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Preservative efficacy testing by a rapid screening method for estimation of D-values

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Synopsis

This report describes a rapid screening method for estimating D-values to determine whether products are adequately preserved. Estimated D-values (ED-values) are determined using aerobic plate counts of test organisms immediately after inoculation into test samples and at 24 hr for pathogenic microorganisms or at 7 days for non-pathogenic bacteria, yeasts, or molds. Products are judged to be adequately preserved if they meet the acceptance criteria of the linear regression method.

There was excellent agreement between D-values and ED-values for 60 sets of data (correlation coefficient = 0.98). The mean D-values and ED-values for the 60 samples differed by 0.5 hr (6.6%) even though the D-values ranged from <0.1 hr to 39 hr. Where differences were observed, the ED-values generally were larger (i.e., more conservative) than D-values for the same samples. The rapid screening method offers about 50% savings in the labor and materials required for preservative efficacy testing by the original linear regression method.

INTRODUCTION

Preservative efficacy testing is used to determine whether experimental formulas, stability test samples, and finished products are adequately preserved. The goal of preservative efficacy testing is to determine the type and minimum effective concentration of preservatives required for adequate preservation of the formula during manufacturing, distribution, and use by consumers.

The methods of preservative efficacy testing currently in use include official methods such as the United States Pharmacopeia (USP) method (1) and the British Pharmacopeia (BP) method (2); trade association methods such as the Cosmetic, Toiletry & Fragrance Association (CTFA) method (3); and rapid methods such as the linear regression method (4). The procedures used in these methods are similar; however, the times at which samples are taken for analysis and the interpretation of test results—the acceptance criteria by which products are judged to be effectively preserved— are different (5). The acceptance criteria of the USP, BP, and CTFA methods were converted to decimal reduction times (D-values) by Orth (5,6). Use of D-values enables a laboratory to determine the effect of the product preservative system on rates of death of test organisms, to compare rates of death in different products tested in different labs, to use

statistical controls of preservative efficacy tests, to determine synergistic effects of formula components, to set rational acceptance criteria, and to determine required D-values (4–9).

As originally outlined, the linear regression method specified that aerobic plate counts (APCs) be performed immediately after inoculation and at various times afterwards—typically at 2, 4, and 24 hr for bacteria and at 4, 8, and 24 hr for yeasts and molds. Additional samples were taken at 3, 5, or 7 d after inoculation unless the previous APC was <10/ml (4). These APCs were then used to determine the D-values for each test organism in product samples.

It would be desirable to be able to determine the rate of microbial death—to determine D-values—using fewer APCs than required by the original linear regression method (4) because this would be less labor-intensive and would save on the cost of materials. This is now possible. This report describes a rapid screening procedure that allows calculation of D-values from two APC data points for each test organism. Our laboratory has performed several hundred rapid screening tests by use of this procedure to determine whether cosmetic and OTC-drug formulas meet acceptance criteria (5).

EXPERIMENTAL

TEST ORGANISMS

The American Type Culture Collection (ATCC) organisms used in this study were received directly from the ATCC and consisted of *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *P. cepacia* (ATCC 13945), *Escherichia coli* (ATCC 8739), *Aspergillus niger* (ATCC 16404), and *Candida albicans* (ATCC 10231). The *Bacillus* sp. was isolated from a product.

The bacteria were maintained by weekly transfer on tryptic soy agar (TSA) with 0.07% lecithin and 0.5% Tween 80 (TSALT). *A. niger* and *C. albicans* were maintained by weekly transfer on potato dextrose agar (PDA). The bacteria and *C. albicans* were incubated at 32°C, and *A. niger* was incubated at 25°C. The bacteria were grown for 24 hr on TSALT agar prior to use in preservative efficacy testing. *C. albicans* was grown for 24 hr on PDA, and *A. niger* was grown for 7 d on PDA prior to use in preservative efficacy testing.

TEST SAMPLES

The test samples used in this study included proprietary formulations of o/w emulsions (creams, lotions, facial moisturizers), anionic surfactants (shower gels and cleansers), and OTC-drug products (sunscreens, anti-dandruff shampoos).

TEST PROCEDURES

Preservative efficacy tests were performed using saline suspensions from surface growth of each test organism as described elsewhere (4), with the following exceptions: 1) bacterial and *C. albicans* cultures were incubated at 32°C, and 2) APC determinations were made using single surface-spread plates of each organism or each group of organ-

isms in "pooled" inocula. Pooled inocula consisted of saline suspensions prepared using organisms that had similar maximum acceptable D-values (i.e., *P. aeruginosa* and *S. aureus; E. coli* and *P. cepacia*) and/or recovery media (i.e., *C. albicans* and *A. niger*).

DETERMINATION OF D-VALUES

D-values were calculated as described previously (4). Estimated D-values (ED-values) were calculated from the same data using APCs taken immediately after inoculation (i.e., at time = 0 hr) and at 24 hr for site-significant organisms (i.e., pathogens/opportunistic pathogens) or 7 d for organisms that are not site-significant (i.e., non-pathogenic bacteria, yeasts, and molds). Thus, ED-values were equal to the negative reciprocal of the slope of a survivor curve constructed from APCs immediately after inoculation of test organisms into test samples and at 24 hr (for *S. aureus, P. aeruginosa,* or *C. albicans*) or 7 d (for *E. coli, P. cepacia, Bacillus,* and *A. niger*). D-values and ED-values, which were calculated using APCs of <10/g, were expressed as "less than" a specific time (i.e., <3.5 hr). The "less than" signs were not used in determining mean APC values.

STATISTICS

Significant differences between D-values and ED-values were assessed by an independent t-test using Sigmaplot 4.0 (Menlo Park, CA). Linear regressions were determined using a hand-held calculator (4).

RESULTS AND DISCUSSION

Screening studies have been used in our laboratories for estimating D-values for several years; however, ED-values obtained with the screening method have not been compared with D-values obtained by the linear regression method for cosmetic and OTC-drug products. D-values were determined using APCs at several times for each test organism. Direct comparison of D-values with ED-values was possible because both were calculated from the same experimental data: D-values were determined using 0, 2, 4, and 24 hr or 7 d APCs; ED-values were determined using 0 and 24 hr or 7 d APCs.

Table I compares 60 D-values and ED-values calculated using data obtained during preservative efficacy tests of cosmetics and OTC drugs including facial moisturizers, night creams, sunscreens, facial toners, shower gels, and antidandruff shampoos that were challenged with several different test organisms. The D-values ranged from <0.1 hr (i.e., where the population of *P. cepacia* was not detected at the 2-hr reading, so that the D-value had to be estimated) to 39 hr. The ED-values ranged from <3.0 hr to 42 hr. The Student's t-test showed that the mean D-value (6.9 hr) and the mean ED-value (7.4 hr) were not significantly different ($p > 0.10$). When the D-values were <10 hr, the differences in D-values and ED-values for the same experimental data ranged from 0 hr (where the values were the same) to 4.9 hr in a night cream (where the D-value was <0.5 hr because *P. aeruginosa* died so quickly that no viable cells were recovered at 2 hr and the ED-value was <5.4 hr because no viable cells were recovered at 24 hr). The differences in D-values and ED-values for the same experimental data ranged from 0 to

Table I
Comparison of D-Values and ED-Values for Cosmetic and OTC-Drug Products

Test product	Test organism	D-value	Correlation coefficient	ED-value
Shower gel	*S. aureus*	2.1	−0.965	<3.1
Cleanser	*S. aureus*	7.5	−0.997	7.7
Cleanser	*S. aureus*	6.8	−0.989	7.3
Night cream	*P. aeruginosa*	<0.5	−1.00	<5.4
Sunscreen	*S. aureus*	<2.9	−0.991	<3.0
Sunscreen	*Bacillus*	5.2	−0.967	4.5
Sunscreen	*C. albicans*	9.0	−0.993	8.6
Sunscreen	*A. niger*	<4.5	−0.999	<4.6
Facial toner	*S. aureus*	25	−0.994	27
Facial toner	*S. aureus*	30	−0.978	27
Sunscreen	*S. aureus*	<3.0	−0.996	<3.2
Sunscreen	*C. albicans*	<4.0	−0.995	<4.4
Sunscreen	*A. niger*	3.5	−0.934	<4.8
Moisturizer	*S. aureus*	<3.1	−0.993	<3.3
Moisturizer	*Bacillus*	7.1	−0.943	6.2
Moisturizer	*E. coli*	2.4	−1.00	3.2
Moisturizer	*P. aeruginosa*	1.0	−1.00	<3.8
Moisturizer	*C. albicans*	4.9	−1.00	4.7
Moisturizer	*A. niger*	<4.7	−0.995	<4.9
Facial toner	*S. aureus*	33	−0.974	31
Shower gel	*S. aureus*	<3.0	−0.992	<3.2
Face product	*S. aureus*	39	−0.978	42
Antidandruff shampoo	*S. aureus*	8.6	−0.999	9.1
Antidandruff shampoo	*C. albicans*	11	−0.995	11
Sunscreen	*S. aureus*	4.0	−0.995	4.2
Sunscreen	*S. aureus*	<2.9	−0.999	<3.3
Moisturizer	*S. aureus*	<3.0	−0.99	<3.2
Moisturizer	*E. coli*	11	−0.96	9.7
Moisturizer	*P. aeruginosa*	1.0	−1.00	<3.5
Moisturizer	*P. cepacia*	1.0	−1.00	<3.9
Moisturizer	*C. albicans*	4.2	−1.00	<3.9
Moisturizer	*A. niger*	<4.5	−0.99	<4.7
Facial toner	*S. aureus*	3.7	−0.993	3.9
Facial toner	*S. aureus*	6.7	−0.999	6.8
Facial toner	*S. aureus*	3.9	−0.999	4.3
Sunscreen	*C. albicans*	1.8	−1.00	<4.7
Sunscreen	*A. niger*	<5.1	−0.99	<5.1
Night cream	*S. aureus*	17	−0.998	18
Night cream	*Bacillus*	10	−1.00	10
Night cream	*P. cepacia*	<0.1	−1.00	<3.4
Night cream	*C. albicans*	4.9	−0.994	4.8
Night cream	*A. niger*	<4.5	−0.993	<4.6
Sunscreen	*S. aureus*	<3.0	−0.991	<3.2
Shower gel	*S. aureus*	15	−0.999	14
Shower gel	*S. aureus*	<3.0	−0.991	<3.2
Shower gel	*S. aureus*	7.3	−0.969	7.1
Moisturizer	*S. aureus*	5.1	−0.99	5.4
Moisturizer	*Bacillus*	8.9	−0.99	8.4
Moisturizer	*E. coli*	5.5	−0.99	5.5
Moisturizer	*C. albicans*	2.6	−1.00	<3.5
Moisturizer	*A. niger*	3.0	−0.99	<4.6

Table I (continued)

Test product	Test organism	D-value	Correlation coefficient	ED-value
Sunscreen	*S. aureus*	<3.1	−0.990	<3.4
Sunscreen	*Bacillus*	6.8	−0.999	7.0
Sunscreen	*E. coli*	6.6	−0.989	6.4
Sunscreen	*P. aeruginosa*	<0.6	−1.00	<3.6
Sunscreen	*C. albicans*	3.8	−0.99	<3.8
Sunscreen	*A. niger*	7.3	−0.99	7.4
Sunscreen	*S. aureus*	5.1	−0.997	5.3
Sunscreen	*Bacillus*	11	−0.99	9.9
Sunscreen	*E. coli*	6.3	−0.97	5.8

D-values and ED-values are in hr. Correlation coefficient is for linear regression used in determining D-values for each sample.

3 hr when the D-values were >10 hr. The mean D-values obtained by use of both methods differed by 0.5 hr (6.6%).

The greatest differences in D-values and ED-values were seen in samples in which the test organisms died so quickly that D-values were indeterminant for both the linear regression method (because no viable organisms were recovered at 2 hr) and the rapid screening method. For example, the D-value and ED-value for *P. aeruginosa* in a night cream were <0.5 and <5.4 hr, respectively. The ED-values were larger than D-values estimated by the linear regression method because data points at 2 and 4 hr allowed closer discrimination of the time at which the test population of organisms was killed as a result of exposure to the preservative system of the product, which enabled a more accurate determination of the D-values than estimating D-values using two APCs.

When the D values were indeterminate, (e.g., <0.5 hr, <2.9 hr, <4.5 hr), ED-values were greater than D-values in all but one sample, in which case they were equal. This is desirable for use of the rapid screening method because obtaining ED-values that are larger than the D-values demonstrates that the rapid method generally errs on the conservative side. (Note: The estimated rates of death provided by indeterminant ED-values make it more difficult for the test product to meet acceptance criteria, which means that the rapid screening method provides a conservative estimate of the rates of death of the test organisms).

The ED-values obtained by using the rapid screening method were plotted as a function of the D-values in a scatter diagram (Figure 1). The proximity of the data points to a line with a slope of 0.95 hr/hr illustrates the close agreement of the ED-values with the D-values. Linear regression analysis of these data gave a correlation coefficient of 0.98, which indicates excellent goodness of fit of the data to the linear regression. The ED-values tend to level off at around 4 hr. This is due to the rapid screening method being unable to give ED-values much less than 4 hr when using approximately 10^6 organisms/g test sample and determining APCs at time 0 and 24 hr (see below).

The survival curve slope method allowed determination of antimicrobial synergism of formula components and preservatives in multicomponent systems (6). This method allowed determination of D-values and survival times (STs) when APC sampling times are known. In addition, it allowed estimation of the maximum possible D-value (MPD-

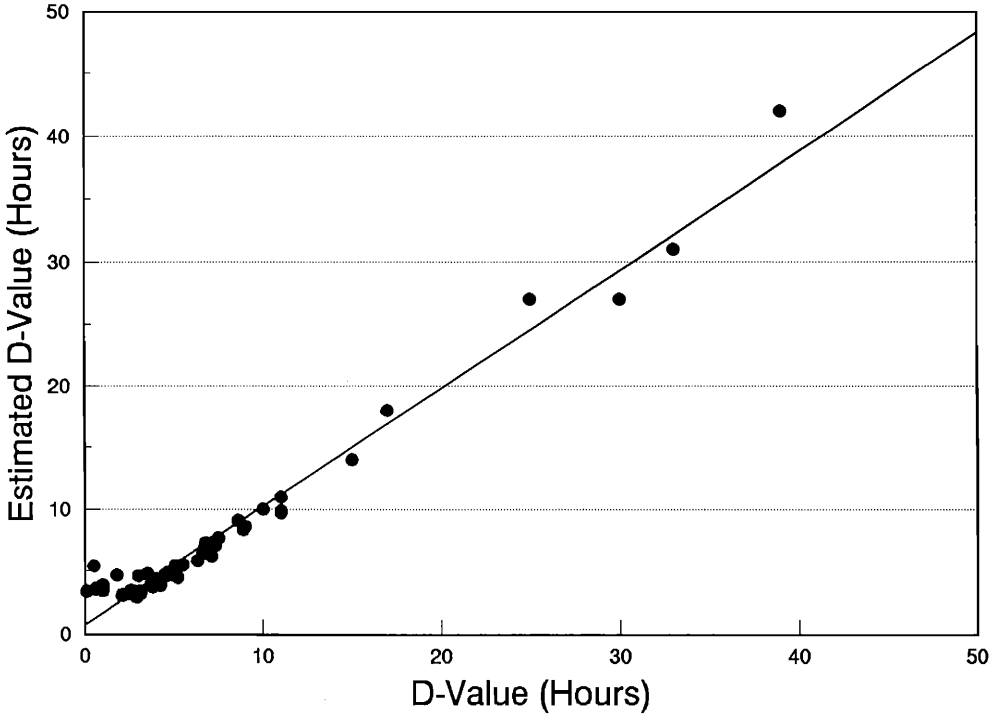

Figure 1. Scatter diagram of the ED-values obtained by the rapid screening method and actual D-values obtained by the linear regression method of 60 cosmetic and OTC-drug samples challenged with different test organisms.

value) when the minimum possible ST (MPST) and the APC at time 0 hr are known. It is apparent that products meet preservative system acceptance criteria if the D-value or MPD-value is less than the D-value of the acceptance criteria (i.e., $\leq$4 hr for (opportunistic) pathogens, $\leq$28 hr for nonpathogens, and bacteriostatic/bactericidal for *Bacillus*).

Use of sufficient concentration of microorganisms in the inoculum (i.e., $>10^6$ organisms/g in the sample) and sampling at the time to determine the ST or MPST provide sufficient information to determine whether the product meets the acceptance criteria for specific test organisms when using the rapid screening method. The ED-values provided by the rapid screening method are based on MPST and should meet acceptance criteria of the linear regression method.

In addition to use of APCs at time 0 and at later times, ED-values have been calculated for scores of samples using a "virtual" survivor curve (6). A virtual survivor curve is made using the APC of the inoculum for each test organism to calculate the initial APC, as is done by the USP method (1), and at other times to obtain ED-values to meet acceptance criteria (i.e., APCs at 24 hr for pathogens and at 7 d for nonpathogenic bacteria, yeasts, and molds). The estimation of D-values using two APCs provides substantial time and material savings over the original linear regression method because a number of samples can be inoculated without the requirement for determining APCs of each test organism in each sample immediately after inoculation and at intermediate time points.

ED-values may be determined using saline suspensions of pure cultures or mixed inocula (i.e., where more than one test organism was used in the saline suspension added to a test sample). We have found that use of pooled inocula is convenient when using organisms with similar ED-values (i.e., *P. aeruginosa* and *S. aureus; E. coli* and *P. cepacia*) and/or recovery media (i.e., *C. albicans* and *A. niger*).

Microbiologists generally use duplicate plates for each dilution when determining APCs. This is done to improve the reliability of the plating method and to help overcome human factors, which decrease the precision of the method (i.e., inaccuracies in pipeting). Preliminary data in our laboratory indicate that use of single plates and duplicate plates give essentially the same D-values. The difference in D-values was <0.5 hr for those tests in which the maximum D-values were no larger than 12 hr. Although the differences in D-values may be greater with larger D-values, it is believed that the percentage difference would be $<10\%$ as long as the assays are "in control" (7–9). Laboratories should implement statistical control procedures to ensure that assays are in control. Our preliminary data suggest that in some instances a laboratory may be able to use single plates for rapid screening studies.

This work describes a rapid screening method for estimating D-values. This method is similar to the linear regression method; however, intermediate samplings and APCs are omitted. This allows determination of an ED-value using APCs at 0 hr and 24 hr (for pathogens) and at 0 hr and 7 d (for non-pathogenic bacteria, yeast, and molds). When using an inoculum of $>10^6$ organisms/g product, recovery of <10 organisms/g product at 24 hr or 7 d indicates that the MPST is $\leq$24 hr or $\leq$168 hr, respectively, and that the ED-value is $\leq$4 hr or $\leq$28 hr, respectively.

The reliability of the rapid screening method was good over the range of D-values one finds in both satisfactorily and unsatisfactorily preserved products (i.e., D-values <4 hr to 39 hr). There was excellent agreement between ED-values and D-values (correlation coefficient = 0.98), and the difference between mean D-values and mean ED-values was well below 10%. This is considered to be suitable for a rapid screening method. Where differences in estimated and D-values were observed, the ED-values generally were larger (i.e., more conservative) than D-values for the same samples.

The physicochemical make-up of each formula, the type of packaging, and conditions of use by consumers determine the risk of microbial contamination and spoilage (10). Several formulas frequently must be tested in the process of selecting the final formula. The rapid screening method provides a convenient means of selecting the preservative system of a product and offers about 50% savings in terms of labor and materials required for testing. It is recommended that ED-values be confirmed by determining D-values for finished formulations.

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REFERENCES

(1) Microbiological Tests, Antimicrobial Preservatives—Effectiveness, *United States Pharmacopeia XXII*, United States Pharmacopeial Convention, Rockville, MD, pp. 1478–1479 (1990).

(2) Efficacy of antimicrobial preservatives in pharmaceutical products, *British Pharmacopoeia 1988, Vol. II.*, Her Majesty's Stationery Office, London, England, pp. A200–A208 (1988).
(3) Preservation Subcommittee of the CTFA Microbiological Committee, A guideline for the determination of adequacy of preservation of cosmetics and toiletry formulations, *TGA Cosmet. J.*, **2**, 20–23 (1970).
(4) D. S. Orth, Linear regression method for rapid determination of cosmetic preservative efficacy., *J. Soc. Cosmet. Chem.*, **30**, 321–332 (1979).
(5) D. S. Orth, Standardizing preservative efficacy test data, *Cosmet. Toiletr.*, **106**(3), 45–48,51 (1991).
(6) D. S. Orth, C. M. Lutes Anderson, D. K. Smith, and S. R. Milstein, Synergism of preservative system components: Use of the survival curve slope method to demonstrate anti-*Pseudomonas* synergy of methyl paraben and acrylic acid homopolymer/copolymers *in vitro*, *J. Soc. Cosmet. Chem.*, **40**, 347–365 (1989).
(7) D. S. Orth and L. R. Brueggen, Preservative efficacy of cosmetic products. Rechallenge testing and reliability of the linear regression method, *Cosmet. Toiletr.*, **97**(5), 61–65 (1982).
(8) D. S. Orth, "Principles of Preservation," in S. P. Denyer and R. M. Baird, Eds., *Guide to Microbiological Control in Pharmaceuticals* (Ellis Horwood, London, 1990), pp. 241–250.
(9) D. S. Orth, Preservative efficacy testing: Rationale, acceptance criteria, and determination of synergism. Oral presentation at *The First Pan European Conference on the Significance and Validation of Test Methods for the Efficacy of Preservatives in Cosmetics, Toiletries and Pharmaceuticals*, held November 20–21, 1991, at the Commonwealth Institute, London, England.
(10) D. S. Orth, The required D-value: Evaluating product preservation in relation to packaging and consumer use/abuse. *Cosmet. Toiletr.*, **107**, 39–43 (1992).