Preformulation Method for Parenteral Preservative Efficacy Evaluation

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Abstract □ A method is described for rapidly and reliably evaluating parenteral preservative efficacy. Solutions containing antimicrobial preservatives were challenged with microorganisms, sampled from 0.5 to 6 h following introduction of the challenge, cultured, and counted for surviving microbial cells. Data were analyzed by computer according to two models: linear and quadratic. Decimal reduction times (D values) were calculated for each microbial challenge in each preservative solution. A D value of ≤2 h for bacteria predicts that the preservative system will pass the British Pharmacopoeia (BP) preservative efficacy test, a more rigorous test than the USP test. Fourteen preservative systems were tested in both neutral isotonic saline solutions and neutral regular insulin solutions. D values and correlation coefficients for both models were calculated. The ranking of preservative effectiveness in neutral saline solutions closely correlated with the results found using neutral regular insulin solutions. The most effective preservative systems were found to be 0.3% m-cresol and various combinations of m-cresol and phenol. The advantages and limitations of this method are discussed.

Keyphrases □ Preservatives—parenteral formulations, efficacy, preformulation determination □ Parenteral formulations—efficacy of preservatives, preformulation determination □ Antimicrobial screening—preservatives, parenteral formulations, efficacy, preformulation determination

The British Pharmacopoeial (BP) test for preservative efficacy requires that the number of bacteria recovered per milliliter in injectable preparations intended for multiple use be reduced by a factor of 10^3 within 6 h following challenge (1). This requirement imposes a far stricter criterion for antimicrobial action than the USP preservative efficacy test, which requires the bacterial concentration to be reduced by a factor of 10^3 14 d following microbial inoculation (2). A large number of preservatives at bacteriostatic concentrations will pass the USP test but will fail the BP test. Parenteral drug manufacturers who wish to market their products in countries abiding by BP requirements must reformulate their products to obtain a preservative system which, in fact, is self-sterilizing to pass the BP preservative test.

There is a need for a rapid, reliable, and inexpensive screening method to determine effective preservative systems in parenteral products. Several methods for screening and evaluating preservative efficacy have been reported (3–6); most employed the construction of time-log survivor curves during a short-time exposure period. However, previous studies have been limited in the number of preservative systems evaluated and have not attempted to correlate results from a test model to results obtained from an actual parenteral drug product. In this work, neutral isotonic sodium chloride solution was selected as the model parenteral solution, while neutral regular insulin was chosen as the actual drug product. The use of neutral isotonic saline was based on the assumption that bacteria would not be adversely affected by such an inoffensive formulation and, thus, bacterial destruction would be solely the result of preservative activity.

An additional unique feature of the work reported herein is the application of a computer statistical program generating two types of models fitted to the experimental data. The use of models to analyze the microbial survivor data facilitates the decision-making process in the selection of appropriate preservative systems in the formulation development of multidose parenteral products.

EXPERIMENTAL SECTION

Solution Preparation—The preservatives used in this study were USP grade, tested by and obtained in-house: phenol (0.2% and 0.5%), m-cresol (0.1–0.3%), benzyl alcohol (1% and 2%), methylparaben (0.1% and 0.2%), propylparaben (0.01% and 0.02%), and chlorobutanol (0.5%). Preservative combinations studied were phenol-m-cresol, methyl- and propylparabens, and methylparaben-benzyl alcohol. A total of 14 preservative systems were evaluated.

Solutions in isotonic saline were prepared using sterile glass flasks and sterile 0.9% NaCl irrigating solution[1]. The accurately weighed or measured volume of preservative was dissolved in the saline solution, and the pH was adjusted to 6.8–7.4 using sterile filtered 1.0 M NaOH or 1.0 M HCl. Solution volume was then adjusted quantitatively to the correct volume with saline.

Insulin solutions were prepared and sterile-filtered using a regular insulin formulation without a preservative. Preservatives were accurately weighed or measured and added to the insulin solution. All insulin solutions were pH 6.9–7.4.

Test Organisms—The test organisms used in this study were *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), *Candida albicans* (ATCC 10231), and *Aspergillus niger* (ATCC 16404). The bacterial cultures were grown for 18–24 h in soybean-casein digest agar[2] and the growth washed from the slants with 10 mL of sterile saline (0.85% NaCl). The stock suspensions were prepared by dilution with sterile saline to give a cell concentration of ~5×10^8 organisms/mL. Sufficient suspension was added to the test solution to obtain ~1×10^6 organisms/mL.

Yeasts and molds were grown on Sabouraud dextrose agar plates[2] at 25°C until sufficient growth appeared. Cells were washed from the plates with sterile saline, sedimented by centrifugation, and the sediment was suspended in sufficient sterile saline to yield ~5×10^8 organisms/mL. Suspensions were further diluted to give ~1×10^6 organisms/mL when added to the test solutions.

Test Procedure—A 0.1-mL amount of the test organism suspension was added to each 5-mL portion of the test solution. After mixing on a vortex mixer, an initial sample was taken for the time zero plate count. A saline control sample prepared in the same manner was also tested; this was used as the time zero count in some instances because of the very rapid bactericidal effect of some test solutions. Test samples were then retested after incubation, typically at 0.5, 1, 2, 4, and 6 h for bacteria and at 4, 8, and 24 h for yeasts and molds. All test samples were held at room temperature unless otherwise specified.

All dilutions were prepared in phosphate buffer solution, pH 7.2 ± 0.1. After shaking, 0.1- and/or 1.0-mL aliquots were transferred into duplicate petri plates. Letheen agar[3] was used as the test medium: it contains polysorbate 80 and lecithin, known to inhibit most phenolic and paraben antimicrobial agents (7, 8). Tests in our laboratories confirmed the effectiveness of this medium. Bacterial and yeast plates were incubated for 48 h at 35°C and mold plates at 25°C for 4 to 7 d. Average plate counts were calculated for each sample.

Data Analysis—The data analysis was accomplished *via* a computer program utilizing the statistical software package SAS[4]. When values of the variables "Hours" and "Counts" were entered, the program generated two types of fitted models relating log (count + 1) to hours and produced plots of the fitted equations superimposed by the original observations. The two

[1] Travenol Laboratories, Deerfield, Il.
[2] Difco, Detroit, Mich.
[3] BBL, Cockeysville, Md.
[4] Statistical Analysis System; SAS Institute, Cary, N.C.

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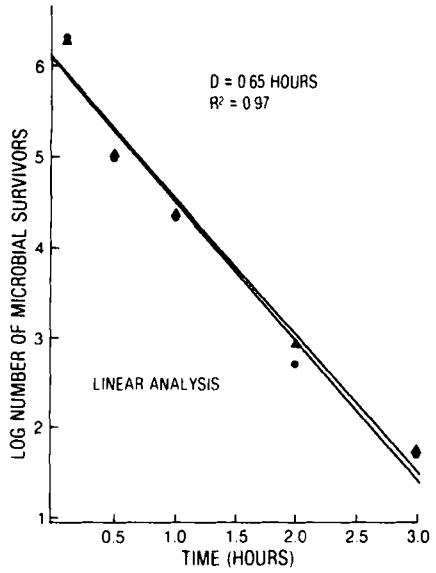

Figure 1—*Linear regression fit of the log number of microbial survivors versus time as calculated by the SAS linear analysis method. Data points represent two separate experiments;* D *and* R^2 *values are the average of two linear regression analyses.*

models used were a linear model and a quadratic model. The linear model is given by:

$$y = \alpha + \beta x + \epsilon \qquad \text{(Eq. 1)}$$

where y = log (count + 1) at hour x, α is the intercept, β is the slope of the line, and ϵ is the experimental error associated with the observation of y. The quadratic model is given by:

$$y = \alpha + \beta x + \gamma x^2 + \epsilon \qquad \text{(Eq. 2)}$$

where the additional term γx^2, represents the departure from linearity of the log (count + 1). The purpose of these model-fitting programs is to calculate estimates of the parameters α, β, and γ in the two theoretical models.

The *D* value refers to the decimal reduction time describing the death rate of the microorganisms; it represents the length of time in which a 1-log reduction in the number of survivors will be achieved. Since the term "*D* value" has heretofore been used in conjunction with a model in which the death rate is independent of the current number of survivors, it has been convenient to calculate it by the formula:

$$D = \frac{U}{\log N_0 - \log N_u} \qquad \text{(Eq. 3)}$$

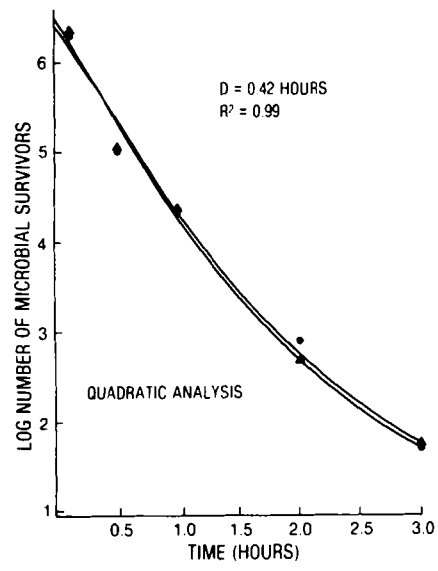

Figure 2—*Linear regression fit of the log number of microbial survivors versus time as calculated by the SAS quadratic analysis method. Data points represent two separate experiments;* D *and* R^2 *values are the average of two linear regression analyses.*

Table I—Comparison of *D* Values for the USP Challenge Organisms in Neutral Saline Solutions Containing Various Preservative Systems

Organism	Linear Method		Quadratic Method	
	D, h	R^2	*D*, h	R^2
	0.2% Phenol			
S. aureus	10.9	0.96	15.6	0.99
P. aeruginosa	7.0	0.99	4.2	1.00
E. coli	26.2	0.96	27.4	.95
A. niger	250.0	0.84	81.3	1.00
	0.3% *m*-Cresol			
S. aureus	0.7	0.98	0.4	0.99
P. aeruginosa	1.9	0.39	0.6	0.57
E. coli	1.8	0.82	0.8	0.97
A. niger	3.2	0.98	2.3	0.99
C. albicans	9.0	0.87	3.5	1.00
	0.2% Phenol + 0.3% *m*-Cresol			
S. aureus	0.5	0.78	0.2	0.95
P. aeruginosa	1.3	0.81	0.5	0.98
E. coli	2.5	0.92	3.6	0.97
A. niger	3.1	0.99	2.1	1.00
C. albicans	2.8	0.84	1.5	0.90

where U is the time exposure interval at constant temperature, N_0 is the initial number of surviving microorganisms, and N_u is the number of surviving microorganisms after exposure period U. This equation is applicable to our model, in which the log of the number of survivors is treated as a linear function of time.

A typical semilogarithmic plot of the number of living microorganisms in a preserved solution *versus* time is shown in Fig. 1. If the R^2 value was ≥0.9, the linear model shown in Fig. 1 was considered sufficient for determining the *D* value of the microorganism in the preserved parenteral system. Preservative systems especially effective in inactivating microorganisms tended to yield curvilinear plots of log count *versus* time. The rate of kill during the first 30–60 min was more rapid than the rate of kill at later times. Fitting the data according to a linear model generally resulted in a poorly fit straight line with an R^2 of ≤0.9. Also, the calculated *D* value often underestimated the actual rate of kill produced initially.

A better fit of the data was achieved by employing the quadratic equation. According to this model, a hyperbolic fit of the data is obtained, as shown in Fig. 2. The *D* value shown here corresponds to the length of time before the first log reduction in count. The fit of the data using the quadratic model improved the R^2.

RESULTS

Preliminary results comparing *D* values of USP challenge organisms in neutral saline solutions containing various preservative systems are reported in Table I. The *D* values for the mold and yeast were higher than those for bacteria. However, both USP and BP requirements for yeast and mold survival in preserved solutions are less stringent than those for bacterial survival. Of the three bacterial challenges, *E. coli* was most resistant to the antimicrobial agents tested. However, *E. coli* is not required by the BP test for injections. Either *S. aureus* or *P. aeruginosa* can be used as a prototype challenge organism to provide early predictions regarding the success or failure of a preservative system to pass the BP preservative efficacy test. *S. aureus* was selected as the model challenge organism, primarily because of the data reported in Table II. In solutions containing lower concentrations of preservatives, the *D* values for *S. aureus* were greater than those for *P. aeruginosa*.

D values for *S. aureus* in 14 different preservative systems were compared in both neutral isotonic saline and neutral regular insulin solution (Table III). The combination of 0.2% phenol + 0.3% *m*-cresol consistently produced the lowest *D* values, while 0.2% phenol consistently produced the highest *D* values. Preservative systems ranked 1–5 in Table III passed the BP requirement of reducing the bacterial population by 1 log in 2 h in both neutral saline and

Table II—Comparison of *D* Values of Three Bacterial Challenges in Phenol and *m*-Cresol Preservative Systems in Neutral Isotonic Saline Solutions

Preservative System	D_{lin}, h		
	S. aureus	*P. aeruginosa*	*E. coli*
0.2% Phenol + 0.3% *m*-Cresol	0.5	1.3	2.5
0.2% Phenol + 0.1% *m*-Cresol	3.8	1.5	7.5
0.2% Phenol	10.9	7.0	26.2
0.5% Phenol	1.1	1.3	2.5
0.2% *m*-Cresol	5.7	1.1	8.3
0.3% *m*-Cresol	0.7	1.9	1.8

Table III—D Values and Correlation Coefficients (R^2) from Linear and Quadratic Data Analyses for Saline and Insulin Solutions Containing Different Preservative Systems Ranked According to Their Effectiveness Against *S. aureus*[a]

Preservative System	Linear Analysis						Quadratic Analysis					
	Saline Solution			Insulin Solution			Saline Solution			Insulin Solution		
	Rank	D_{lin}, h	R^2	Rank	D_{lin}, h	R^2	Rank	D_{quad}, h	R^2	Rank	D_{quad}, h	R^2
0.2% Phenol + 0.3% *m*-Cresol	1	0.5	0.78	1	0.5	0.79	1	0.2	0.95	1	0.2	0.96
0.3% *m*-Cresol	2	0.7	0.98	2	0.6	0.76	2	0.4	0.99	2	0.3	0.94
0.5% Phenol	3	1.1	1.00	3	0.8	0.93	5	1.2	1.00	4	0.4	0.99
2.0% Benzyl Alcohol	4	1.6	0.68	3	0.8	0.74	3	0.6	0.94	2	0.2	0.97
0.2% Phenol + 0.2% *m*-Cresol	5	1.7	0.93	5	1.3	0.75	4	0.8	1.00	4	0.4	0.96
0.2% Methylparaben + 0.02% Propylparaben	6	1.8	0.99	10	3.0	0.92	6	1.7	1.00	8	1.6	0.99
0.1% Methylparaben + 1.0% Benzyl Alcohol	7	2.7	0.94	6	1.4	0.90	7	1.7	0.97	7	0.7	0.91
0.2% Phenol + 0.1% *m*-Cresol	8	3.8	0.99	8	2.2	1.00	8	3.6	1.00	10	2.2	1.00
0.2% *m*-Cresol	9	5.7	0.92	8	2.2	0.94	9	4.9	0.96	8	1.6	0.98
0.1% Methylparaben + 0.01% Propylparaben	10	7.4	0.97	13	12.3	0.91	10	8.3	0.98	13	12.0	0.95
0.1% Methylparaben	11	8.9	0.95	12	9.5	0.86	11	11.0	0.95	12	7.6	0.94
0.5% Chlorobutanol	12	10.1	0.81	7	1.8	0.80	—	—	—	6	0.7	0.99
1.0% Benzyl Alcohol	13	10.7	0.93	11	4.2	0.83	13	12.2	0.96	11	3.4	0.94
0.2% Phenol	14	10.9	0.96	14	16.2	0.96	14	15.6	0.99	14	12.9	0.96

[a] All data are the average of two experiments.

neutral regular insulin solutions. D values, generally, were lower in insulin solution compared with saline. Thus, the neutral isotonic saline solution conservatively estimated the preservative effectiveness against *S. aureus* observed in neutral regular insulin solutions.

Fits of the linear models of log count *versus* time were very good ($R^2 \geq 0.93$) for the majority of the saline solutions tested. Fits were not as good for the insulin solutions using the linear method, but the quadratic model improved the fits. Additionally, the quadratic model ranked the effectiveness of the various preservative systems in nearly the same order as that found using the linear model. Thus, the choice of the model did not cause any major change in the identification of best and worst systems.

DISCUSSION

The logarithmic order of death of microorganisms (9) facilitates the study of the effectiveness of destroying microorganisms within a convenient time frame. Simple techniques of microbiological assay and computer data analysis permit the generation of microbial kinetic data easily and rapidly. The methodology described in this paper can be used to evaluate preservative effectiveness in a given formulation as a function of any number of variables: type and concentration of preservatives, combination of preservatives, microbial challenge organism, solution pH, formulation differences, and temperature. The results reported in this paper show that D values can be easily obtained and used to compare the effectiveness of different preservative systems. Computers aid in the analysis of data that employ different models for an adequate fit. From the data one can select the best preservative system for the formulation being developed.

Like most experimental screens, this method has certain limitations and precautions. The initial microbial concentration cannot be determined accurately from the test sample because of the immediate action of the antimicrobial agent. Initial values must be determined using a control solution with no antimicrobial activity. At least three, preferably four, samples should be taken from the test solution for microbial analysis at various times over a maximum of 6 h. In the studies reported here, four samples were taken.

Rapid-acting preservative systems require sampling times at intervals much sooner and shorter than for long-acting preservative systems. For example, rather than taking samples at 1, 2, 4, and 6 h following the addition of the microbial challenge, rapid-acting preservative systems need to be sampled at 0.25, 0.5, 1, and 2 h after time zero. Many preservative systems caused a precipitous initial reduction in the microbial population followed by a more gradual decline. This produced curvature in the log count *versus* time graph, which necessitated the use of the quadratic model for analyzing the data. The D value calculated gave a more representative estimate of the destructive action of the preservative system than that obtained from the standard linear model.

It may not be advisable to study only a single species challenge in a screening test. Of course, the fewer the types of microorganisms used, the more expedient the test. Ideally, the most resistant microorganism in the test system is identified at the beginning of the screening study. The hazard of employing a single challenge to evaluate a large number of preservative systems is the potential for a different microbial species than that used as the challenge to be significantly more resistant in a particular preservative system. For example, *S. aureus* might be the most resistant microorganism in phenolic preservative systems, while *E. coli* might be more resistant in paraben preservative systems. It is because of these possibilities that the most effective preservative system selected as a result of its activity against one microorganism should subsequently be studied in the presence of all other compendial challenge microorganisms. This screening method is not a substitute for the USP and/or BP preservative efficacy tests, but rather serves to reduce the potential number of preservative systems to be tested by the compendial methods.

The D values reported in this study represented the average of two experiments. Preliminary investigation found that the standard deviations calculated from five replicate D value experiments of the same organism in separately prepared solutions containing the same preservative system ranged from 4.2 to 8.7%. This seemed adequate for a preformulation screening test, especially one involving a plate count microbiological test procedure.

Several of the data sets showed significant departures from linearity ($p \leq 0.01$ for the improvement in fit gained by including a quadratic term in the model). By fitting both linear and quadratic models and comparing the rankings of D values from these models, it was possible to verify that the ranking of these preservative systems was not dependent on the choice of model. Although the linear models proved to be adequate for the screening of preservatives, a quadratic model based on an adequate number of time points should be considered whenever an accurate estimate of the actual value of D is desired.

While this method provides an excellent initial estimate of preservative activity in parenteral solutions, it is not intended to evaluate other issues concerning antimicrobial preservatives, such as stability and compatibility characteristics in the final formulation and package system. The most promising systems identified by this method will be studied in greater detail for compliance to compendial standards of effectiveness, stability, and compatibility in the newly developed parenteral formulation.

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