Preservative Efficacy Testing: Accelerating the Process

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ABSTRACT: *A preservative efficacy testing method, called the accelerated double challenge, can assesses the ability of a product or material to resist microbial contamination in only 14 days. It also maintains a high degree of correlation with longer-term preservative challenge protocols.*

The accurate and reproducible determination of the degree of microbial contamination resistance of preserved personal care formulations is a critical element in the development of safe and effective consumer products. Various approaches to preservative efficacy testing (PET) have been developed throughout the years by regulatory agencies, standards organizations, industry organizations and individual companies. Of the various protocols and approaches proposed and developed, the microbial challenge test has evolved as the most commonly used and accepted evaluation criterion.

The fundamental principle of the microbial challenge is based on the concept of measuring the survival ability of selected microorganisms that are purposely introduced into a preserved test product system. Conventional PETs or preservative challenge test methods generally require microbial assays at multiple test points throughout extended periods of time. Test durations typically range from a minimum of 28 days to 12 or more weeks.

The prolonged four-week test cycle originally evolved via the United States Pharmacopeia (USP) for application in the pharmaceutical industry. It has since been adopted in one form or another for evaluating the efficacy of preservatives in cosmetic and other consumer product formulations. The belief is that by extending the test time, slow-growing or preservative-damaged microorganisms would have ample opportunity to recover and grow if they were capable, according to a revitalizing phenomenon known as "the phoenix effect."

> Late detection of a potentially problematic issue could ultimately have a major negative impact on the formulation development process.

Based on considerable research and the accumulation of extensive comparative test data during the past 10 years, the current authors have determined that these conventionally held concepts and beliefs regarding preservative test duration may not be the only reliable approach for measuring the microbial resistance of preserved formulations. This paper will present an alternative testing approach using a conventional microbial challenge technique that is capable of reducing the test cycle time from four weeks to 14 days without the loss of sensitivity or impeding the predictability of long-term preservative efficacy effects. Because standard microbiological techniques similar to those employed in longer-term generic challenge protocols are involved, no special equipment or training is necessary to perform the assay. In fact, this accelerated double challenge (ADC) assay is currently being used in its basic form or in variations at numerous laboratories for a variety of applications (see **Current ADC Applications**).

CURRENT ADC APPLICATIONS

Currently, the ADC method or one of its variations is being applied at more than 40 companies worldwide to evaluate a variety of cosmetic, personal care, household and industrial products at various stages of product development:

- New product preservative system development
- Product preservative stability studies
- Package compatibility studies
- Batch scale up or pilot batch evaluation
- Process modification preservative effects
- Formula change/modification effects
- Post in-use study preservative evaluation
- Alternative ingredient preservative effects
- Verification of preservative system integrity
- Raw ingredient susceptibility studies

Table 1. Method requirements

Method	Test Duration	Sampling Times
AOAC	28 days	7, 14 and 28 days
CTFA	28 days or 56 days*	2, 7, 14, 21 and 28 days
ASTM	28 days or 56 days*	7, 14, 21 (optional) and 28 days
USP	28 days	14 and 28 days
JP	28 days	14 and 28 days
EP/BP	28 days	2, 7, 14 and 28 days
ADC	14 days	1, 3, 7 and 14 days

*Additional 28 days for reinoculation

Standard PET Methods

Standard PET methodologies have been developed by numerous organizations and countries, including the Cosmetic, Toiletry, and Fragrance Association (CTFA), the American Society for Testing and Materials (ASTM), the Association of Analytical Communities (AOAC), Japanese Pharmacopoeia (JP), European Pharmacopoeia/British Pharmacopoeia (EP/BP) and the USP.

Duration: These standard methodologies recommend PETs that require minimum test durations of 28 days after inoculation. In the case of the CTFA and ASTM methods, an additional 28 days (i.e., a 56-day test duration) is proposed if a reinoculation step is included. The test duration requirements and suggested test sampling times for each of these standardized methodologies are presented in **Table 1**.

Although the sequence of individual test days may vary somewhat from protocol to protocol, the key indicator points where specified reductions are required are essentially the same; i.e., 7 and/or 14 days with no increase in the recovered number of microorganisms within 28 days. In terms of test applicability to specific product forms, the pharmacopoeia methods generally have application for the full range of parenteral, ophthalmic, topical and oral preparations.

Application: The AOAC, ASTM and CTFA methods are somewhat more restricted in their application. The CTFA method is recommended for the evaluation of water-miscible topical cosmetics, toiletries and eye-area products, as well as for a number of OTC formulations such as sunscreens and antidandruff preparations.

The AOAC protocol, on the other hand, specifies application for "non-eye area, water-miscible cosmetic and toiletry formulations" only. No recommendation is suggested for eye-area preparations, although there does not appear to be any significant reason why the method could not be applied to such formulations. It is possible that the method has not been validated for those product forms or that the proposed criteria of acceptability may not be applicable.

The ASTM method, which has recently been re-approved, was designed primarily to determine the suitability of preservatives for use in cosmetic products. Although it was not specifically developed for the evaluation of preservative effectiveness in cosmetic products, it has been used for this purpose.

Organisms: In regard to the organisms recommended for inclusion in the various preservative efficacy tests, all, with the exception of the EP/BP protocol, include the same five indicator organisms: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Aspergillus niger*. The EP/BP excludes *Escherichia coli*. The CTFA, AOAC and ASTM methods that are only applicable to cosmetic formulations go one step further and suggest a variety of additional microorganisms and, if appropriate, environmental isolates.

The AOAC method specifies pooling groups of like organisms for inoculation whereas the CTFA and ASTM methods allow either inoculation of product with individual challenge organisms or inoculation of test product with compatible mixed cultures. The various challenge organism options for each of the specified methodologies are presented in **Table 2**.

In-house PET Methods

Although a number of cosmetic and personal care companies have been known to use the pharmacopoeia or either the CTFA or AOAC test methodologies, the vast majority of companies have developed and adopted their own in-house PET protocols. Oftentimes, these procedures can be far more stringent than those specified in the standard methodologies and may even include simulated or real-time "in-use" preservative challenge evaluations.

In most cases, however, these methods are essentially modifications or variations of the standard PET procedures. Some of the more common differences from the standard PETs include: test points, test duration, specified challenge organisms, inoculum content/mixtures, reinoculation and criteria of acceptability. It is interesting to note that even with all of these variations of the basic theme, most protocols in use, whether they are in-house or standard methods, are capable of identifying both well-preserved products and those that have weak preservative systems. The potential issue with some of these methods generally arises in detecting formulations that have a marginal spectrum of activity, weaknesses in activity against a particular category of organisms such as mold, or have a reduced antimicrobial capacity or robustness.

With a single challenge protocol and inappropriately designated test points, these difficulties could go undetected or, at best, not recognized until very late in the testing cycle. Since the duration of many of the more rigorous in-house test protocols generally is in excess of the

Table 2. Challenge test organisms

For bacteria, the abbreviated genus names are *Acinetobacter*, *Burkholderia*, *Escherichia*, *Klebsiella*, *Pseudomonas* and *Staphylococcus*. The exceptions are *Enterobacter gergoviae* and *Enterobacter cloacae*.
For fungi, the abbreviated genus names are *Aspergillus*, *Candida* and *Eupenicillium*.

Method	Bacteria	Fungi
AOAC	1. *S. aureus*, *S. epidermidis* 2. *K. pneumoniae*, *E. coli*, *E. gergoviae* 3. *P. aeruginosa*, *B. cepacia*, *A. baumanii*	*A. niger*, *C. albicans*
CTFA	1. *S. aureus* or *S. epidermidis* 2. Two from: *K. pneumoniae*, *E. coli*, *E. gergoviae*, *E. cloacae*, *Proteus* species 3. *P. aeruginosa* and one from: *B. cepacia*, *P. fluorescens*, *P. putida*, *Flavobacter* species, *Acinetobacter* species	*A. niger* or *P. luteum* *C. albicans* or *C. parapsilopsis*
ASTM	*S. aureus*, *P. aeruginosa*, *B. cepacia*, *E. coli*, *E. gergoviae*	*A. niger*, *C. albicans*, *E. levitum*
USP	*S. aureus*, *P. aeruginosa*, *E. coli*	*A. niger*, *C. albicans*
JP	*S. aureus*, *P. aeruginosa*, *E. coli*	*A. niger*, *C. albicans*
EP/BP	*S. aureus*, *P. aeruginosa*	*A. niger*, *C. albicans*
ADC	*S. aureus*, *P. aeruginosa*, *B. cepacia*, *E. coli*	*A. niger*, *C. albicans*, *Penicillium* species

28-day minimum, as specified in the conventional methodologies, and in many cases as long as 12 or more weeks, the late detection of a potentially problematic issue could ultimately have a major negative impact on the formulation development process.

Chief concerns are the ability of accelerated tests to predict long-term preservative efficacy and the degree of correlation of these tests to conventional preservative efficacy methods.

PET Primary Objectives

The primary objective of any PET is to accurately and reproducibly measure the ability of a product or formulation to resist both normal and abnormal microbial insult. This objective may or may not necessarily be compatible with the preconceived notion of passing some arbitrary criteria of acceptability, such as those specified in some of the standard methodologies, but rather with the goal of ensuring that the product is properly and effectively preserved. The selected methodology should be capable of predicting both the risk potential for product recontamination as well as the long-term continued efficacy of the preservative system during its shelf and use life. In order to accomplish this, the method must encompass a means of measuring and determining two key preservative challenge predictive elements—rate of kill or death rate, and robustness or capacity to continue to resist subsequent recontamination.

Accelerated Testing Benefits and Concerns

The benefits of using an accurate and verifiable accelerated challenge protocol are numerous.[1] Reducing the time to evaluate the preservative capability of new or revised formulations allows for significantly more flexibility within the development cycle. Successes or potential failures can become obvious in a more reasonable period of time, allowing for rapid feedback to development personnel.

Data developed in a shorter time frame allows for formulation adjustments or modifications to be initiated weeks before data from longer-term testing can be made available. The selection of alternative preservatives and preservative systems can be further facilitated by allowing for many more formula variations to be evaluated over a shorter period of time. The dollar and labor savings involved in using protocols having reduced testing time can obviously be substantial[2] because decisions and product direction can more efficiently be managed.

Chief among the concerns of employing accelerated testing protocols are questions concerning the ability of accelerated tests to predict long-term preservative efficacy and the degree of correlation of these tests to conventional standard or in-house preservative efficacy methods. The issues related to slow-growing

fungi and damaged organisms also have been bothersome, as have the potential difficulties involved in properly interpreting test data.

Some published accelerated protocols introduce foreign materials to the test product system[3] or serial dilute the formulation.[4] Each of these approaches radically alters the substance and integrity of the formulation so that the test material may differ from the original test formulation in its chemical makeup or its physical makeup or both. Although these approaches may be suitable for selected product forms, there are questions about the general reliability of testing significantly modified product systems and meaningfully relating the data developed to predicting intact product contamination.

An alternative approach for rapid PET, based upon linear regression (D-values), was proposed by Orth in a series of publications.[5–6] The basic concept of this direction definitely has merit; however, the protocol addresses only rate of kill, which is only one of the two key predictive preservation test elements. Product preservative system robustness or the capacity of the product to resist recontamination does not appear to be easily measured or predicted using the D-value approach as defined.

Accelerated Double Challenge PET

In order to meet the requirements for an all-inclusive predictive accelerated PET protocol, a method must be capable of determining and measuring both the rate of kill and the robustness or resistance capacity of a product preservative system. Through extensive research, numerous method comparison studies, and real-time practical product preservative evaluations, Bio-Control has established that the accelerated double challenge (ADC) test[a] is a meaningful predictive tool that measures both kill rate and product robustness.

The method originally was developed to provide a rapid screening procedure for the identification of poorly preserved or marginally preserved cosmetic formulations. Once the accuracy and reproducibility of the process were established for generic cosmetic creams, lotions and liquids, the method was applied with appropriate variations to other product and material forms such as w/o emulsions, wet wipes,[7] household products and preserved raw materials. In all these cases, the ADC protocol rapidly—in 14 days—assessed the ability of a product or material to resist microbial contamination while maintaining a high degree of correlation with longer-term preservative challenge protocols.

The protocol: The ADC protocol allows for flexibility with regard to the use of pure cultures, mixed cultures or pooling groups of like organisms and any of these inoculation approaches can be applied to the protocol. Although the basic procedure includes the standard list of indicator organisms, the final selection of challenge organisms can vary and include environmental or product contaminants as well as other organisms that may have significance for the product form being evaluated.

In most cases, the current authors found that using separate, previously determined compatible, mixed cul-

[a] *The Bio-C Accelerated Double Challenge test*

tures of bacteria and fungi provides meaningful and reproducible results with conclusions comparable to those of pure-culture or pooled like-culture techniques and with a reduction in labor and materials.

The basic ADC protocol involves preparing aliquots of each test preparation and inoculating them with separate mixed cultures of bacteria and fungi or, alternatively, with individual pure cultures or pooled like-cultures of the selected challenge microorganisms. Test units are then inoculated at time 0 in a manner similar to that used for other recognized challenge protocols. Inoculum levels are ~10^6 for the mixed or pure bacterial cultures and ~10^5 for the mixed or pure fungal cultures.

What distinguishes the ADC protocol from other similar challenge protocols is the reinoculation of the same sample test units at seven days using the same concentrations of inoculum. The reinoculation at seven days is designed to severely stress the product preservative system in an effort to determine the product's robustness or innate ability to resist significant repetitive microbial insult.

ADC was significantly more sensitive in detecting marginally or weakly preserved formulations.

Conventional plate counts to determine survivors are performed using appropriate neutralizing diluents and recovery media on each of the inoculated test units after 1, 3, 7 and 14 days of incubation at room temperature. Evaluating the inoculated test samples at days 1, 3 and 7 is, in addition to the 7-day re-challenge, the other key predictive element and sensitive comparative indicator for determining and measuring the rate of kill or potency of a product preservative system.

The ADC method uses three data points such as 1, 3 and 7 in developing rate-of-kill trends. Use of three points enhances the reliability of the data obtained—especially in the case of non-linear death curves/rates—and allows for a more informative picture when comparing the preservative efficacy of two or more test product variations.

The total test duration is 14 days not counting an additional five days for the incubation of the fungal plates.

Results: ADC results generally are presented as the number of surviving organisms present at each time interval per gram of product inoculated.

Acceptability: Because the ADC protocol is significantly more rigorous than the standard recognized methodologies, the interpretation of the data developed may be somewhat more challenging. A tester may use any reasonable interpretive guideline appropriate to the tester's needs and the product form being evaluated, but the authors' experience with the protocol suggests the following minimum criteria of acceptability:

- Bacteria (pure or mixed cultures): recovery of <10 cfu per gram 7 days after the first inoculation plus a 5 log reduction after the second inoculation at 14 days.
- Fungi (pure or mixed cultures): a 3 log reduction 7 days after both the first and second inoculations with no increase in counts.

Reliability: Based on criteria for predicting product preservative efficacy, the conclusions drawn from the ADC-generated preservation data were essentially comparable to the conclusions drawn from data developed using the standardized methodologies. For those situations where the data or conclusions drawn did not correlate, the differences were largely a result of the greater rigorousness of the ADC method.

ADC was significantly more sensitive in detecting marginally or weakly preserved formulations. Products that were marginally preserved failed the ADC criteria of acceptability while passing the longer-term single inoculation protocols. In-house data from a number of studies on different product forms suggest that there appears to be a >99% positive correlation with the longer four-week test protocols such as the CTFA or USP. Some of those studies are described next.

Comparative Studies: USP versus ADC

The authors undertook two studies whose purpose was to verify that the accelerated double challenge preservative evaluation testing procedure was equivalent to or better than the 28-day single challenge mixed culture USP protocol for the demonstration of preservative effectiveness in multiple dosage cosmetic and OTC product forms. The selected product forms were an SPF cream in the first study and a medicated lotion in the second. Each study was conducted in triplicate.

Methods and materials: The protocols described here are procedure briefs and were to be used for the purposes of these studies only. The procedures are not intended to be detailed representations of the official protocols that they represent.

For the modified USP (MUSP) protocol, aliquots of each model test preparation were inoculated with separate mixed cultures of bacteria and fungi at time 0 only. Plate counts to determine survivors were performed at time 0 and after 7, 14, 21 and 28 days of incubation at room temperature. Results are presented as the number of surviving organisms per gram of tested product present at each time interval. Inoculum levels were ~10^6 for the individual bacteria and ~10^5 for the individual fungi. Specific inoculum counts for the inoculations at time 0 are presented later in **Tables 3** and **5**. The reader should note that USP requires sampling and testing only at days 14 and 28 for Category 2 products. For the purposes of these studies, two additional test times at 7 days and 21 days were included.

For the ADC protocol, aliquots of each model test preparation were inoculated with separate mixed cultures of bacteria and fungi. Samples were inoculated at time 0 and reinoculated at 7 days. Plate counts to determine survivors were performed at time 0 and after 1, 3, 7 and 14 days. Results are presented as the number of surviving organisms per gram of tested product present at each time interval. Inoculum levels were ~10^6 for the mixed bacteria and ~10^5 for the mixed fungi. Specific inoculum counts for both the time 0 inoculation and the 7 day reinoculation are presented later in **Tables 4** and **6**.

For the inoculata in both protocols, the following microorganisms were used:

- *Pseudomonas aeruginosa* ATCC #9027
- *Escherichia coli* ATCC #8739
- *Staphylococcus aureus* ATCC #6538
- *Candida albicans* ATCC #10231
- *Aspergillus niger* ATCC #16404.

For the MUSP protocol, individual pure cultures of each of the organisms were used. With the ADC protocol, separate mixed cultures of the same bacteria and fungi were used.

Results: The results from the comparative challenge studies conducted in triplicate are presented in **Tables 3** and **4** for the SPF cream and **Tables 5** and **6** for the medicated lotion. Microbial counts are recorded as the number of cfu's recovered per gram of inoculated test material.

Discussion of the results from the SPF cream: Based on the results obtained from the three MUSP trials (**Table 3**), the SPF cream passed the USP criteria of acceptability and appears to be adequately

Table 3. MUSP Protocol: SPF cream trials (all microbial counts in cfu's per gram of inoculated test material)

Microbe	Inoc	7 Days	14 Days	21 Days	28 Days
Trial 1					
P. aeruginosa	2,100,000	10	<10	<10	<10
E. coli	1,320,000	<10	<10	<10	<10
S. aureus	1,200,000	<10	<10	<10	<10
A. niger	210,000	720	240	<10	<10
C. albicans	320,000	600	200	<10	<10
Trial 2					
P. aeruginosa	2,100,000	<10	<10	<10	<10
E. coli	1,320,000	<10	<10	<10	<10
S. aureus	1,200,000	<10	<10	<10	<10
A. niger	210,000	610	280	<10	<10
C. albicans	320,000	560	190	<10	<10
Trial 3					
P. aeruginosa	2,100,000	<10	<10	<10	<10
E. coli	1,320,000	<10	<10	<10	<10
S. aureus	1,200,000	<10	<10	<10	<10
A. niger	210,000	590	310	<10	<10
C. albicans	320,000	640	240	<10	<10

Table 4. ADC Protocol: SPF cream trials (all microbial counts in cfu's per gram of inoculated test material)

Microbe	Inoc	1 Day	3 Days	7 Days*	Reinoc	14 Days
Trial 1						
Bacteria	2,300,000	81,000	1,500	10	1,900,000	880
Fungi	310,000	2,250	1,100	460	205,000	150
Trial 2						
Bacteria	2,300,000	51,000	1,200	<10	1,900,000	900
Fungi	310,000	1,600	900	480	205,000	100
Trial 3						
Bacteria	2,300,000	64,000	1,350	<10	1,900,000	850
Fungi	310,000	1,400	980	360	205,000	300

* Reinoculated at 7 days

preserved. A 6 log reduction in numbers was observed for each of the bacterial species within 14 days and there was no increase in microbial counts thereafter. In terms of the fungi, both the *C. albicans* and the *A. niger* demonstrated a 3 log reduction in 14 days with no increase thereafter.

The data generated in the three ADC trials (**Table 4**) demonstrated similar results at the 7-day test point; however, it also identified an apparent weakness in antibacterial preservative capacity after reinoculation. A 6 log reduction in 7 days after the first inoculation for the mixed bacteria was observed, whereas only a 4 log reduction in bacteria was apparent after the second inoculation.

Fungal reductions for the two methodologies were essentially the same with 3 log reductions demonstrated in both situations. Both protocols demonstrated excellent procedural reproducibility as was evident from the triplicate data generated. Comparatively, one could conclude that, in this particular study, the ADC protocol was more capable of defining product preservative system vulnerabilities than was the MUSP single challenge methodology.

Discussion of the results from the medicated lotion: Data generated by the three MUSP trials on the medicated lotion (**Table 5**) clearly demonstrate that this model formulation is well-preserved and easily passes the acceptability criteria for adequate preservation. A 6 log reduction in numbers was observed for each of the bacterial species within 14 days and there was no increase in microbial counts thereafter. In addition, a very respectable 4 log reduction was observed for both of the fungal inocula.

Data developed in the three ADC trials (**Table 6**) also appears to support the well-preserved status of this formulation even after the second microbial challenge. Excellent 6 log bacterial reductions were observed 7 days after each of the mixed bacteria inoculations whereas a similarly acceptable 4 log reduction was observed after each inoculation for the mixed fungi. From the additional kill time data points at days 1 and 3, there is also the suggestion that the fungal rate of kill, although acceptable, may be somewhat slow with low level survivors detectable at each test point through day 7. Depending on product recommended use characteristics and final package configuration, this slower rate of kill could be a potential vulnerability.

Conclusions: Based on the data developed during the comparative challenge testing trials to determine equivalency between the USP 28-day PET and the described 14-day ADC PET, it appears that both methods are essentially equivalent and arrive at similar conclusions in determining preservative system effectiveness for well-preserved product systems.

With marginal or reduced-capacity systems, however, it appears that the ADC PET protocol has the capability of detecting preservative vulnerabilities or potential weaknesses in efficacy that could go undetected by using only the USP methodology. This additional ability to detect and measure robustness and rate of kill can be an invaluable tool when evaluating the preservative capability of multiple dosage cosmetic and OTC product forms.

Table 5. MUSP Protocol: Medicated lotion trials (all microbial counts in cfu's per gram of inoculated test material)

Microbe	Inoc	7 Days	14 Days	21 Days	28 Days
Trial 1					
P. aeruginosa	2,100,000	<10	<10	<10	<10
E. coli	1,320,000	<10	<10	<10	<10
S. aureus	1,200,000	<10	<10	<10	<10
A. niger	210,000	50	30	<10	<10
C. albicans	320,000	10	<10	<10	<10
Trial 2					
P. aeruginosa	2,100,000	20	<10	<10	<10
E. coli	1,320,000	<10	<10	<10	<10
S. aureus	1,200,000	<10	<10	<10	<10
A. niger	210,000	60	20	<10	<10
C. albicans	320,000	<10	<10	<10	<10
Trial 3					
P. aeruginosa	2,100,000	10	<10	<10	<10
E. coli	1,320,000	<10	<10	<10	<10
S. aureus	1,200,000	<10	<10	<10	<10
A. niger	210,000	60	30	<10	<10
C. albicans	320,000	30	10	<10	<10

Table 6. ADC Protocol: Medicated lotion trials (all microbial counts in cfu's per gram of inoculated test material)

Microbe	Inoc	1 Day	3 Days	7 Days*	Reinoc	14 Days
Trial 1						
Bacteria	1,300,000	50	<10	<10	2,200,000	<10
Fungi	370,000	5,000	1,200	50	210,000	120
Trial 2						
Bacteria	1,300,000	100	10	<10	2,200,000	<10
Fungi	370,000	1,600	900	20	210,000	60
Trial 3						
Bacteria	1,300,000	60	<10	<10	2,200,000	<10
Fungi	370,000	1,400	980	40	210,000	80

*Reinoculated at 7 days

Summary

On the basis of the comparative studies described here and the established history of successful application of the accelerated double challenge test to the evaluation of multiple product forms, it would appear that this methodology provides a reliable and viable alternative option for conducting preservative efficacy testing.

The method is relatively easy to conduct using traditional microbiological practices and requires no specialized equipment. Obviously, it is strongly suggested that before adopting this or any other testing methodology, appropriate qualification and comparative verification studies must be conducted.

The current urgency to replace preservatives viewed as unacceptable by consumers places high value on the use of accelerated preservative efficacy testing methods, such as the one described here, to bring reformulated products more quickly to the marketplace.

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