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Developing a Test Method for Determining the Effectiveness of Antimicrobial Preservatives

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Abstract: Antimicrobial preservatives are commonly used in cosmetic products in order to reduce their microbial contamination. The effective concentration of these products is one of the most critical points in formulation of cosmetics. There are some standard test methods for determining the efficiency of antimicrobial preservatives in cosmetics and even in other products such as wood. No standard test method was found for determining the effectiveness of antimicrobials before adding to the final product in our searches. Minimum Inhibitory Concentration (MIC) method described for antimicrobial drugs was considered as a supportive tool for establishing a practical and proper method for preservatives. We assessed the differences between antimicrobials which are used as drugs or preservative and made some modification in the basic method. The modified method was use by 3 laboratories on the same samples and results with slightest differences showed that it might be practical as a standard test method. Further studies on reproducibility and repeatability are to be investigated.

Key words: Antimicrobials, preservatives, effectiveness, Minimum Inhibitory Concentration (MIC)

INTRODUCTION

Since, recent times, chemical compounds have been used to preserve different types of products. Among the products are microbiologically safe and stable. Antimicrobial preservatives are used in products which should be preserved, cosmetics and toiletry are much important, because of their nature that is sensitive to microbial contamination, location of their use, such as eye or damaged skin and long period of use. In addition to their principal ingredients of oil and water, cosmetics often also contain such substances which provide a source of carbon for microorganisms and proteins derivatives which provide the source of nitrogen for them.

The objective of cosmetic preservation is to ensure that reduce the likelihood of microbial growth in aqueous systems and to reduce the chance of microbial survival in anhydrous products that may be contaminated or moistened during use. Repeated application of some products such as creams, shampoos or eye products may cause them to be contaminated with microorganisms. There was a dramatic increase in usage of preservatives in recent years. Scientists and formulators have been aware of risks of preservative, they try to decrease its usage in products.

An ideal preservative should have certain characteristic. It should have a broad spectrum of activity against organisms and be compatible with different ingredients of a product and its packaging. The preservative should be active in the complete formulation with its lowest concentration and be effective and stable over the range of pH values normally encountered in cosmetics. The safety of preservatives is always the most important characteristic of a preservative. Since, these are biologically active products, they all have potential for being toxic, or irritating or sensitizing. The cost of a preservative is essential for manufacturers, so they would like to use as low as active ingredients (Brannan, 1995; Kabara, 1996; Mitsui, 1997).

In order to maintain control over preservation during use, manufacturers should consider alternative forms of packaging which will help prevent product contamination under normal conditions of use. The goal of preservative efficacy testing is to select the most appropriate preservative as well as the minimum concentration of it in a formulation (Steinberg, 1996).

The goal of the formulators is to have the minimum amount but most effective preservation system in their formulations. In order to achieve such a goal, they should know the minimum inhibitory concentration of the preservatives against certain group of microorganisms, before adding it to the formulation (Brannan, 1997; Pharmacopeia, 1998; USP, 1998).

For determining the efficacy of preservatives in the formulation, there are different test methods such as challenge test, determining D value and capacity test. We

have established the challenge test for determining the efficacy of preservatives in cosmetic products as a national standard (ISIRI 5874, 2003).

In current study we tried to establish a standard test method for determining the effectiveness of preservatives before introducing them to the formulation. Although, in most credentials the MIC of preservatives against variety of microorganisms has been mentioned, we have found no standard test method. Researchers and formulators need to have a certain test method in order to inspect the effectiveness and efficiency of purchased preservatives as fast as possible. For that reason we focused our efforts on developing a standard test method for determining the effectiveness of preservatives based on the MIC method described for antimicrobial drugs, but the conditions were optimized for antimicrobial preservatives.

MATERIALS AND METHODS

Samples: Samples consisted of 2 commercial preservative, containing following active ingredient as stated by the manufacturers (the commercial names are reserved):

- Parabens.
- Isothiazolinones.

The samples were prepared as recommended by the manufacturers.

Test organisms:

- *Staphylococcus aureus* PTCC 1112 (ATCC 6538).
- *Escherichia coli* PTCC 1338 (ATCC 8739).
- *Pseudomonas aeroginosa* PTCC 1074 (ATCC 9027).
- *Candida albicans* PTCC 5027 (ATCC 10231).
- Aspergilus niger, isolated from a food sample in the laboratory for food microbiology and identified by Iranian national standard 997 (ISIRI 997, 1995).

Test method: The MIC test method according to the method described in CLSI was chosen as the supportive method. In order to find if the method with the modifications is applicable in different laboratories, we used it in 3 laboratories at the same time, with the same materials and on the samples as well. The modifications were as following:

Antimicrobial dilutions

Stock preparation note: Since, 1 mL of microorganism suspension was to be inoculated to each concentration and the final concentration in each tube would be half of what had been predicted, each tube should contain double strength of the desired concentration. Therefore, in the following steps, each concentration should be multiplied by two (CLSI, 2005).

The stock solution was prepared by diluting the antimicrobial preservative in water or other solvents as recommended by the manufacturer. If the solvents other than water were needed, the solvent was added to water as much as needed to solve the antimicrobial powder. The stock solution should contain 2 fold of the highest concentration to be tested. According to the test methods for determining the efficacy of antimicrobial disinfectants and antiseptics, the highest concentration was suggested to be 2 fold of the amount recommended by the manufacturer, or allowed by the authorized references, whichever was greater (Steinberg, 1996).

In the case of first sample contained parabens as active ingredient, 0.3-1% was recommended; we have chosen 1% as the highest concentration. In the case of the second sample which contained Isothiazolinones as active ingredient, 1.5±0.1% was recommended by the manufacturer, we used 1.6% as the highest concentration.

The stock solution for the first sample was prepared by adding 8 mL of the preservative to 100 mL of solvent. Since it was rarely soluble in water and soluble in ethanol, it was dissolved in a little amount of ethanol and then water was added to 100 mL. The given concentration of stock solution was 8%.

The stock solution for the second sample was prepared by adding 6.4-100 mL water. Since this preservative system is highly soluble in water, there was no need to use any other solvent other than water. The given concentration of stock solution was 12.8%.

Number of concentrations tested: Serial dilutions for the particular antimicrobial preservatives contained four concentrations as a minimum requirement. These are the highest concentration containing 2 fold of the maximum amount to be tested, the maximum amount as well as at least 2 concentrations lower than that to the concentration which is half of the maximum amount, according to the standard test methods for antimicrobial disinfectants (CLSI, 2005).

Preparing the serial dilutions: The required volumes of broth depending on the desired dilutions were distributed into the tubes. The first dilution (the highest concentration) was made by adding equal volume of stock solution to the broth in the first tube. The further dilutions were made by adding 1 mL of previous dilution to the next tube containing 1 mL broth (where 1:2 was to be used) or the calculated volume of previous dilution to the next tube containing the required volume (for example: 1.5 mL of

previous dilution to 2 mL broth, in case of 3:4). It was considered that final concentration of antimicrobials in each tube after adding 1 mL microbial suspension would be half of which it contained.

The final volume of broth and antimicrobial preservative in each tube should be 1 mL, so the excessive amounts were discarded.

The dilutions for the first sample were as following:

- 2, 1, 0.75, 0.5, 0.3, 0.15, 0.075 and 0.05%

The dilutions for the second sample were as following:

- 3.2, 1.6, 1.2, 0.8, 0.4, 0.2, 0.1, 0.075 and 0.05%

While 5 test organisms were to be tested, 5 series of each antimicrobial dilution should be prepared.

Preparing test organism suspensions: The lyophilized microorganisms obtained from culture collections were reconstituted according to the manufacturer's instructions and stock cultures were prepared. Working cultures were prepared from the stock cultures. Overnight cultures of bacteria at 30°C were prepared using working cultures on TSA, whereas for yeast a longer incubation time (48 h) at 25°C was needed (DIN EN 12353, 2006). As soon as the fungal spores were formed on SDA (after 5-7 days incubation at 25°C), they were harvested for the test (ISIRI 5874, 2003).

The suspensions of test organisms were prepared in peptone water using colonies taken from the overnight cultures. The absorbances of suspensions were adjusted to 0.08-0.1 in 625 nm using a spectrophotometer with a 1 cm light path and matched cuvette. In this turbidity the bacterial suspensions contain about 1×10^8 cells mL^{-1} and suspension of *Candida albicans* contains about 1×10^7 cells mL^{-1} (ISIRI 5874, 2003).

Number of fungal spores was adjusted to 1×10^4 mL^{-1} using Heamocytometer slide (ISIRI 5874, 2003).

Counting test organisms: For verifying the number of cells in suspensions, they were counted using pour plate method. In order to perform counting, certain dilutions of each suspension was made. Generally dilutions up to 10^{-6}-10^{-7} which yields a countable number of colonies were prepared. One milliliter of last dilutions (it means: 10^{-5}-10^{-7}) were inoculated in Petri dish, after adding appropriate volume of culture media (TSA) and mixing well, the agar plates were incubated at the given temperature (30°C for bacteria and 25°C for yeast) for adequate time (24-48 h for bacteria and 48-72 h for yeast). After incubation the number of organisms per mL was calculated according to the related national standard (ISIRI 2325, 2000).

Inoculating into the antimicrobial dilutions: One milliliter of a 10^{-2} dilution of each bacterial suspension and 10^{-1} dilution of yeast (contains about 1×10^6 CFU mL^{-1}) were added to each series of tubes containing preservative dilutions. After inoculation, each tube contained 0.5×10^6 CFU mL^{-1} of test organisms (ASTM, E., 640, 1978; CTFA, 1993; ISIRI 5874, 2003).

As same as above, 1 mL of suspension containing fungal spores (contains about 1×10^4 spores mL^{-1}) was added to the fifth series of tubes containing preservative dilutions. After inoculation, each tube contained 0.5×10^4 spores mL^{-1} (Kabara, 1996).

Using controls: One mL of a 10^{-2} dilution of each bacterial suspension and 10^{-1} dilution of yeast (contains about 1×10^6 CFU mL^{-1}) were added to tubes each one containing 1 mL broth without antimicrobial preservative. These tubes were growth control (positive control).

Each series contained a control negative which was consisted of a tube containing a certain amount of antimicrobial agent and broth without test organisms.

Incubating the test tubes: All of antimicrobial tubes containing bacteria were incubated at 30°C for 24 h. The antimicrobial tubes containing yeast and fungal spores were incubated at 25°C for 48-72 h, which depends on observing growth in the control positive tube (ISIRI 5874, 2003).

RESULTS

The MIC was considered the lowest concentration of antimicrobial in which, growth of test organisms was completely inhibited. The growth inhibition was detected by the unaided eye. The growth in the inhibited tubes was compared with the positive and negative controls (CLSI, 2005).

Determining the cidal activity of antimicrobials (MBC): In order to determine the bactericidal, yeasticidal or fungicidal concentrations of antimicrobials, from the MIC and the next concentrations were inoculated onto the TSA plates. Plates inoculated by bacterial MIC were incubated at 30°C for 24 h and plates of yeasts and fungi were incubated at 25°C for 48-72 h. Growth on the surface of plates was assessed. The plates related to the lowest concentration, on which no growth had been observed, were considered as MBC. While inhibition of growth in

Table 1: MICs resulted from collaborative study in 3 laboratories

Laboratory	Test microorganism/sample	*Staphylococcus aureus* PTCC 1112 (%)	*Escherichia coli* PTCC 1338 (%)	*Pseudomonas aeroginosa* PTCC 1074 (%)	*Candida albicans* PTCC 5027 (%)	*Aspergilus* Niger
1	Parabens	0.075	0.075	0.15	0.075	0.15
1	Isothiazolinones	0.05	0.05	0.1	0.075	0.075
2	Parabens	0.075	0.075	0.15	0.075	0.15
2	Isothiazolinones	0.05	0.05	0.1	0.075	0.1
3	Parabens	0.075	0.075	0.15	0.075	0.15
3	Isothiazolinones	0.05	0.05	0.1	0.075	0.075

cosmetics is enough and killing microorganisms are not required, determining the MBC is not necessarily needed (CLSI, 2005).

Antimicrobial preservative dilutions: The MICs resulting from the laboratories participating in the study are shown in Table 1.

Results showed that there were no significant differences between MICs obtained by different laboratories. This method with the mentioned modifications could be used for determining the antimicrobial preservatives MIC. According to the results, a national standard was established for determining the MIC of preservatives used in cosmetics, while it could be applied for food preservatives.

DISCUSSION

Because of drastic differences between antibiotics and antimicrobial preservatives, the following modifications were made in the CLSI method. The results have shown that the method is an applicable method for determining the minimum inhibitory concentration of antimicrobial preservatives used in cosmetics and food as well. The modifications have been discussed as following:

Culture media: In CLSI Mueller-Hinton broth is recommended as the medium for susceptibility testing: but since this medium is not a routine culture medium in laboratories for cosmetic microbiology, we use Tryptic Soy broth instead of Mueller-Hinton broth. It has been shown for years that Tryptic Soy broth yields satisfactory growth of most microorganisms which may be existed in food, cosmetic or health care products (Merck Microbiology Manual, N.D), as well as it supports the growth of many fastidious microorganisms. In addition, it has no inhibitory ingredient that may interfere with the antimicrobial preservatives.

Test microorganisms: The antimicrobial preservatives used in cosmetics and toiletries should inhibit the growth of microorganisms which may exist in these products, the health indexes were chosen as test microorganisms. Since, this method should be used as a standard method, certain test microorganisms should be introduced to the users of this standard method. So, we have chosen *Staphylococcus aureus* as representative a gram positive group, *Escherichia coli* from fermentative gram negative group, *Pseudomonas aeroginosa* from non fermentative gram negative group, *Candida albicans* as the representative of yeasts which may exist in cosmetics and *Aspergilus niger* as a contaminant fungus. All of strains were ones which are used for antimicrobial tests and taken from known culture collections.

Number of cells per mL of antimicrobials was 0.5×10^5 as described in CLSI. Since, antimicrobial preservatives may encounter high contaminations in cosmetic products and while in challenge test in cosmetics the antimicrobials challenged with 10^6 cells mL^{-1} of microorganisms, we used the same number of test organisms instead of 10^5 CFU mL^{-1} (ASTM, E., 640, 1978; CTFA, 1993; DIN EN1656, 2000; DIN EN1657, 2005; DIN EN 12353, 2006; ISIRI 5874, 2003).

Incubation temperature: As described in CLSI, the tubes containing antimicrobials and microorganisms suspension should be incubated in 35°C for 16-20 h. This temperature is suitable for pathogens, but in cosmetic microbiology, the pathogens are not necessarily be detected, instead the microorganisms that may grow in the product or are cause of spoilage, are to be investigated, so the elevated temperatures are not needed. We used the incubation temperature 30°C for bacteria and 25°C for yeasts and fungi (DIN EN1656, 2000; DIN EN1657, 2005; DIN EN 12353, 2006; ISIRI 5874, 2003).

Antimicrobial serial dilution: As described in CLSI, the stock solutions are to be prepared at concentrations of at least 1000 μg mL^{-1} of antimicrobial agent (for example: 1280 μg mL^{-1}) or 10 times the highest concentration to be tested, whichever is greater. The first concentration in our test was the antimicrobial preservative concentration recommended by the manufacturer. In the cases that such a statement was not existed, we used the maximum amount for each preservative which was allowed according to the national laws and regulations, or international or other

certified references. One of the most important differences between antimicrobial drugs and antimicrobial preservatives is the amount used by consumers. In the case of antibiotics a 1:2 dilution of the drug is prepared, but in the case of preservatives, the 1:2 dilutions may not be practically applied, so we prepared the dilutions by the ratios other than 1:2. In this way, we had dilutions such as 2:3 or 3:4; therefore, there were fewer differences between the concentrations and formulators can benefit from fewer changes (Pharmacopeia, 1998; Steinberg, 1996; USP, 1998).

CONCLUSION

The amount of preservative which should be added to the final formulation is important not only for health of consumers, but also regarding its costs for the manufacturer. This standard test method could be applied to determine the minimum amount which might be added to the formulation and helps cosmetic manufacturers and researchers to use the proper and adequate amounts of antimicrobials. The further investigations on reproducibility and repeatability of this method are to be investigated.

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