.477	0.301
Methyl and Propyl paraben	0.602	0.301	0.000	0.301	0.477
Control	0.903	0.477	0.602	0.778	0.845

As per the result showed in Table 4 lauric-p-nitro anilide and stearic-p-chloro anilide were found to be active against *E. coli* on 14th as well as on 28th day and met the requirement for preservative efficacy testing as per USP 2004.

Table 4. Bacterial count of *E. coli* in Aluminium Hydroxide Gel – USP supplemented with preservatives.

Preservative added	Log CFU/ml (Time in days)				
	0	7	14	21	28
Lauric acid	0.845	0.000	0.000	0.301	0.954
Lauric-p-nitroanilide	0.602	0.000	0.000	0.000	0.301
Stearic acid	0.000	0.000	0.000	0.903	1.000
Stearic-p-chloroanilide	0.698	0.301	0.301	0.000	0.301
Methyl and Propyl paraben	0.778	0.000	0.602	0.302	0.698
Control	0.845	0.602	0.778	0.954	1.041

In case of *C. albicans,* lauric-p-nitro anilide showed no increment in log CFU/ml from 7th to 28th days, so it passes the preservative effectiveness testing and results are comparable to that of standard. There is decrease in log CFU/ml from 7th to 14th and 21st to 28th days in case of stearic-p-chloroanilide, so it also passes preservative effectiveness testing against *C. albicans* (Table 5).

Table 5. Fungal count of *C. albicans* in Aluminium Hydroxide Gel - USP supplemented with preservatives.

Preservative added	Log CFU/ml (Time in days)				
	0	7	14	21	28
Lauric acid	0.000	0.000	0.602	0.778	0.000
Lauric-p-nitroanilide	0.301	0.000	0.000	0.000	0.000
Stearic acid	1.111	0.698	0.301	0.954	0.602
Stearic-p-chloroanilide	0.903	0.602	0.477	0.778	0.698
Methyl and Propyl paraben	0.301	0.698	0.602	0.778	0.000
Control	0.477	0.778	0.845	0.845	0.903

In case of *A. niger* generally there is no increase in log CFU/ml on 14th and as well as on 28th day for lauric-p-nitro anilide, stearic-p-chloro anilide and lauric acid from its previous values, so they pass preservative effectiveness test as per USP 2004 and showed better activity as compare to standard preservatives (Table 6).

Table 6. Fungal count of *A. niger* in Aluminium Hydroxide Gel-USP supplemented with preservatives.

Comp.	Log CFU/ml (Time in days)				
	0	7	14	21	28
Lauric acid	0.301	0.778	0.602	0.477	0.301
Lauric-p-nitroanilide	0.602	0.301	0.000	0.000	0.000
Stearic acid	0.477	0.301	0.000	0.301	0.477
Stearic-p-chloroanilide	0.602	0.477	0.301	0.301	0.301
Methyl and Propyl paraben	0.301	0.301	0.698	0.000	0.477
Control	0.698	1.079	0.954	1.000	1.079

The microorganisms are resistant to antimicrobials and in some cases are able to degrade many commonly used preservatives especially *p*-hydroxybenzoates e.g. parabens (Close and Nielsen 1976). The anilides used in the present study has the advantage over the parabens due to their chemical nature. The parabens, basically of ester category are susceptible to hydrolysis at a

faster rate than the anilides. Due to the above facts the anilides used in the present study especially the lauric *p*-nitroanilide showed the potential to be used as an alternative to the existing preservatives.

Conclusion

The selected anilide derivatives were found to be effective against all selected strains and showed preservative efficacy comparable to that of standard and even better in case *A. niger*. The study showed the preservative potential of lauric-*p*-nitroanilide and stearic-*p*-chloroanilide in the pharmaceutical preparation.

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