

Development of a Universal Diluting Fluid for Membrane Filtration Sterility Testing

DAVID W. PROUD* AND SCOTT V. W. SUTTON

Bausch and Lomb Inc., Rochester, New York 14692-0450

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A universal diluting fluid (UDF) was developed for use in membrane filtration sterility testing. Diluting fluid is used to free the filter membrane of preservative residues which could inhibit microbial recovery. Current procedures employ one of several fluids described by the *United States Pharmacopeia*. UDF was designed to utilize the neutralizing capabilities of a combination of inactivating agents while minimizing any inherent microbial toxicity. This formulation eliminates the need for multiple types of preservative-specific diluting fluids. Its neutralizer effectiveness was examined against several common preservative agents used in health care products. UDF provides significantly better microorganism recovery than dilution alone among a wide range of preservative classes.

Determination of final product sterility is an essential step in the manufacturing of pharmaceutical and health care products. The *United States Pharmacopeia XXII* (USP) describes a membrane filtration sterility test procedure (14). In this assay a large volume of solution is filtered through a membrane to trap microbial contaminants. The membrane is subsequently rinsed with a diluting fluid to cleanse it of residual solution and adhering preservatives. Insufficient rinsing of the membrane may cause the growth inhibition of contaminating microorganisms (2, 3, 7), potentially leading to a false sterility finding for the product.

The USP recommends the use of 0.1% meat peptone in water, called diluting fluid A (DFA), as a general-purpose rinsing fluid (14). If necessary, a nontoxic neutralizing agent may be added. Our laboratory has confirmed that the exclusive use of DFA is insufficient for the neutralization of some preservatives. However, preparing specific diluting fluids for each preservative class is a cumbersome, costly, and time-consuming endeavor. This study evaluates a universal diluting fluid (UDF), based upon Dey-Engley neutralizing broth (4, 5), that is capable of inactivating a broad range of preservatives. UDF eliminates the need to prepare several individual types of rinsing solutions, each tailored to inactivate a different preservative class (13).

The evaluation was conducted by applying the concepts of neutralizer efficacy and toxicity. Neutralization efficacy is defined as the ability of a treatment to inactivate preservatives, allowing the uninhibited recovery of microorganisms in comparison with recovery in the absence of preservatives (1, 5, 6, 8-10, 12). Neutralizer toxicity is the degree to which the treatment constrains microbial growth. UDF contains a combination of inactivating agents at concentrations minimizing any inherent toxicity. DFA and UDF were prepared in deionized water, adjusted to pH 7.4 ± 0.1 with 1 N sodium hydroxide, and sterilized by autoclaving. Both diluting fluids contain peptic digests of animal tissue (1 g/liter). Only UDF contains the following neutralizing agents (grams per liter): polysorbate 80, 1.0; lecithin, 1.0; sodium bisulfite, 1.0; sodium thioglycolate, 0.5; magnesium sulfate, 0.6; and sodium thiosulfate, 2. Additionally, UDF is buffered with 0.5 g

of monobasic sodium phosphate per liter and 1.4 g of dibasic sodium phosphate per liter.

Test solutions contained preservative agents representing a broad range of commonly used functional groups (13). Each was formulated in a borate-buffered saline base consisting of the following (grams per liter) in deionized water: boric acid, 8.5; sodium borate, 0.9; sodium chloride, 4.9; and disodium EDTA, 1. Glutaraldehyde (2 g/liter), methyl and propyl parabens (1.8 and 0.2 g/liter, respectively), and polyaminopropyl biguanide (PAPB) (3 g/liter) were formulated to the use concentrations described by Wallhäuser (16). Thimerosal (0.2 g/liter), sodium hypochlorite (2 g/liter), and benzalkonium chloride (BAK) (0.2 g/liter) were prepared at concentrations found in commercial products. Thymol (0.7 g/liter) was formulated at its maximum solubility. The relative efficacies of UDF and DFA were compared by using standardized liquid suspensions of *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, and *Clostridium sporogenes* ATCC 11437 as recommended in the USP bacteriostasis and fungistasis tests (14). Cultures were obtained from Biological & Environmental Control Laboratories, Toledo, Ohio. One liter of test solution was filtered under vacuum through a polyvinylidene difluoride filter membrane (pore size, 0.45 μm ; diameter, 47 mm; Millipore Corp., Bedford, Mass.) of a membrane filter assembly. The membrane was rinsed with two 100-ml aliquots of either DFA or UDF. A portion (100 ml) of the same diluting fluid was inoculated with low levels of challenge organism (30 to 80 CFU) and poured through the same filter membrane. The membrane was aseptically removed with tweezers and placed on the solidified surface of a tryptic soy agar (Difco Laboratories, Detroit, Mich.) petri plate. To facilitate the statistical analyses, it was necessary to quantify the recovery of challenge organisms by using agar rather than monitoring growth in a liquid medium. The entire procedure was repeated without the test solution to investigate potential microbial toxicity caused by the diluting fluids. To confirm organism viability, the same quantity of challenge inoculum was dispensed into an empty petri dish and covered with molten tryptic soy agar held at 45°C. All *B. subtilis* and *C. albicans* plates were incubated at 20 to 25°C for 48 to 72 h. *C. sporogenes* plates were incubated anaerobically at 30 to 35°C for 48 to 72 h. Each population consisted of at least three replicates.

* Corresponding author.

TABLE 1. Neutralizer efficacy. Statistical analysis of index microorganisms recovered from filters exposed to preservative after dilution with DFA or UDF^a

Preservative	<i>P</i> value of diluting fluid comparisons for:					
	<i>B. subtilis</i>		<i>C. albicans</i>		<i>C. sporogenes</i>	
	DFA	UDF	DFA	UDF	DFA	UDF
BAK	<0.01	>0.25*	<0.01	>0.25*	>0.25*	>0.25*
Glutaraldehyde	>0.25	>0.25*	>0.25*	>0.25*	>0.25*	>0.25*
PAPB	<0.01	>0.25*	>0.25*	>0.25*	0.05	>0.25*
Parabens	<0.01	>0.25*	<0.25*	<0.25*	0.25*	>0.25*
Hypochlorite	0.1	>0.25*	>0.25*	>0.25*	0.25*	>0.25*
Thimerosal	0.1	>0.25*	<0.01	>0.25*	>0.25*	>0.25*
Thymol	0.05	>0.25*	>0.25*	>0.25*	0.25*	>0.25*

^a ANOVA and Dunnett's tests were used to determine significance of differences between test and control populations. These populations were comprised of recovery using diluting fluid in the presence or absence of preservative. Entries marked with asterisks were determined by ANOVA only. A significant difference is denoted by $P \leq 0.05$.

Statistical analyses were performed on colony counts transformed to \log_{10} values. Data from the five populations examined (viability, UDF plus test solution, UDF minus test solution, DFA plus test solution, and DFA minus test solution) were initially analyzed by analysis of variance (ANOVA) (18). Dunnett's test (18) was applied if a significant difference was found by ANOVA ($P \leq 0.05$). UDF minus test solution served as the control population. Significance for Dunnett's test was determined at $P \leq 0.05$. Differences were considered biologically significant when recovery was less than 75% of control levels. The neutralizer toxicity of a diluting fluid was determined by comparing the mean CFU recovered from the viability plates with the mean CFU recovered from membranes treated with a diluting-fluid rinse only. Neutralizer efficacy was measured by comparing the mean CFU from filter membranes exposed to diluting fluid plus test solution with those from membranes rinsed with diluting fluid only. These analyses are shown in Table 1.

Comparisons of population means for neutralizer toxicity are presented in Fig. 1 as percentages of the viability control. The improved recovery of the bacterial spores by UDF is of interest. Colony counts from filters treated with UDF were not significantly lower than the recovery from filters given DFA treatment. We therefore conclude that neither DFA nor UDF is toxic in this assay configuration.

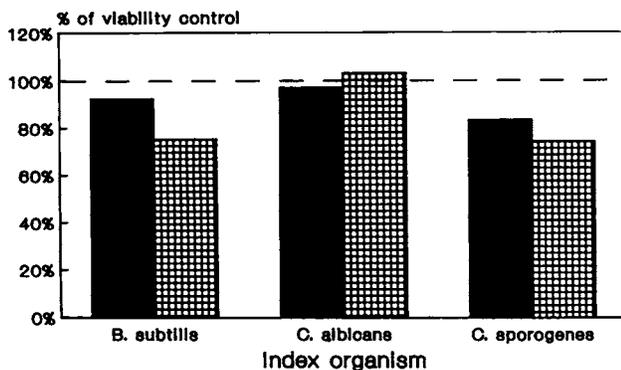


FIG. 1. Neutralizer toxicity of UDF and DFA. The histogram shows organism recovery after treatment with UDF (■) and DFA (▨) measured as a percentage of the viable count.

Neutralizer efficacy mean comparisons of specific index organisms are presented in Fig. 2 as percentages of the DFA controls. This control population was chosen as the most accurate estimate of the viable count in the system, since it incorporates any loss of cells to the filtration vessel. These graphs (Fig. 2) refer to the recovery of *B. subtilis*, *C. albicans*, and *C. sporogenes* (panels A to C, respectively). UDF-treated filter membranes provided improved recovery of each index organism with all test solutions. Recovery of index organisms from DFA-treated membranes was significantly lower than the control value with several test solutions. Recovery of *B. subtilis* from DFA-treated membranes was significantly lower than the control value when testing BAK-, PAPB-, thymol-, and paraben-preserved solutions. *C. albicans* recovery was significantly reduced with DFA for thimerosal- and BAK-preserved solutions, and *C. sporogenes* recovery was significantly lower for the BAK-preserved solution. The combination of dilution and chemical inactivation offered by UDF provides a wider range of neutralizer effectiveness than does simple dilution with DFA.

Detection of microbial contamination is the basis for performing any sterility test. This basic premise is compromised, however, if the test procedure inhibits the recovery of microbial contaminants (2, 3, 7). One cause of the growth inhibition may be the adherence of residual preservative to the filter membrane (2, 15). The condition is exacerbated by index organisms that might be highly sensitive to selected preservatives. Preservatives may be diluted or flushed from the filter with rinsing. Also, filtration through a low-binding filter material, such as polyvinylidene difluoride, may help to minimize this adherence (11, 15). Nonetheless, in some cases dilution alone is insufficient and chemical inactivation is necessary. The effectiveness of UDF arises from a combination of chemical inactivation and mechanical dilution of the preserved solution.

Improved recovery of *B. subtilis* and *C. sporogenes* with UDF may also be due to the presence of the surfactant polysorbate 80. The spores of these bacteria are significantly more hydrophobic than the vegetative cells (17). Cell loss may occur through adsorption to the filtration vessel when rinsing with DFA. The surfactant would minimize this effect.

This study clearly demonstrates the advantages of using UDF for USP sterility testing. One would expect greater success with bacteriostasis and fungistasis testing and improved recovery of contaminating microorganisms over a wide range of preservative classes. This broad efficacy eliminates the need for multiple, specific diluting fluids tailored to individual preservatives.

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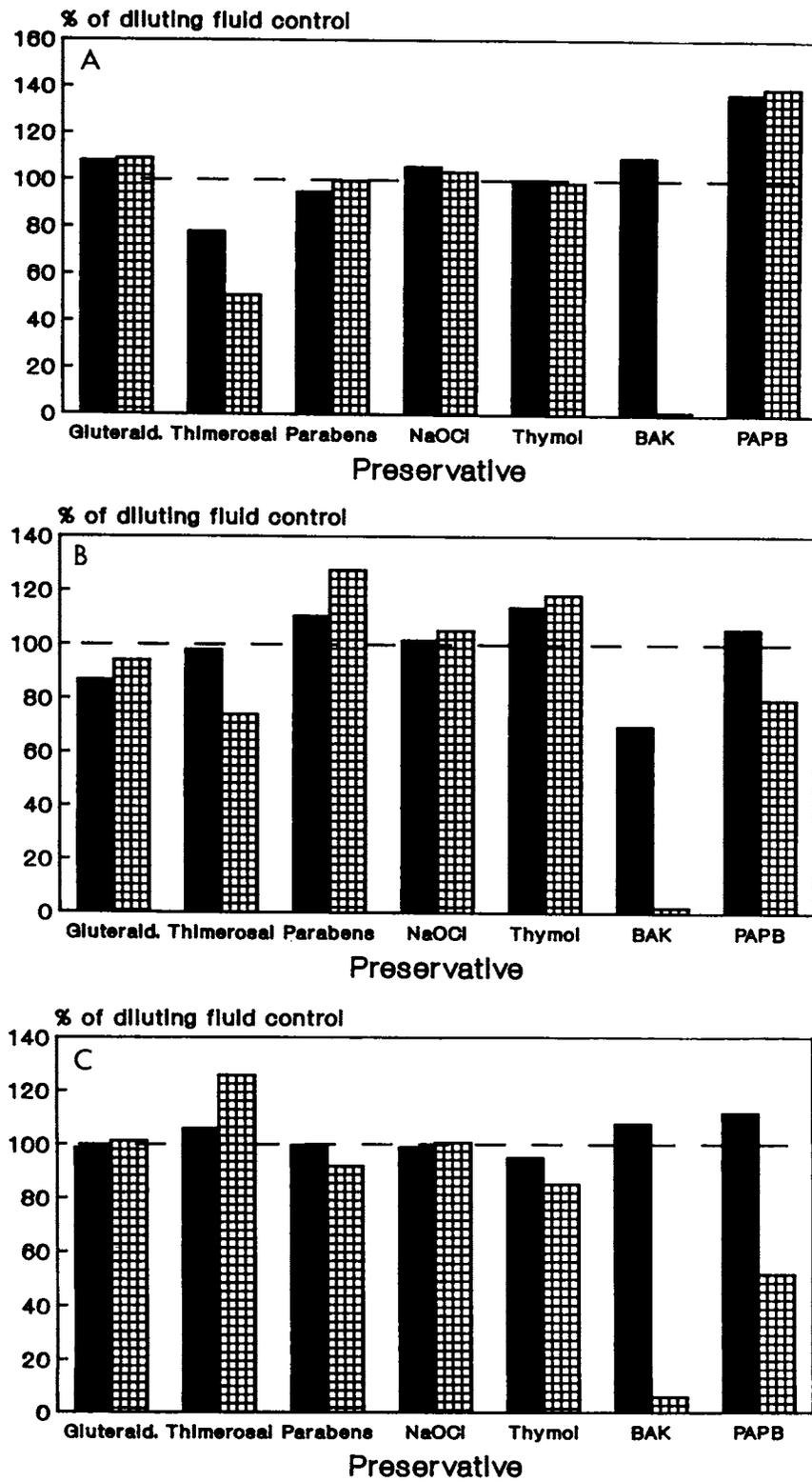


FIG. 2. Neutralizer efficacy. The histograms show recovery facilitated by UDF (■) and DFA (▣) of *C. albicans* (A), *C. sporogenes* (B), and *B. subtilis* (C) after treatment with diluting fluid with or without test solution.

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