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D-Value Determinations Are an Inappropriate Measure of Disinfecting Activity of Common Contact Lens Disinfecting Solutions

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Determination of a D value for specific test organisms is a component of the efficacy evaluation of new contact lens disinfecting solutions. This parameter is commonly defined as the time required for the number of surviving microorganisms to decrease 1 logarithmic unit. The assumption made in establishing a D value is that the rate of kill exhibits first-order kinetics under the specified conditions. Such exponential kill rates are seen with thermal contact lens disinfection systems. A comparison of the death rate kinetics for a variety of chemical contact lens disinfecting solutions was undertaken to ascertain the suitability of D-value determination for these chemical disinfectants. The active agents of these different solutions included hydrogen peroxide, thimerosal, chlorhexidine, tris(2-hydroxyethyl)tallow ammonium chloride, thimerosal, polyaminopropyl biguanide, and polyquaternium-1. The solutions were challenged with 10^6 CFU of either *Pseudomonas aeruginosa*, *Serratia marcescens*, or *Staphylococcus hominis* per ml, and survival rate was determined. This study clearly demonstrates the nonlinear nature of the inactivation curves for most contact lens chemical disinfecting solutions for the challenge organisms. D-value determination is, therefore, an inappropriate method of reporting the biocidal activity of these solutions.

The disinfection efficacy of various contact lens disinfecting solutions is currently measured in D values. The use of this measurement grew out of thermal disinfection studies from the dairy and food industry (3, 4, 8). The rate of kill (the $\log_{10}$ number of surviving bacterial cells versus time) in the presence of constant heat was an exponential function. Therefore, when the $\log_{10}$ numbers of the survivors were plotted against time, the relationship was described by a straight line. The D value is broadly defined as the time required for the number of viable bacteria to decrease 1 logarithmic unit (or the negative reciprocal of this slope). This D value is then used as a predictor for responses beyond the data to estimate the time required for disinfection (10^{-3} CFU/ml) or sterilization (10^{-6} CFU/ml). The underlying assumption when utilizing this measure is that the relationship between the $\log_{10}$ number of survivors and time is linear (1, 5, 27, 28). The early theoretical work in this field clearly describes the restriction that the D value has meaning only for those situations where the kinetics of kill are first order (3–5, 7, 8, 27–29).

This assumption holds true, in general, for thermal disinfection of contact lenses (9, 10). However, the application of D-value determination is gaining acceptance in the pharmaceutical industry (13, 18–21) as a measure of chemical disinfection efficacy in the absence of demonstrated first-order kill kinetics. In addition, D values have been suggested as rapid indicators of preservative efficacy (2, 19, 20). Although the rate of bacterial death in some chemical preservative formulations may follow first-order kinetics (6), this seems to be the exception rather than the rule (11, 16, 22, 23, 31).

There are three methods for the determination of D value from kinetic kill information (32). The most commonly used is the Stumbo or end-point method utilizing only initial and terminal time points. In this method, a known amount of bacteria is exposed to the disinfecting conditions for a specified amount of time, and the number of survivors is determined. The D value is defined as the ratio of time to the $\log_{10}$ of the reduction in CFU per milliliter. The second method involves averaging multiple end-point D values, one for each sampling, derived over the course of an experiment. The third, statistical, approach utilizes linear regression analysis of multiple data points.

All three of these methods will provide similar values for rate of kill when the kinetics of kill are first order. If the relationship between $\log_{10}$ number of survivors and time is not linear, however, very different measurements will result. These differences will have a direct effect on the final marketed product. Current guidelines set the recommended soak time for contact lens disinfecting solutions as equivalent to 9 D values (32).

Although a straight line has only a single slope, a curvilinear line has many. Accordingly, the three methods for D-value determination of contact lens disinfecting solutions have the potential for disparate and inaccurate representation of disinfecting efficacy in cases of nonlinear kinetics.

The present study evaluated several different, currently marketed contact lens disinfecting solutions. They were tested for disinfecting efficacy against *Staphylococcus hominis*, *Pseudomonas aeruginosa*, and *Serratia marcescens*, organisms representative of ocular bacterial pathogens. Previous reports provided D values for several of these solutions without consideration of linearity (21–24), a necessary precondition. In the present study, the inactivation curves

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TABLE 1. Commercial soft contact lens disinfecting solutions tested

Solution	Active agent(s)
Flex-Care (for Sensitive Eyes)	Chlorhexidine (0.005%) Edetate disodium (0.1%)
Soft-Mate Disinfecting Solution	Chlorhexidine (0.005%) Edetate disodium (0.1%)
Bausch & Lomb Sterile Disinfecting Solution	Chlorhexidine (0.005%) Thimerosal (0.001%) Edetate disodium (0.1%)
Allergan Oxysept Disinfecting Solution	Hydrogen peroxide (3%)
Allergan Hydrocare Cleaning and Disinfecting Solution	Tris (2-hydroxethyl) tallow ammonium chloride (0.013%) Thimerosal (0.002%)
Bausch & Lomb ReNu Multi-Purpose Solution	Polyaminopropyl biguanide (Dymed) (0.00005%)
Opti-Free Rinsing, Disinfecting & Storage Solution	Polyquaternium-1 (Polyquad) (0.001%) Edetate disodium (0.05%)

were statistically analyzed for linearity and equivalence among the different methods of D-value determination.

MATERIALS AND METHODS

Test organisms. Bacteria were grown to confluence on slants of Trypticase soy agar (Difco Laboratories, Detroit, Mich.) and harvested into phosphate-buffered saline (PBS; pH 7.2). Samples were washed twice in PBS and then standardized to 10^8 CFU/ml by turbidimetric reading (Spectrometer 20; Bausch & Lomb, Rochester, N.Y.). Fresh suspensions of test organisms were prepared each day. Test organisms included *S. hominis* (ATCC 17917), *P. aeruginosa* (ATCC 15442), and *S. marcescens* (ATCC 14041).

Test solutions. A variety of ophthalmic disinfecting solutions were utilized in this study. A list of these is provided in Table 1 with a description of the active agents in each formulation. Each solution was assigned a unique solution code for identification in Fig. 1 and Tables 2 to 4. These solutions were not arranged in order in Table 1 in the same sequence as the codes were assigned.

Procedure. Samples (10 ml) of commercial soft contact lens disinfecting solutions (Table 1) were inoculated with 10^6 CFU of the specific test bacterium per ml in polystyrene tubes (Falcon Plastics—Becton Dickinson Labware, Oxnard, Calif.). The inoculated disinfecting solutions were then incubated at 20 to 25°C, and aliquots of the bacterial suspension were removed at different times and diluted 1:10 into Dey-Engley Broth (Difco). Duplicate samples from each solution were then plated in Dey-Engley agar. Each solution was therefore tested in duplicate, with two platings performed at each time point for each solution. Several samples were taken during the course of each disinfecting period. The total time spent in the disinfecting solution was determined by the soaking time recommended by the manufacturer for soft contact lens disinfection. The Dey-Engley agar plates were incubated at 30 to 35°C for 48 to 72 h. This extended incubation period was employed to allow growth of chemically damaged bacteria. The numbers of survivors were calculated by averaging the numbers recovered from the duplicate plates.

Hydrogen peroxide solutions were neutralized before plating by being diluted 10-fold into peroxide neutralizing buffer. This buffer consisted of 1% peptone, 0.8 mM phosphate buffer (pH 7.2), and 1.2×10^4 U of thymol-free catalase from bovine liver (Sigma Chemical Co., St. Louis, Mo.) per ml.

Analysis of data. All determinations were made by using normalized data points where the number of survivors was described as the $\log_{10}$ value of the fraction remaining of the original inoculum, normalized to an inoculum of 1.000×10^6. The time course incorporated at least four time points except where a rapid rate of kill precluded repeated sampling. Two replicates (each was plated in duplicate and the results were averaged) were compiled on each of two separate days, resulting in four data points.

(i) Average D value. D values were determined independently for each datum point as the time of exposure divided by the reduction in number of microorganisms ($\log_{10}$). Average D values were calculated by determining the mean for the D values from each datum point. The time course experiment was terminated at the point which described no surviving bacteria. This end point was defined by the assay as ≤ 3 CFU on a dilution plating of 10^{-1} from the initial inoculum.

(ii) End-point D value. The end-point D value was reported as the determination of D value for the final time point by the method outlined above. The final time point was defined as the first time point that showed no surviving bacteria (i.e., ≤ 30 CFU/ml).

(iii) Regression. The D value was taken as the negative reciprocal of the slope of the regression line without requiring the intercept to pass through the origin. The coefficient of determination value was derived by determining the ratio of the regression sum of squares to the total sum of squares (33). This value describes the proportion of the total variation of survivors described by the fitted regression line.

(iv) F test for linearity. Analysis of variance was performed on the data to determine their linearity as described by Zar (33). The ratio of deviations from linearity mean square to within-groups mean square will equal 1 if the data are described by a straight line. This ratio will be greater than 1 if the data are not described by a straight line because deviations from linearity will exceed the variability of the data. Probability that the data are described by a straight line was derived from a table of critical F values.

Graphic depiction of deviations from linearity were plotted as residuals from the regression against the estimate. An unequal distribution of the residual values around zero is indicative of heteroscedasticity.

(v) T test for similarity of slopes. The slope of the linear regression line for each survivor curve was compared with the hypothetical slopes of the lines determined by the end-point D values and averaged end-point D values. The slopes of the hypothetical lines were taken to be the negative reciprocal of the respective D values. Student's t statistic was used to test the comparisons at P equal to 0.05 as described by Zar (33).

RESULTS

Figure 1a, c, and e provide examples of inactivation curves prepared during this study. They are presented only to illustrate the analysis. The linear regression line is a predictor of values in this data set within the limits of the observations for all three of these solutions with *S. marcescens*. However, a significant amount of heteroscedasticity is demonstrated in the residual plots of solutions 2 and 7. A measure of the proportion of the variability in number of survivors explained by the linear regression line is the coefficient of determination.

Table 2 provides derived values for the coefficient of determination values (R^2) of the data fitted to a straight line. The R^2 value is a measure of the amount of variability explained by the model. A test of goodness-of-fit of this linear model is the F test provided by analysis of variance (33). The probability that the data are described by linear regression is shown in Table 2. Few of the survivor curves derived in this study are linear in nature as shown by values of $P \leq 0.05$. The number of data points utilized in the analysis is shown as N (Table 2).

The three methods for determination of D values for ophthalmic disinfecting solutions assume linear kinetics. If the kinetics are not linear, then these different methods may yield differing values. Table 3 provides a comparison of D values derived by these methods in an assessment of currently marketed disinfectants. As seen in Table 3, the different methods for D-value determination can yield very different results for a given contact lens solution. The statistical significance of these differences was measured by a t test described by Zar (33). Significant differences exist among the three methods for D-value determination.

DISCUSSION

Determination of efficacy of a new soft contact lens disinfecting solution includes both a carrier test and a suspension efficacy test. The carrier test is performed in the presence of organic soil by inoculating soft contact lenses with 10^6 CFU of the challenge organisms. The recommended disinfection or cleaning regimen is then performed after a brief incubation period.

The suspension test is performed with an inoculum of 10^6 CFU of the challenge organism without organic matter. The parameter reported is the D value or amount of time required to reduce the levels of recoverable microorganisms by a factor of 10. This determination assumes first-order kinetics. For example, the D value could be extremely misleading as a predictor of efficacy if the amount of time required to reduce the viable microorganisms from 10^6 to 10^5 CFU/ml was different from that required to reduce the same bacterial suspension from 10^4 to 10^3 CFU/ml.

Accurate determination of the rate of cell survival depends upon neutralization of the disinfecting agent at a specific time point. Low levels of residual disinfectant could inhibit growth, leading to an overestimation of kill (26). Similarly, the agents used to neutralize the disinfectant must not inhibit growth. Neutralization was accomplished in this study through dilution and plating in Dey-Engley media (15, 17), a process which effectively neutralizes all contact lens disinfecting solutions tested (30) except for the 3% hydrogen peroxide system. This solution was neutralized in peroxide neutralizing buffer before being plated.

The general protocol for determination of kill kinetics (32) differs from other suspension tests in several details. This general protocol avoids the problems inherent in determining a phenol coefficient as described by the procedures of Rideal and Walker (25) and Chick and Martin (12) and by a variety of international procedures (for a review, see reference 14) for solutions differing in mode of action and dilution coefficients. This test also differs in the bacteria chosen for demonstration of efficacy. These bacteria include *S. marcescens*, *P. aeruginosa*, and *S. hominis*. The only concentration of disinfectant evaluated is that of the full formulation, distinguishing it from the use-dilution methods. Finally, it departs from the qualitative tests in that specific numbers of organisms are determined, giving useful information on the kinetics of bacterial kill.

The so-called D-value protocol is designed to provide kinetic information on disinfecting efficacy and, therefore, to serve as a predictor for the recommended soak time. The recommended soak time for contact lens disinfection is the extrapolated period of time sufficient for a 10^9 reduction (i.e., 9 D values) of microorganisms from the initial 10^6 inoculum (32). This predictive power is presumed to be provided by use of the D value. Therefore, in those cases where kill kinetics are nonlinear, the validity of the predicted extent of disinfection may be compromised. Several investigators have presented D values for contact lens disinfecting solutions despite the nonlinearity of the data (21–23). This inaccuracy for contact lens disinfecting solutions was first noted by Houlsby et al. (16). The fallacy of applying D values to these types of data was discussed fully in the early literature on D values (3–5, 7, 28, 29).

An example of the graphic evaluation of the linearity of the data is shown in Fig. 1 for *S. marcescens* and three disinfecting solutions. In this analysis, the residuals of the regression analysis are plotted against the estimates. The residual data points for solutions 2 and 7 are not uniformly scattered about the estimate but rather show clear correlation with the estimates (Fig. 1b and d). This heteroscedasticity decisively demonstrates the inappropriateness of forcing a linear model through these data. An example of homoscedasticity is provided by solution 3 (Fig. 1f). These data are well described by the regression line, and all variability from the line is due to the inherent variability of the system within the observed range. Even this example, however, shows increasing heteroscedasticity with lower estimates. This may indicate that the fewer the remaining organisms, the greater the departure from linearity.

Table 2 examines the linear nature of the data for the disinfecting solutions and organisms within the time points. The correlation of determination values describes the proportion of the survivor variability predicted by the regression line. The lower values (i.e., less than 0.8) reflect poor correlation with the linear regression. These low R^2 values could be due to data points that are essentially linear but are scattered about the regression line. Alternately, the data

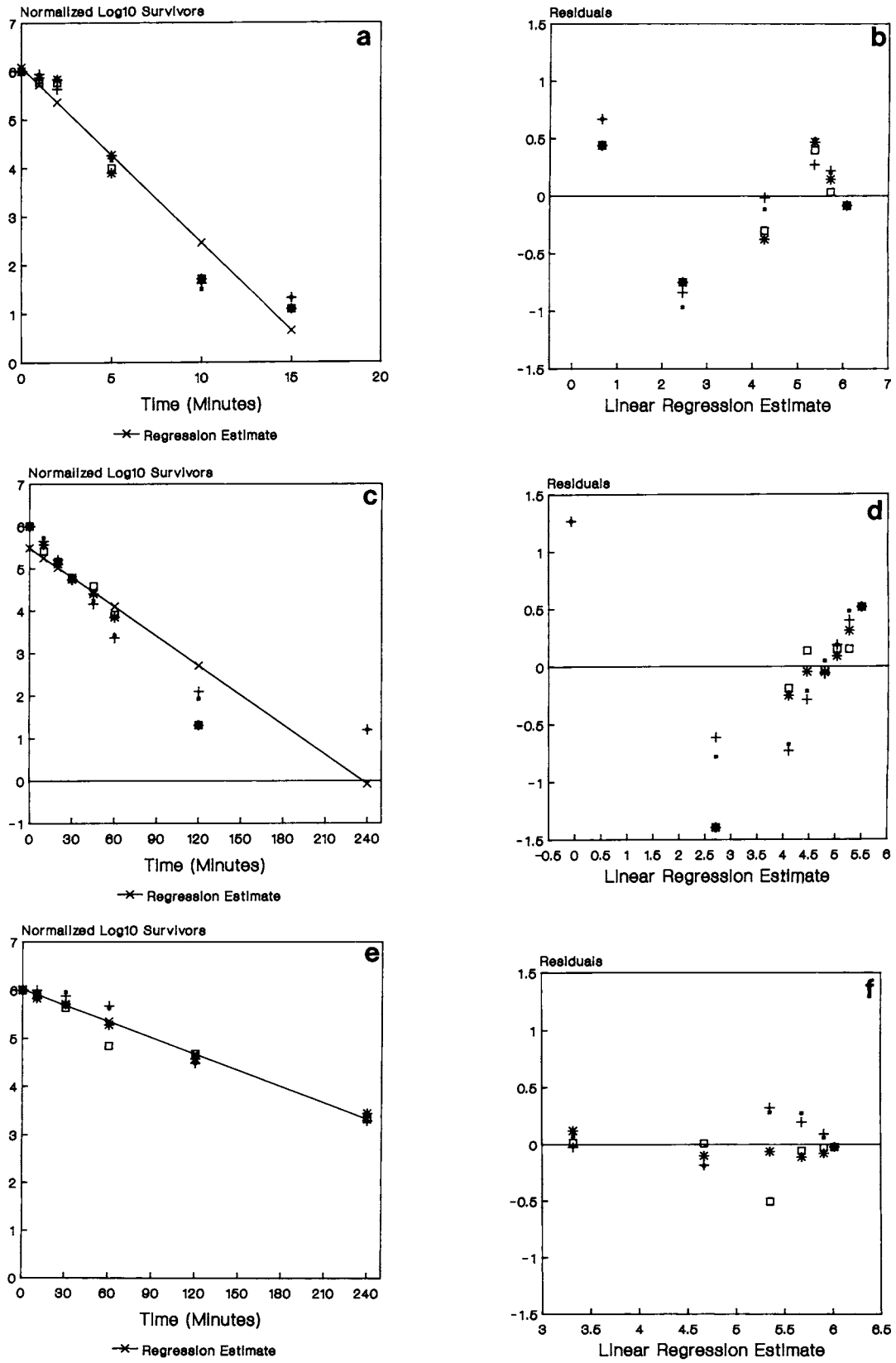

FIG. 1. Linear regression modeling of data. Examples of data from the experiments with *S. marcescens* and solutions 2 (a and b), 7 (c and d), and 3 (e and f). These data were plotted with the regression lines shown (graphs a, c, and e) as well as the data points from four experiments. The residual plots against the estimate are shown in panels b and d, demonstrating the heteroscedasticity of the residuals. The residual plot f demonstrates homoscedasticity. See text for details.

TABLE 2. Linearity of survivor curves[a]

Organism	Solution	R^2	P by F test	N
P. aeruginosa	1	0.637	<0.0005	16
	2	0.621	0.0025	14
	3	0.929	0.025	12
	4	0.867	>0.25	20
	5	0.742	<0.0005	32
	6	0.655	<0.0005	16
	7	0.812	<0.0005	10
S. hominis	1	0.902	<0.0005	12
	2	0.817	0.025	20
	3	0.888	0.05	20
	4	0.647	>0.25	26
	5	0.308	0.005	22
	6	0.659	<0.0005	22
	7	0.943	<0.0005	20
S. marcescens	1	0.909	0.25	20
	2	0.846	<0.0005	30
	3	0.968	>0.25	24
	4	0.806	>0.25	24
	5	0.823	>0.25	24
	6	0.694	0.0025	30
	7	0.947	<0.0005	24

[a] The R^2 values were derived by least-squares analysis of the data. The F test utilized analysis of variance to determine the ratio of mean squares for the deviations from linearity among groups to variation within groups (33). The size of the population (*N*) is provided.

could be of high quality but are not best described by a straight line. The F test with analysis of variance was used to measure the probability of linearity.

The coefficient of determination and the F test measure two different properties of the data. The R^2 value is a measure of the amount of variability explained by the model, while the F test compares the variability of the data with deviations from the regression model. If the data are linear in nature, then the inherent variability of the data will be equivalent to the deviations from linearity. If the data are not linear, then the deviations from linearity mean square will exceed that within groups.

It is striking that, for the seven disinfecting solutions analyzed with three different organisms, there were 15 cases in which the data were not significantly linear in nature ($P \leq 0.05$). That is, most of the survivor curves evaluated in this study were curvilinear within the time points examined. Several of the solutions studied displayed a high coefficient of determination for the linear regression, while the F test indicated nonlinear data. These findings underscore the inaccuracy of extrapolations beyond the observed data from a regression line in a biological system.

The effects of nonlinear kill kinetics on D-value determination are evident in the divergent values reported in Table 3. The three methods for determination of D values do, in theory, provide consistent results. However, they all assume first-order kill kinetics. In the absence of a straight-line relationship between $\log_{10}$ survivors and time of exposure to the disinfectant, these methods are no longer consistent. For example, solution 4 and solution 5 might be compared for disinfection efficacy on the basis of reported D values. In this comparison, solution 4 is superior as a disinfectant for *P. aeruginosa* when the analysis is performed by linear regression. If, however, the data analysis is performed by the end-point averaging analysis, then solution 4 is determined to be a much less efficacious solution.

TABLE 3. Derived D values for solutions tested

Organism	Solution	D values (min) as determined by[a]: Linear regression	End point analysis	Avg for curve
P. aeruginosa	1	0.8	0.7	0.4
	2	0.5	0.5	0.3
	3	0.3	0.3	0.2
	4	29.2	29.8	28.5
	5	68.6	58.8	25.8
	6	8.0	6.3	4.3
	7	0.2	0.2	0.2
S. hominis	1	0.6	0.7	3.2
	2	1.1	1.1	0.8
	3	1.1	1.1	1.4
	4	463.6	409.2	298.5
	5	81.2	76.9	30.0
	6	15.1	12.2	7.6
	7	1.0	1.1	1.2
S. marcescens	1	3.1	3.2	5.6
	2	43.2	50.0	29.0
	3	88.6	91.0	103.8
	4	383.3	421.8	402.6
	5	138.9	142.1	223.3
	6	55.3	58.8	26.2
	7	2.8	3.1	4.9

[a] The reported D values were calculated by the three currently accepted methods. Linear regression analysis of the data was performed by least-squares analysis (33). End-point analysis was performed by the method of Stumbo (27). Averaged D values were calculated by treating each datum point as the end point and averaging the resultant values. The analysis concluded at the first time point resulting in complete kill (defined as ≤3 CFU on a 10^{-1} dilution).

The differences among these D-value determinations are significant on two levels. In the first, there is a statistically significant difference among the values, particularly between the linear regression value and the averaged D value. This is demonstrated in Table 4 by use of a one-tailed *t* test. The averaged D-value method gives a high level of false values with curvilinear data as compared to the linear regression method. The second level of significance revolves around the use of these differing D values as predictors of efficacy. A statistical significance might not be demonstrable between a D value of 138.9 and 223.3 min for *S. marcescens* in solution 5 with these data. However, this ambiguity in

TABLE 4. Results of *t* test between linear regression D values and other estimates of D values[a]

Solution	P					
	P. aeruginosa		*S. hominis*		*S. marcescens*	
	End point	Avg	End point	Avg	End point	Avg
1	0.25	<0.0005	>0.25	<0.0005	>0.25	<0.0005
2	>0.25	0.01	>0.25	0.005	0.1	<0.0005
3	>0.25	0.25	>0.25	0.05	>0.25	0.1
4	>0.25	>0.25	>0.25	0.1	>0.25	>0.25
5	0.25	<0.0005	>0.25	<0.0005	>0.25	0.025
6	0.25	0.025	>0.25	0.05	>0.25	<0.0005
7	>0.25	0.25	>0.25	0.05	0.1	<0.0005

[a] The agreement between the linear regression slope and the hypothetical slopes as determined by end-point analysis and averaged end-point analysis was evaluated by a *t* test as described by Zar (33).

interpretation of the same data set is not an satisfactory situation for comparing disinfecting efficacies.

The D value is a useful predictor of efficacy and appropriate to use where the kill kinetics are linear, as is the case with thermal disinfection (9, 10, 26, 27). D values are inappropriate and may be misleading, however, in situations where the relationship between the $\log_{10}$ number of survivors and time of exposure to the disinfecting condition is not first order (3–5, 7, 8, 27–29). Lacking demonstrable linear kill kinetics, the D value cannot be utilized as a predictor of disinfection efficacy for chemical contact lens disinfecting solutions.

Disinfecting efficacies of currently marketed contact lens disinfecting solutions are not described by first-order rate kinetics. Since the validity of the D value as a predictor of efficacy depends upon first-order kinetics, the D value is not an appropriate measure of the disinfecting efficacy of contact lens disinfecting solutions. In addition, the three currently accepted methods to approximate a D value are not applicable without linear kinetics and can lead to conflicting estimations of efficacy.

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