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EVALUATION OF PRESERVATIVE EFFECTIVENESS OF 3, 5 DINITROBENZOIC ACID DERIVATIVES IN ALUMINIUM HYDROXIDE GEL– USP

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ABSTRACT

The potential derivatives of 3, 5 Dinitrobenzoic acid from our previous study were subjected to preservative efficacy testing. Aluminium Hydroxide Gel – USP was used as a pharmaceutical product and *Staphylococcus Aureus, Bacillus subtilis, Escherichia coli, Candida albicans and Aspergillus Niger* were used as representative challenging microorganisms for antimicrobial effectiveness testing as per USP 2004. The 3, 5 Dinitrobenzoic acid derivative, 3,5 Dinitrobenzyl anilide has better preservative efficacy than 3, 5 Dinitrobenzoic acid as well as the standard preservatives, methyl paraben and propyl paraben .

Keywords: 3, 5 Dinitrobenzoic acid; hydrazide; anilide; Preservative efficacy.

INTRODUCTION

High degree of water availability in pharmaceutical products may give rise to their contamination by microorganisms which may cause spoilage of the product along with loss of therapeutic properties and, if they are pathogenic, serious infections can arise [1, 2]. During past 20 years, the frequency of systemic infection has increased dramatically along with the number of invasive, mostly opportunistic, fungal species carrying infectious diseases. [3]. Therefore, preservatives are being added to the preparations to prolong their shelf life by preventing the microbial attack[4]. 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.)[5, 1].

Experimental

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 ranbaxy, Mumbai.

Method

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

Preparation of Aluminum Hydroxide Gel-USP [6].

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. Preservative Evaluation of Novel 3, 5 Dinitrobenzoic acid Derivatives.

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

Sr. No	Preservatives	Amount (gm)
1.	3,5 Dinitrobenzoic acid	0.19
2.	3,5 Dinitrobenzyl hydrazide	0.28
3.	3,5 Dinitrobenzyl anilide	0.35

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 (Table 1) were calculated with reference to the amount of methyl paraben (0.0013 mol) and added into the pharmaceutical products.

Preservative efficacy testing in pharmaceutical products [7]

Aluminum hydroxide gel prepared with different preservatives was sterilized in autoclave at 120°C for 15 minutes. The products were then inoculated separately with 2 x 10^4 CFU/mL of *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Candida albicans* and *Aspergillus niger and* stored at room temperature (25°C). The CFU/mL of the product was determined at an interval of 0, 7, 14, 21 and 28 days. The experiment was performed in triplicate. The log values of number of colonies of microorganisms per ml along with their log standard deviation values (Table 2 – Table 6) in Aluminum hydroxide gel was calculated and compared as per the guidelines of USP 2004.

Preparation of inoculums

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 inoculums 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

The log results are shown in Table 2. The parent compound 3,5 Dinitrobenzoic acid was effective upto the limit prescribed by USP on 21th day (0.000 ± 0.00) but it could not pass the limit on 28th day (0.699 ± 0.04). The derivatives 3,5 Dinitrobenzyl hydrazide and 3,5 Dinitrobenzyl anilide were found to be effective on 21th day (0.000 ± 0.00, 0.000 ± 0.15) respectively) and 28th day (0.301 ± 0.08, 0.499 ± 0.08 respectively) as the log results were found to be in accordance with limit prescribed in the USP. The standard preservative was active on 21th day (0.000 ± 0.00) but fails to meet the required limit on 28th day (0.778 ±

0.03). In the present study, even though the B. subtilis is not specified as a test organism for the preservative efficacy testing in USP, it has been selected as a test organism being it is mentioned in the Indian Pharmacopoeia as a possible aerobic microbial contaminant of pharmaceutical substances [8]. Further, the Bacillus species synthesize a necrotic enterotoxin, possibly in conjunction with the primary haemolysin which may be responsible for non gastrointestinal bacillus infection [9].

Tab. 2. Bacterial count (CFU/mL) of *B. subtilis* in Aluminium Hydroxide Gel USP supplemented with preservatives.

Comp.	LOG CFU/Ml (time in days)				
	0	**7**	**14**	**21**	**28**
3,5 Dinitrobenzoic acid	0.000 ± 0.00	0.000 ± 0.15	0.000 ± 0.00	0.000 ± 0.00	0.699 ± 0.04
3,5 Dinitrobenzyl hydrazide	0.000 ± 0.15	0.000 ± 0.00	0.000 ± 0.15	0.000 ± 0.00	0.301 ± 0.08
3,5 Dinitrobenzyl anilide	0.301 ± 0.08	0.000 ± 0.00	0.499 ± 0.09	0.000 ± 0.15	0.499 ± 0.08
Methyl paraben and propyl paraben	0.602 ± 0.05	0.499 ± 0.09	0.000 ± 0.00	0.000 ± 0.00	0.778 ± 0.03
Control	0.699 ± 0.04	0.602 ± 0.05	1.110 ± 0.02	0.301 ± 0.08	0.845 ± 0.03

For *S. aureus*:

As per the results given in Table-3, 3,5 Dinitrobenzoic acid was found to be active against S. aureus on 14th (0.000 ± 0.17) as well as 28th (0.602 ± 0.05) day. The 3,5 Dinitrobenzyl hydrazide (0.000 ± 0.17, 0.000 ± 0.00) and 3,5 Dinitrobenzyl anilide (0.000 ± 0.00) showed results better than 3,5 Dinitrobenzoic acid , standard methyl paraben(0.699 ± 0.09) and are within the pharmacopoeial limits.

Tab. 3. Bacterial count (CFU/mL) of *S. aureus* in Aluminium Hydroxide Gel USP supplemented with preservatives

Comp.	LOG CFU/Ml (time in days)				
	0	**7**	**14**	**21**	**28**
3,5 Dinitrobenzoic acid	0.30 1 ± 0.08	0.00 0 ± 0.00	0.00 0 ± 0.17	0.60 2 ± 0.04	0.60 2 ± 0.08
3,5 Dinitrobenzyl hydrazide	0.30 1 ± 0.05	0.00 0 ± 0.17	0.00 0 ± 0.00	0.00 0 ± 0.00	0.00 0 ± 0.00
3,5 Dinitrobenzyl anilide	0.60 2 ± 0.05	0.30 1 ± 0.05	0.00 0 ± 0.17	0.00 0 ± 0.00	0.00 0 ± 0.00
Methyl paraben and propyl paraben	0.60 2 ± 0.05	0.30 1 ± 0.08	0.30 1 ± 0.08	0.47 7 ± 0.08	0.69 9 ± 0.09
Control	0.69 9 ± 0.04	0.60 2 ± 0.05	1.11 0 ± 0.02	0.30 1 ± 0.08	0.84 5 ± 0.03

For *E. coli:*

3,5 Dinitrobenzoic acid (0.000 ± 0.17 (21th day), 0.699 ± 0.08 (28th day)) meets the pharmacopoeial limits and 3,5 Dinitrobenzyl hydrazide (0.000 ± 0.00 (14th day), 0.602 ± 0.08 (28th day)) showed results better than 3,5 Dinitrobenzoic acid and meets the required limits.

Tab. 4. Bacterial count (CFU/mL) of *E. coli* in Aluminium Hydroxide Gel USP supplemented with preservatives

Comp.	LOG CFU/Ml (time in days)				
	0	7	14	21	28
3,5 Dinitrobenzoic acid	0.301 ± 0.08	0.301 ± 0.17	0.000 ± 0.17	0.000 ± 0.00	0.699 ± 0.08
3,5 Dinitrobenzyl hydrazide	0.000 ± 0.00	0.000 ± 0.17	0.000 ± 0.00	0.301 ± 0.05	0.602 ± 0.08
3,5 Dinitrobenzyl anilide	0.699 ± 0.04	0.301 ± 0.08	0.000 ± 0.17	0.000 ± 0.08	0.301 ± 0.08
Methyl paraben and propyl paraben	0.778 ± 0.03	0.301 ± 0.08	0.000 ± 0.00	0.602 ± 0.05	0.699 ± 0.09
Control	0.602 ± 0.05	0.778± 0.03	0.845 ± 0.03	0.954 ± 0.03	1.041 ± 0.08

3, 5 Dinitrobenzyl anilide showed results better on 21th day (0.000 ± 0.08) and passes the limit on 28th day (0.301 ± 0.08) as complete microbial inhibition was seen at that period. The standard (0.602 ± 0.05 (21th day), 0.699 ± 0.09 (28th day) fails to meet the limits. The results are shown in Table 4.

For C. albicans:

3,5 Dinitrobenzoic acid (0.000 ± 0.17 (14th day), 0.000 ± 0.00 (28th day) passes the limit of preservative efficacy test and the derivatives 3,5 Dinitrobenzyl hydrazide (0.000 ± 0.00 (14th day), 0.000 ± 0.00 (28th day) and 3,5 Dinitrobenzyl anilide (0.301 ± 0.05 (14th day), 0.000 ± 0.00 (28th day) were found to be active in accordance with USP.

Tab. 5. Fungal count (CFU/mL) of *C. albicans* in Aluminium Hydroxide Gel USP supplemented with preservatives.

Comp.	LOG CFU/Ml(time in days)				
	0	7	14	21	28
3,5 Dinitrobenzoic acid	0.301 ± 0.08	0.301 ± 0.17	0.000 ± 0.17	0.000 ± 0.00	0.000 ± 0.00
3,5 Dinitrobenzyl hydrazide	0.477 ± 0.03	0.000 ± 0.17	0.00 ± 0.05	0.00 ± 0.08	0.000 ± 0.00
3,5 Dinitrobenzyl anilide	0.00 ± 0.17	0.00 ± 0.08	0.000 ± 0.05	0.000 ± 0.00	0.000 ± 0.00
Methyl paraben and propyl paraben	0.301 ± 0.17	0.477 ± 0.08	0.699 ± 0.17	0.778 ± 0.05	0.000 ± 0.00
Control	0.477 ± 0.08	0.778± 0.17	0.845 ± 0.03	0.954 ± 0.03	1.079 ± 0.08

The standard (0.699 ± 0.17 (14th day), 0.000 ± 0.00 (28th day) also meets the limits and the test compounds showed results better than standard. The results are presented in Table 5.

For *A. niger*:

3,5 Dinitrobenzoic acid (0.778 ± 0.05) 14th day, (0.301 ± 0.17) 28th day, 3,5 Dinitrobenzyl hydrazide (0.699 ± 0.05) 14th day, (0.000 ± 0.00) 28th day and 3,5 Dinitrobenzyl anilide (0.301 ± 0.00) 14th day, (0.000 ± 0.00) 28th day, were found to be active against the fungus.

Tab. 6. Fungal count (CFU/mL) of *A. niger* in Aluminium Hydroxide Gel USP supplemented with preservatives

Comp.	LOG CFU/Ml (time in days)				
	0	7	14	21	28
3,5 Dinitrobenzoic acid	0.477 ± 0.03	0.699 ± 0.17	0.778 ± 0.05	0.301 ± 0.17	0.301 ± 0.17
3,5 Dinitrobenzyl hydrazide	0.477 ± 0.03	0.477 ± 0.03	0.699 ± 0.05	0.000 ± 0.08	0.000 ± 0.00
3,5 Dinitrobenzyl anilide	0.301 ± 0.17	0.699 ± 0.08	0.477 ± 0.05	0.301 ± 0.00	0.000 ± 0.00
Methyl paraben and propyl paraben	0.301 ± 0.17	0.477 ± 0.08	0.699 ± 0.17	0.000 ± 0.00	0.477 ± 0.05
Control	0.477 ± 0.08	0.778 ± 0.01	0.845 ± 0.03	1.080 ± 0.08	1.099 ± 0.17

The test compounds 3,5 Dinitrobenzyl hydrazide and 3,5 Dinitrobenzyl anilide were more active than 3,5 Dinitrobenzoic acid and comparable to standard methyl paraben (0.699 ± 0.17), 14th day, 0.477 ± 0.05, 28th day. The results are presented in Table 6.

Conclusion

The selected derivatives were found to be effective against all selected strains and showed preservative effectiveness comparable to that of standard and even better in case *A. niger* and *S. aureus*. The study showed the preservative potential of 3,5 Dinitrobenzyl hydrazide and 3,5 Dinitrobenzyl anilide in the pharmaceutical preparation.

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