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REPRINTS

A Rapid Screening Test For Ranking Preservative Efficacy

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Microbial contamination of pharmaceutical and cosmetic products is of concern despite the fact that numerous official and unofficial tests are available to predict preservative efficacy (**USP, CTFA, ASTM**). Ramp and Witkowski (1975) proposed more extensive testing than the method described in the **USP** with respect to increasing (a) the spectrum of challenge organisms and (b) the duration of the experiment from 28 to 63 days. This approach, in which increased sensitivity is attempted, is costly and does not address the real need for rapid assessment during product development.

The official **U.S. Pharmacopeia** Preservative Efficacy Test requires more than four weeks to complete. It is a "Pass/Fail" antimicrobial test rather than a quantitative determination of preservative capacity. Quick semiquantitative screening tests are needed for the early stages of formulation development. Such tests can (a) expedite selection of the effective preservative or formulation, (b) allow a rational determination of optimum concentration and (c) monitor preservative concentration in a new formulation for a long-term stability study program.

During the early stages of formulation development, a simple and rapid quantitative test is essential for preservative selection. Not only can substantive judgments be made on the chemical agent to be used, but additional data can be obtained on concentration optima, and stability. Several rapid methods have been reported (Alegnani 1973; Prince 1975; Davies **et al.** 1976; Orth 1979; Hob **et al.** 1979), but the majority of these rapid tests involve construction of time/log survivor curves during a short-term exposure period and require considerable amounts of sample and media.

A presumptive challenge test based upon a 24-hour tube dilution experimental model is described on these pages. Data from these tests, compared with those obtained from the standard USP challenge test and from a rapid kill curve model test employing short term rate of death, showed that conventional formulations, with varying degrees of efficacy as judged by the MIC or D-value methods, passed the USP challenge test with equal merit. Since the USP test predicts neither for failure in the field nor failure in production, it is essential to incorporate a safety or "overkill" factor into the product. The MIC and rapid kill techniques allow for this approach since they were able to rank efficacy of otherwise similar formulations. Ideally, a formulation with the highest endpoint in the MIC dilution test and the lowest D_{10}-value in the rapid kill test will pass a **USP**-type challenge test with greater assurance that eventual microbial failure will not occur. This approach is similar to the concept now employed for validation of sterilization processes. A USP-type antimicrobial effectiveness test, like a sterility test, should provide confirmation and not **proof** that a product is adequately preserved.

The proposed rapid test can be used to compare and rank preservative efficacy of a given set of samples with that of a known or standard formulation, or help predict the optimum concentration of antimicrobial agent prior to costly product development. In combination with D_{10}-value analysis from a rapid kill curve model, the presence of synergism from mixed antimicrobial systems can be detected. The presumptive challenge test reported here is an adaption of the minimum inhibitory concentration (MIC) procedure, which is widely used for antibiotics by the clinical microbiologists and for drug screening by industrial microbiologists. Formulations with various preservatives were studied in an attempt to decrease the time of the test as well as to determine if one could detect synergisms in the case of mixed agents.

The panel of microorganisms used were those listed in USP XX for challenge study: **Aspergillus niger** ATCC 16404, **Candida albicans** ATCC 1023.1, **Escherichia coli** ATCC 8739, **Pseudomonas aeruginosa** ATCC 9027, and **Staphylococcus aureus** ATCC 6538.

Two types of samples were used in this study. The first consisted of commercially available pharmaceutical products (Bacteriostatic water for injection,

TABLE 1
USP PRESERVATIVE EFFICACY CHALLENGE STUDY OF
BACTERIOSTATIC WATER FOR INJECTION

MICROBES	TIME	A PARABENS 0.132%	B BENZYL ALCOHOL 0.9%	C PARABENS 0.2%	D PARABENS 0.132% + BENZYL ALCOHOL 0.9%	SALINE CONTROL
A. NIGER ATCC 16404	ZERO	6×10^5	8.6×10^5	6.4×10^5	6.8×10^5	6.0×10^5
	1 wk	$<10^1$	$<10^1$	$<10^1$	$<10^1$	1.2×10^6
	2 wks	$<10^1$	$<10^1$	$<10^1$	$<10^1$	8.0×10^4
	3 wks	$<10^1$	$<10^1$	$<10^1$	$<10^1$	2.0×10^5
	4 wks	$<10^1$	$<10^1$	$<10^1$	$<10^1$	1.2×10^5
C. ALBICANS ATCC 10231	ZERO	2×10^5	5.5×10^5	3.4×10^5	2.2×10^5	1.8×10^5
	1 wk	$<10^1$	$<10^1$	$<10^1$	$<10^1$	3.0×10^5
	2 wks	$<10^1$	$<10^1$	$<10^1$	$<10^1$	5.0×10^5
	3 wks	$<10^1$	$<10^1$	$<10^1$	$<10^1$	3.0×10^5
	4 wks	$<10^1$	$<10^1$	$<10^1$	$<10^1$	2.2×10^5
P. AERUGINOSA ATCC 9027	ZERO	5×10^6	3.1×10^6	2.5×10^6	1.0×10^6	1.0×10^7
	1 wk	$<10^1$	$<10^1$	$<10^1$	$<10^1$	$>1.0 \times 10^7$
	2 wks	$<10^1$	$<10^1$	$<10^1$	$<10^1$	1.0×10^8
	3 wks	$<10^1$	$<10^1$	$<10^1$	$<10^1$	1.0×10^8
	4 wks	$<10^1$	$<10^1$	$<10^1$	$<10^1$	2.0×10^8

Note: All formulations appear to be equally effective.

Invenex, with methylparaben 0.12% plus propylparaben 0.012%; and bacteriostatic water for injection, **Penta Products** with benzyl alcohol 0.9%). The second consisted of samples prepared by Syntex product development (bacteriostatic water with the following antimicrobials: methylparaben 0.12% and propylparaben 0.012%; benzyl alcohol 0.9%; methylparaben 0.18%; and propylparaben 0.02%).

Methods And Materials

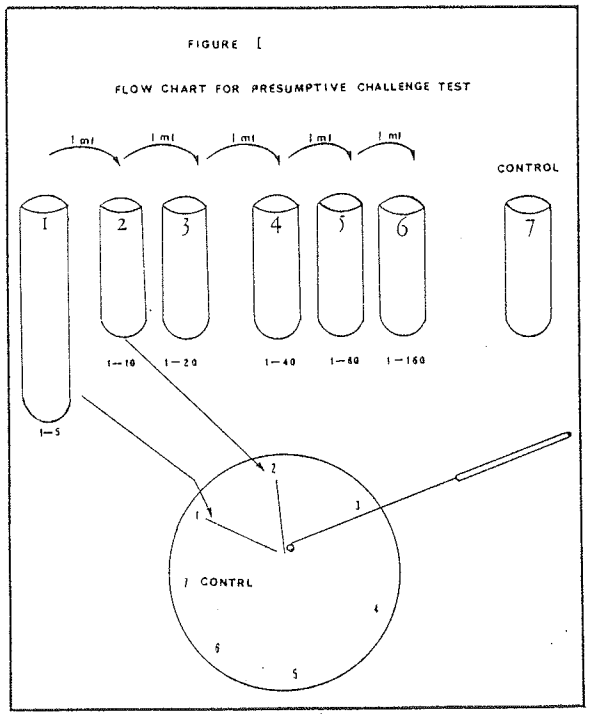
Two types of diluents were used for these studies. Saline, 0.85%, was used for the presumptive challenge test and the USP challenge study. Letheen Broth (DIFCO Laboratories) was used so as to provide rapid neutralization of carry-over preservative for the rapid kill curve study. Letheen agar was used as the solid media for the presumptive challenge test and STAT agar (trypticase soy agar + 2% Tween 80 + 0.5% lecithin for the rapid kill curve study). Tryptic soy agar or trypticase soy agar (TSA) (DIFCO and BBL, respectively) were used for the USP challenge study.

The presumptive challenge test is designed to test the ability of a range of saline dilutions of the product to prevent growth of approximately 10^6 organisms after 24 hours under non-nutrient conditions. Thus, preservative concentration and not exposure time is the variable.

Procedure for Presumptive Challenge Test (MIC assay) (see Figure 1).

1. Stock inocula for each of the cultures to be assayed were prepared by suspending in saline the growth form 18 to 24 hour TSA cultures of bacteria, 24 to 48 hour old **TSA** cultures of yeast, or 10 to 14 day potato dextrose or Sabouraud agar cultures (the **A. niger** saline was supplemented with 0.1% triton X-100 to facilitate harvest of mold spores). The viable microbial population of each inoculum was adjusted by plate count to 10^8 cfu/ml. This was labeled **stock inoculum** for each microorganism in the panel.

Separate **inoculated diluent** was prepared by



adding 1.0 ml of the appropriate **stock inoculum** to 100 ml saline (approximately 10^6 cfu/ml). The inoculated diluent thus prepared was thoroughly mixed before dispensing and served as the product diluent for the test.

2. A series of eight sterile tubes were arranged for each microorganism in the panel. Four ml of **inoculated diluent** was added to the first tube and 1.0 ml to each of the small tubes.

3. The test was conducted by adding 1.0 ml of the test sample or product to each of the 4.0 ml tubes containing **inoculated diluent** (1-5 dilution of product). One ml of this suspension then was added to the first small tube containing inoculated diluent and mixed well. Thereafter, 1.0 ml portions were serially transferred as two-fold dilutions to the geometric level desired. The last tube served as the preservative-free growth control. Test samples and controls were incubated at 35°C for 24 hours, after which time the presence or absence of survivors was determined by surface streak onto sectored Letheen agar plates which were incubated at 35°C for 24-48 hours. At the end of the incubation period, the degrees of growth were scored as follows:

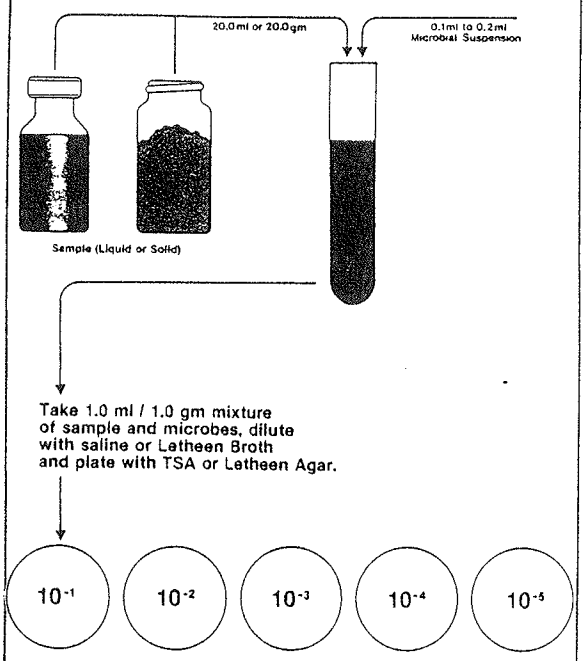
		Interpretation
0	= no growth (active endpoint)	Active
+	= ≤15 colonies (borderline)	Active
++	= 50% of control	Borderline
+++	= 75% of control	Inactive
++++	= 100% of control	Inactive

Procedures for Rapid Kill Curves and the USP Challenge Test (See Figure 2)

The rapid kill curve and USP challenge tests differed mainly in respect to sampling times; the samples were challenged and enumberated in an identical manner. Stock inoculum was prepared for each of the cultures to be assayed as described above. At zero time, 0.1 ml of bacterial suspension was added to 20 ml of test sample for a zero-time population of approximately 10^6 cfu/ml. For a positive growth control, the same volume of inoculum was added to 20 ml of sterile saline. After each inoculation, the inoculated sample was vigorously shaken with a vortex mixer or stirred in the case of cream. 1.0 ml/gm was removed and transferred to 9.0 ml of Letheen broth for rapid kill curve test and to saline for the USP challenge. From the 10^{-1} dilution so prepared, serial dilutions to 10^{-6} were made the plate counts used to determine survivors (35°C, 24-48 hours). All test samples were incubated at room temperature. The USP test was graded according to the specifications set forth in USP XX. Accordingly, it was viewed in terms of its intent, i.e., to judge the antimicrobial effectiveness of articles in the compendium. Calculations of the D_{10}-value for the rapid kill curve were made according to the STUMBO equation:

FIGURE 2

FLOW CHART FOR USP CHALLENGE AND RAPID KILL CURVE PROCEDURE



$$D_{10} = \frac{\text{time}}{\log_{10} N_0 - \log_{10} N_1},$$

where N_0 = initial cell population,

N_1 = survivors at time (t). Alternatively one can strike off l-log intercepts from the survivor curve and read the D-value from the ordinate.

D_{10} = the time required for a l-log kill.

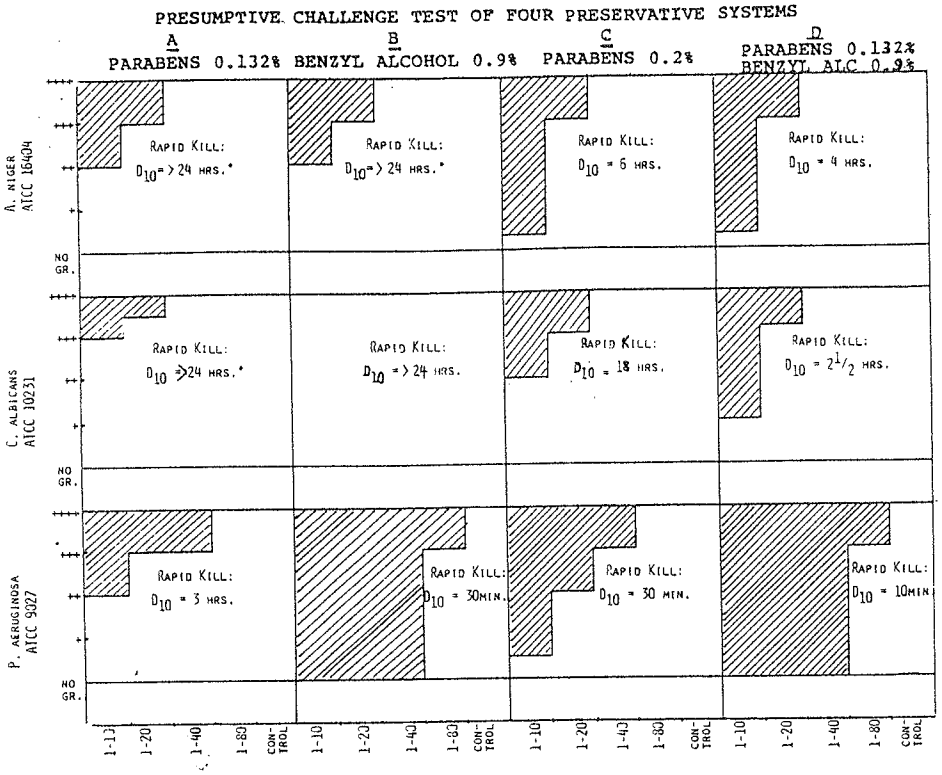
The test samples and the saline positive controls were resampled at the designated time intervals: seven, 14, 21, and 28 days for USP challenge study and several points between zero time and 14 hours for rapid kill curves (e.g. one, two, six, 12-18, and 24 hours).

For baseline data, the USP challenge test was used to screen preservative efficacy of four bacteriostatic waters: (A) parabens 0.132%, (B) benzyl alcohol 0.9%, (C) parabens 0.2% and (D) parabens 0.132% plus benzyl alcohol 0.9%. Although the USP challenge results (Table I) showed that all samples passed the test, no determination of their comparative preservative efficacy was possible due to the method's low sensitivity.

The same bacteriostatic water samples then were tested by the presumptive challenge test. The results of this study (Figure 3) showed that high paraben con-

FIGURE 3

PRESUMPTIVE CHALLENGE TEST OF FOUR PRESERVATIVE SYSTEMS



NOTE:
THE GREATER THE AREA ABOVE THE HISTOGRAM, THE GREATER THE ACTIVITY PREDICTED BY THE PRESUMPTIVE METHOD.

* PRESUMPTIVE CHALLENGE METHOD SHOWS MINIMAL ACTIVITY WHERE RAPID KILL CURVE SHOWS NO ACTIVITY.

centration (0.2%) was more effective than lower concentration (0.132%) against all microorganisms in the panel. Benzyl alcohol (0.9%) was less effective than the parabens at high concentration against **A. niger, C. albicans** and **S. aureus** (not shown). However, benzyl alcohol appeared to be more effective than the high paraben concentration against **P. aeruginosa.** Benzyl alcohol appeared less effective than the paraben concentration against **Candida** but showed similar efficacy against **A. niger;** it appeared to be superior to the low concentration of parabens against bacteria when tested against **Psuedomonas.** However, the combination of parabens at the low concentration of (0.132%) and benzyl alcohol (0.9%) showed efficacy against two out of three organisms than either of the components alone. It is important to point out that the presumptive test determined differences amongst the four formulations not otherwise detected by the USP test.

The rapid kill curve tests (Figures 4, 5 and 6) showed that benzyl alcohol (0.9%) was only slightly more effective than the parabens at low concentration (0.132%) against **A. niger** and **C. albicans** but superior against **P. aeruginosa.** On the other hand, parabens at high concentration (0.20%) were superior to benzyl alcohol (0.9%) against **Aspergillus** and **Candida** and equal against **Psuedomonas.** However, a combination of low concentration of parabens with benzyl alcohol was superior to either alone, a confirmation of the synergistic effect detected when

FIGURE 4

RAPID KILL CURVES OF 4 PRESERVATIVES VS A. niger

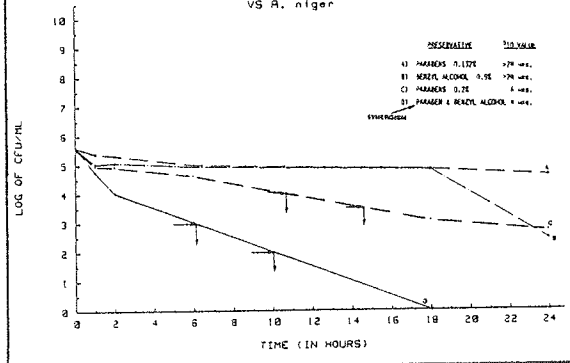
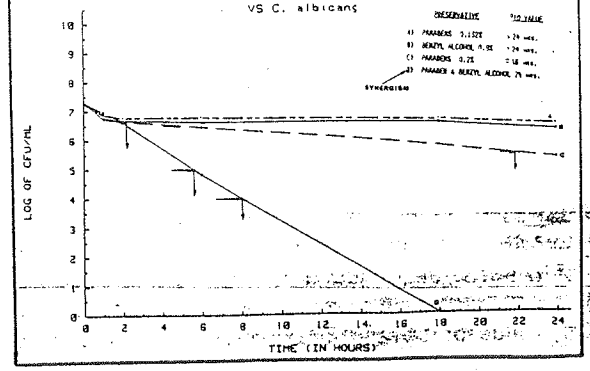


FIGURE 5

RAPID KILL CURVES OF 4 PRESERVATIVES VS C. albicans



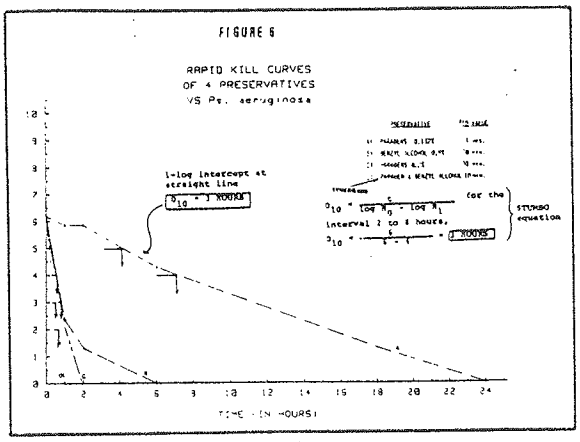
the assays were performed according to the presumptive challenge test.

Data from the presumptive challenge test, the rapid kill curve method and the USP challenge study are compiled and summarized in Table II. The ranking collation shown reveals that the USP test is non-discriminatory. The rapid kill curve method showed "no effect" in 4/12 test cases and one-increment ranking ability. The presumptive MIC test showed "no effect" in only 1/12 test cases and produced 1 as well as 2-increment ranking ability.

Results depicted in Figure 3 show the correlation obtained between the two rapid methods reported here. Both methods were superior to the USP test in that preservative efficacy (or lack of it) were determined in approximately 48 hours. The greater sensitivity of the presumptive challenge technique (MIC) is seen, however, for **Aspergillus niger** and **Candida albicans.** For **A. niger** increasing activity in the presumptive test correlated well with decreasing D-10 values in the rapid kill test. However, the latter test did not detect activity against this organism (D-10 $\geq$ 24 hours) for the 0.132% paraben and 0.9% benzyl alcohol formulations, whereas an antimicrobial effect was seen in the MIC assay. Similarly, for **C. albicans** the rapid kill test did not detect activity (D-10 > 24 hours) for the 0.9% benzyl alcohol formulation. It should be pointed out that both methods rapidly detected the synergistic nature of the combination of parabens (0.132%) and benzyl alcohol (0.9%).

The data reveal that the presumptive challenge MIC test can be used as a quick test during development stages of a large group of samples and that it can also be used as a rapid quality control procedure to compare preservative efficacy of a newly manufactured lot

FIGURE 6



with that of a standard. The data also show that it is a semi-quantitative test and that the results can be correlated with time/log killing of microorganisms in the presence of the same preservative system. It is designed as a rapid method to reduce amount of USP testing, but not as a replacement for the compendial method. The USP test was designed as a quality and/or regulatory assessment of the antimicrobial effectiveness of USP articles.

The rapid kill curve is the best quantitative measurement for determining antimicrobial efficacy of a preservative system in a given formulation. Procedure for this test, with the exception of sampling time intervals and the addition of preservative inactivating agents to the diluent, is essentially identical to the USP challenge study. The rapid kill curve quantitates rate of death in a sample during a defined dosage interval and, like the presumptive test, produced results in three days. Both USP challenge and rapid kill curve

TABLE II

RANKING OF 4 PRESERVATIVE SYSTEMS BY THE USP METHOD AND BY TWO RAPID METHODS

ORGANISM	USP				RAPID KILL CURVE				PRESUMPTIVE MIC TEST			
	A	B	C	D	A	B	C	D	A	B	C	D
A. NIGER	P	P	P	P	0	0	3	3	2*	2*	2*	2*
C. ALBICANS	P	P	P	P	0	0	2	3	1*	0	2*	2*
PS. AERUGINOSA	P	P	P	P	3	4	4	4	2*	3*	3*	4*

A = PARABENS 0.132%
B = BENZYL ALCOHOL 0.9%
C = PARABENS 0.2%
D = PARABENS (0.132%) + BENZYL ALCOHOL (0.9%)

1* = ACTIVITY ONLY AT 1-10 DILUTION
2* = ACTIVITY AT 1-20 DILUTION
3* = ACTIVITY AT 1-40 DILUTION
4* = ACTIVITY AT 1-80 DILUTION

ACTIVITY = GROWTH IN SUBCULTURE FROM 0 to ++ AS OPPOSED TO 4+ CONTROL

RANKING CODE
P = PASS WITH NO RANKING POSSIBLE (POOR SENSITIVITY) (ALL SPECIES <10/g AT 7 DAYS)
0 = ZERO TO MINIMAL ACTIVITY DETECTED (D-10 ≥24 HRS.) (INACTIVE 1-10)
2 = D_{10} = 8-24 HOURS
3 = D_{10} = 2-6 HOURS
4 = D_{10} = 1 HOUR OR LESS

test require 20 ml of sample per microorganism, but the presumptive challenge requires only 1.0 ml of sample per microorganism. Sample size may be a critical problem during product development stage.

It is of interest that survivor curves of some of the preservatives systems studied here did **not** fall into straight lines, as was observed by Orth (1979). These observations and other unpublished data suggest that the lag phase of a preservative system is concentration-dependent. This lag phase (Figure 4) is important from a product development point of view. Further work should be done to define the D_{lag} of each preservative system and whether or not it should be included in the calculations of the D_{10} value that is derived from the straight line portion of the curve. It has been shown elsewhere (Prince, 1980) that calculations employing a 2-point Stumbo equation correlate well with l-log intercepts form a multipoint assay (Figure 6). Thus, the rapid kill curve assay can be truncated by eliminating some points shown in figures 4-6.

In summary, a simple saline tube dilution method, similar to the MIC procedure used by clinical microbiologists, was developed to screen preservative efficacy. Employing this test it became possible to semi-quantitatively rank preservative efficacy of a set of aqueous pharmaceutical formulations. Data from these tests can be correlated with USP challenge and time/log survivor tests. It is hoped that investigations of the type reported here will stimulate flexibility and creativity in the minds of formulation chemists and microbiologists so that research and development tasks will not be characterized by slavish adherence to the strict protocols of regulatory compendia. □

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