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Research Article

ASSESSMENT OF THE HARMONIZED PRESERVATIVES EFFECACY TEST IN ORAL LIQUID PHARMACEUTICAL PREPARATIONS USING REFERENCE AND NONE REFERENCE TEST MICROORGANISMS

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ABSTRACT

The microbial challenge test used for the evaluation of preservatives efficacy in none sterile liquid pharmaceutical preparations has been recently harmonized between the United States, Europeans and Japanese pharmacopoeias. This investigation reports on the assessment of this test using 2 sets of microorganisms. The first was composed of recommended strains derived from the American Type Culture Collection and the second was of clinical isolates with multi drug resistance. Testing was carried out on a prototype antacid preparation in accordance with documented methodology. It is shown that although similar results were achieved by using either sets of cultures, many of the clinical isolates persisted in the challenged product for a longer period of time. In all cases three log reductions were obtained for the challenge organisms within one week of inoculation and remained with no increase till the end of the experiment which lasted for 28 days. It is concluded that clinical isolates with multi drug resistance can be used effectively in the test as the recommended strains provided their adaptability and potentials to grow in the unpreserved product is established. The impact of this investigation on the pharmaceutical industries of the developing countries in regard to registration of new products is discussed.

Keywords: harmonized challenge test, reference organisms, clinical isolates, liquid pharmaceutical preparations, none sterile dosage form

INTRODUCTION

Preservatives are usually incorporated into none sterile drug products such as oral dosage form to control bacteria and fungi that may be inadvertently introduced during manufacture or use by patients[1]. Several techniques can be utilized to evaluate preservative effectiveness during the formulation stage of the product. These include single challenge test, multi challenge test and in use test [2,3]. The use of different concentrations of challenge inoculums' was also employed and was suggested as a predictable technique for the microbiological stability of the product over the expected shelf life [4]. However, for official use, compendial procedures should be followed.

In recent years challenge test for none sterile liquid pharmaceutical preparations has been harmonized between the United States, Europeans and Japanese pharmacopoeias [5]. The harmonization involved almost all aspects related to the test methodology including size of the inoculum, frequency of sampling, recovery media, neutralization procedure and the assessment of results [6]. It was also pointed out that a single challenge with one level of microbial count using reference microorganisms should be employed in the evaluation studies [7]. These details are valuable as they eliminate bias in results that could arise due to the use of different test procedures

Sutton[8] indicated that work done by the compendia in harmonization, assumes the equivalence of test strains from the various culture collections while, in reality this claim of equivalence might not be true beyond the culture collection catalogues. The same author suggested that it would be useful to have current confirmation that isolates from different collections are in fact, the same. Friedel [9] questioned the value of using reference organisms in the challenge test and argued that these organisms have been maintained in cryogenic storage for several decades and therefore, they may have lost the toughness of environmental isolates which can resist the action of antimicrobial agents.

Reference strains in use today, were included for the first time in the USP [10] of 1970 upon recommendations from the Pharmaceutical Manufacturer's Association [11]. Had other organisms been proposed, most probably they would have been adopted instead of those employed at present. In order to establish the persistence of the activity of the challenge organisms, the Phenol Coefficient and the Antimicrobial Resistance tests were then used. The harmonized requirements abandoned these tests and stipulated that all stock cultures should be used within five passages from the original repository stock. This point is difficult to achieve by many pharmaceutical manufacturers in certain countries where national collections of type cultures are not available. This is due to tedious regulations imposed recently on the movement of microbial cultures across boarders.

This investigation was undertaken to establish the difference between the results of the harmonized challenge test using two sets of test organisms. The first was composed of clinical isolates of *Pseudomonas aeruginosa, Staphylococcus aureus* and *candida albicns* (the bacteria were multi antibiotic resistant). While the second was composed of organisms derived from the American Type Culture Collection.

MATERIALS AND METHODS

A prototype antacid containing 36 gram aluminium hydroxide gel, 7 g mannitol, and 0.05 g saccharin was aseptically prepared in 100 ml sterile purified water. It was preserved with 0.2 % (weight / volume) methyl and 0.03 % (w/v) propyl parabens. Both preservatives were dissolved in 1 ml alcohol before adding to the preparation. Other two similar prototype products were prepared; one was devoid of preservatives and the second contained half the amount of preservatives employed above. The final pH of all preparations was 8.1.

Products contamination testing

The absence of microbial contamination from the prototype preparations was established as described in the United States Pharmacopoeia [5]. In brief, 1 ml aliquot of each product was separately inoculated into a flask containing autoclave sterilized Casein Soya Bean Digest (CSBD) broth supplemented with 0.5 % Polysorbate 80 as preservatives neutralizer, incubated at 35 ºC for 48 hours before a loop full of the flask content was taken and streaked onto a plate of CSBD agar. These plates were incubated for 48 hours at 35 ºC before the presence or absence of grown colonies was noted. Positive control composed of the prototype product devoid of preservatives and inoculated with 10^4 colony forming unit (CFU/ ml) of *Pseudomonas aeruginosa* ATCC 9027 was processed along with the samples.

Microbial cultures used

Staphylococcus aereus ATCC 6538 and *P. aeruginosa* ATCC 9027 in addition to *Candida albicans* ATCC 10231 were employed in the

challenge test. Another set of similar organisms isolated from clinical specimens were obtained from the Medical Diagnostic laboratories (Zarqa- Jordan) and were also used. The bacterial isolates were resistance to various antibiotics and were chosen for this work due to the possible linkage between bacterial resistance to preservatives and antibiotics[12-14]. Each culture was independently inoculated into an aliquot of the prepared prototype antacid (unpreserved) to allow for adaptation and this process was repeated for three consecutive subcultures. Recovered organisms from the third subculture were used to construct the growth curve for each isolate in the prototype preparation over a period of five days.

Preparation of Inoculums

Each microbial culture was separately inoculated into CSBD plate, incubated for 24 hours at 32 ºC before grown colonies were harvested with sterile saline. This suspension was standardized using McFarland solution and spectrophotometry to contain 2 x 10^8 Colony Forming Unit / ml as described by Sutton [15]. Cand*ia albicans* was grown at 25 ºC fore 48 hours prior to harvesting as for the other cultures.

Challenge test

The test procedure was similar to that described in the harmonized challenge test and it involved the inoculation of 2 x 10^6 CFU of the test organism (0.1 ml of the second 10 fold dilution prepared from the original cell suspension) into 20 g of the preserved antacid prototype. Inoculated aliquots were incubated in a shaking water bath at 35 ºC for bacteria and 25 ºC for *C. albicans*. Samples were aseptically removed at day 0, 7, 14, 21 and 28 for viable counting using buffered sodium chloride peptone solution (supplemented with 0.5 % polysorbate 80 as preservatives neutralizer) and the pour plate technique. Un-inoculated sample of the test preparation was treated as above to serve as a negative control, whereas, aliquots of CSBD broth inoculated with test bacteria were included to serve as a positive control. The product was considered as adequately preserved when 99.9 % (three log) reduction of the initial inoculums' count was obtained on the 7th day of incubation and remained with no increase up to the 28th day of the experiment. Microbiological culture media used were all derived from Difco – USA. In separate experiments the same tests were performed using antacid prototype with half the amount of preservatives and the use of different concentrations of challenge organisms.

Neutralization efficacy

The prototype antacid suspension used in the test was challenged separately with each tested organism (ATCC cultures and the clinical isolates) to give counts of 10^3 CFU / ml. One ml of the challenged sample was diluted with 9 ml buffered sodium chloride peptone solution with preservatives neutralizers as given above. After one hour, further 10 fold serial dilution using similar buffer were made. All dilutions were kept at room temperature for additional 30 minutes to allow preservative neutralization to occur. Recovery of at least 50 % of each test bacteria on SBCD agar or *C. albicans* on SDA indicated the effectiveness of the neutralization procedure.

RESULTS

Contamination testing of the prepared prototype antacid preparations indicated that all were free from any contaminants. Figure 1 demonstrates the ability of the clinical isolates to grow in the prototype antacid which was free from any preservative. Although it is evident that none of the used cultures lacked the ability to grow in this product, *Pseudomonas aeruginosa* was the most prolific while *C. albicans* was the least.

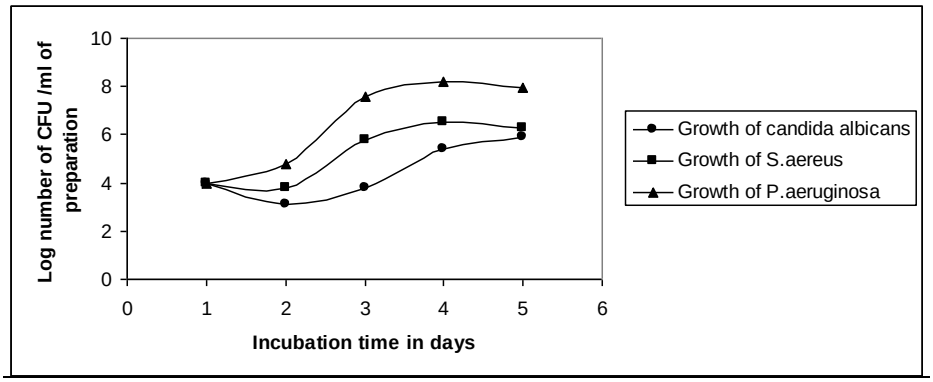

Figure 1: Growth of clinical isolates (*P. aeruginosa, S. aereus* and *C. albicans*) in a prototype antacid preparation devoid of any preservative system.

Results of challenge test conducted with all ATCC cultures indicated that 3 log reductions in the number of the inoculated test organisms was obtained on the seventh day of inoculation and remained without increase till the end of the experiment (Table 1). It is clear from this table that the tested preparation was self sterilizing for *C. albicans* ATCC 10231, as after 1 week of challenge, no survivors could be detected while more than three log reduction was obtained for the bacterial strains used in the test and these survivors did not increase in number through out the period of incubation but in the contrary they were not detectable after 28 days of inoculation. It is important to mention that plate counts were considered as valid only when the number of colonies on the plate exceeded 30 as results of lower counts were not reproducible. When counts below this figure were obtained, they were reported as low to count (LTC).

Table 1: Survival of various reference organisms inoculated into adequately preserved prototype antacid preparation in high challenge level.

Challenge organism	Number of survivors (CFU/ ml) over the incubation period				
	Zero time	7 days	14 days	21 days	28 day
P. aeruginosa ATCC 90279	10^5	65	LTC*	0	0
S. aureus ATCC 6538	10^5	LTC*	0	0	0
C. albicans ATCC 10231	10^5	0	0	0	0

*** Low to count in a reproducible manner**

Table 2 demonstrates the inhibitory effect of the prototype antacid (with preservatives) against all clinical isolates. It is worthy to note that the tested preparation exhibited sterilizing effect against *C. albicans* within 7 days of inoculation whereas, the same effect was recorded for *S. aereus* after 21 days. *P. aeruginosa* was reduced in number by more than 3 logs and was not detected on the 28th day of the experiment.

Table 2: Survival of various clinical microbial isolates inoculated into adequately preserved prototype antacid preparation in high challenge level.

Challenge organism	Number of survivors (CFU/ ml) over the incubation period				
	Zero time	7 days	14 days	21 days	28 day
P. aeruginosa	10^5	80	45	LTC*	0
S. aureus	10^5	63	LTC*	0	0
C. albicans	10^5	0	0	0	0

*** Low to count in a reproducible manner**

The prototype antacid prepared with half the strength of parabens could not withstand the challenge test performed using high microbial inoculums of the clinical isolates (table 3), but the case was different when the product was challenged with low numbers of the same organisms (table 4). The effect of the severity of the challenge can be extrapolated from both tables. Preservatives neutralization studies demonstrated that the neutralizer employed was effective as it was always possible to recover more than 50 % of the inoculated organisms.

Table 3: Survival and growth of various clinical microbial isolates inoculated into partially preserved prototype antacid preparation at high challenge level.

Challenge organism	Number of survivors (CFU/ ml) over the incubation period				
	Zero time	7 days	14 days	21 days	28 day
P. aeruginosa	10^5	7×10^3	2×10^3	1×10^3	4×10^3
S. aureus	10^5	6×10^2	4×10^2	2×10^2	10^2
C. albicans	10^5	2×10^2	LTC*	0	0

*** Low to count in a reproducible manner**

Table 4: Survival and growth of various clinical microbial isolates inoculated into partially preserved prototype antacid preparation at low challenge levels.

Challenge organism	Number of survivors (CFU/ ml) over the incubation period				
	Zero time	7 days	14 days	21 days	28 day
P. aeruginosa	10^3	2×10^2	3×10^1	LTC*	LTC*
S. aureus	10^3	7×10^1	LTC*	LTC*	LTC*
C. albicans	10^3	0	0	0	0

*** Low to count in a reproducible manner**

DISCUSSION

The preservation efficacy test agreed upon by the Americans, Europeans and Japanese pharmacopoeias was evaluated using three recommended test microorganisms and 3 clinical isolates. Findings obtained revealed that using either sets of organisms, results were comparable to each other. This is important as it indicated that reference cultures and none reference organisms could be used effectively in the challenge test.

The preparation which was investigated in this study was an antacid and this was chosen due to the fact that alkaline liquid pharmaceuticals are amongst the most difficult to preserve [16]. Table 1 demonstrates that when the prototype antacid was separately inoculated with the reference ATCC cultures, several survival patterns were recorded. *C. albicans* were not detected after 7 days of inoculation while *S. aereus* was not isolated after 14 days. The only organism which lost its viability after 21 days was *P. aeruginosa* but all organisms exhibited more than three log reduction after 7 days of inoculation. The rapid kill of *C. albicans* in the preparation tested could be explained on the basis of product alkalinity (pH 8.1) which is not conducive for the survival of this organism. On the other hand, the longer survival of *P. aeruginosa* than *S. aereus* is anticipated as the latter is more demanding in regard to nutritional requirement as compared to the former one.

Table 2 illustrates that the clinical isolate of *C. albicans* was the only challenge organism which exhibited identical death rate to the reference strain, whereas the absolute absence of *S. aereus* and *P. aeruginosa* required additional 1 week as compared to the reference strains. Although, slight variation in the kill rate was noted, between reference and none reference organisms, the interpretation of the test results could lead to the same conclusions. The outcome of the test is the appropriateness of the preservative system of the antacid preparation in coping with challenge organisms regardless of being reference or otherwise.

The severity of the challenge to which a liquid pharmaceutical preparation is exposed, determines the persistence of its preservative activity. Table 3 shows that all clinical isolates when inoculated into the inadequately preserved antacid in high numbers, they all survived and no 3 log reduction was noted after seven days of challenge. Table 4 demonstrates that when the partially preserved product was challenged with 10^3 CFU / ml, *C. albicans* was killed in 7 days whereas the bacteria remained detectable up to the 28th day of incubation. Abu Shaqra and Husari [4] demonstrated that while a product could be bactericidal to a small microbial challenge, it can be bacteriostatic or even nutritional to a larger challenge level. However, these authors used a recommended strain of *P. aeruginosa* (ATCC 9027); their results are in agreement with those given in the above tables, despite the use of none recommended strains in the experiment.

It has long been suggested that for a meaningful challenge test, strains must be chosen for their high level of resistance to antimicrobial agents including preservatives [17]. However, antibiotics and preservatives resistance profiles of the contaminants of pharmaceutical products are hardly available in scientific literature; such a literature is found in relation to cosmetics. Osungunna *et al.*[18] demonstrated that 14 isolates recovered from commercial creams and lotions were with multi antibiotic resistant whereas, Flores *et al.*[19] showed that microorganisms resistant to preservatives were capable of deteriorating cosmetic products. The bacterial cultures employed in this work were clinical isolates with multidrug resistance and as results illustrated they were capable of survival in the tested product 7 days more than the reference strains. This is tangible evidence that these isolates were more resistant to the action of parabens by which the tested preparation was preserved than the reference cultures. This is consistent with observation reported by Ferrarese *et al.* [20] who found that environmental and bacterial contaminants of cosmetics showed higher resistance to preservatives than ATCC strains.

Other points which should be considered in choosing the challenge organisms are their ability to adapt and grow in the product under test. This is critical to ensure that inhibition occurs as a result of the stress posed by the preservative system and not due to physical and chemical factors excreted by the product environment. These factors were taken into account and the clinical isolates were allowed to

pass through several subcultures in the product before they were used in the final test. Figure 1 demonstrates that all clinical isolates were capable of proliferating in the prototype antacid in the absence of growth restraints and thus, their inability to grow in the same product when it was supplemented with preservative must have been due to the efficacy of preservation.

It is true that reference strains in the challenge test can decrease bias in results obtained by different laboratories but it is obvious that this is secondary to the primary goal of indicating the microbiological stability of the product. Therefore, the use of organisms with possible resistance to preservative systems should be used as they might give more meaningful results than the reference strains. Such organisms can be isolated from the manufacturing environment, raw materials, water used in formulation in addition to organisms recovered from experimental batches. This investigation has added clinical isolates with multi drug resistance as possible candidates in testing the microbiological stability of liquid pharmaceutical preparations.

In countries where own pharmacopoeias are not present, regulatory bodies rely on guidelines derived from international compendia's, and since for registration purposes challenge test is required, the use of reference cultures becomes mandatory. Constant update of reference microbial cultures is a subject of concern to drug producers as the harmonized preservative efficacy test procedure demands the use of these organisms within five subcultures. Many companies may find this stipulation difficult to achieve and probably use reference organisms that have undergone unlimited numbers of subcultures and in this case, challenge test might be performed using strains thought to be reference, while in fact they are not. It is therefore proposed that, in developing countries where recent update of culture collection is not always feasible, the use of wild test strains should be encouraged and if certain about the authenticity of the reference strains, then why not to be included in the test.

Pharmaceutical companies in America and probably in many developed countries are requested to contribute with their ideas regarding any change in compendial methods or standards [21-23]. This is typically what happened during the preparatory stage of the harmonized microbiological limits which started in the nineties of the last century[24]. The continuous demands of the concerned companies to modify the agreed upon drafts have resulted in the delay of its implementation[25]. Even soon after approval of the harmonized limits, calls for revisions and further clarifications were requested [26]. Because many of the pharmaceutical companies in the developing countries are negatively affected by the harmonized preservative efficacy test, particularly the choice of challenge organisms, it is thought that a statement should be included in future revisions to allow the use of wild organisms provided that their competence for such work is experimentally proven.

CONCLUSION

Results presented in this communication provided strong evidence to the suitability of using microbial cultures other than those recommended by the harmonized challenge test procedure. Criteria for the selection of appropriate test organisms include adaptability and ability to grow in the tested product devoid of preservatives.

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