

Rapid Screening of Preservative Effectiveness in Liquid Pharmaceutical Products using a Flow Cytometric Method Q-443

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ABSTRACT

The United States Pharmacopeia/European Pharmacopeia Antimicrobial Effectiveness Test is used to screen candidate preservatives for multiple-use aqueous liquid products. Problems with the compendial method include a 35-day time to result, significant labor and material costs, and evaluation of a limited number of preservative systems. These factors limit the capacity to select optimal preservative systems, thus delaying product development. The ability of the RBD 3000 flow cytometer (Advanced Analytical Technologies, Inc., Ames IA (AATI)) to address these issues was demonstrated using three over-the-counter nasal solutions containing the active ingredient oxymetazoline hydrochloride or phenylephrine hydrochloride. Each nasal solution was challenged with 10^5 to 10^6 cfu/mL inocula of *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538, and *Candida albicans* ATCC 10231. Each microbial culture was tested prior to inoculation and at 8, 24 and 48 hours post-inoculation. Products were diluted 1:100 in 10 mM phosphate buffer (PB) and tested for Biomass and Dead Cell counts on the RBD 3000 using respective reagent test kits. Also at each time point, the product was diluted in PB with 4% (v/v) Tween 20 and spread-plated on Tryptic Soy Agar for comparison to the RBD 3000 result. By plotting the percent dead cells versus time, the relative effectiveness of the preservative was determined and expressed as a D-value (time for a 90% reduction in count). The D-values for *Ps. aeruginosa* and *S. aureus* in Brands A and B were ≤ 24 hours and Brand C was > 48 hours. The D-value for *C. albicans* was 4 hours for all three brands. Testing protocol was established using 0.01% (w/v) benzoic acid to show proof of concept. This method provides a rapid screening tool for the development of optimal preservative systems for multiple-use aqueous liquid products.

MATERIALS

Bacterial Cultures: *Pseudomonas aeruginosa* #9027, *Staphylococcus aureus* #6538 and *Candida albicans* #10231 (ATCC, Manassas, VA). Tryptic Soy Broth (TSB) (EMD, Gibbstown, NJ) and buffered peptone water (Difco, Sparks, MD) were used for culturing, Tryptic Soy Agar (TSA) (EMD, Gibbstown, NJ) for plating, Phosphate Buffer (PB) with 4% (v/v) Tween 20 (PBT) was used for inactivating preservative in plated dilutions and 0.01% (w/v) benzoic acid (ThermoFisher Scientific, Inc., Waltham, MA) was used for proof of concept testing. **Products:** Three over-the-counter brand name nasal sprays. **Detection:** *FASTEST* Total Viable Organisms (TVO) Kit, Biomass Test Kit, Dead Cell Test Kit and fully automated RBD 3000 (AATI).

METHODS

Evaluation of microbial cultures: Each microorganism was serially diluted in PB. Three 3 mL samples were analyzed on RBD 3000 for TVO, Biomass and Dead Cell Counts. Percent dead was calculated by (Dead Cell count/Biomass count) * 100. The percent dead of the challenge inoculum was $<10\%$ of the biomass cell count. TVO results were used to determine inoculum. **Proof of Concept:** Used 0.01% (w/v) benzoic acid and *Ps. aeruginosa* to establish the RBD 3000 preservative challenge protocol described below. (Table 1 and Figure 1). **Evaluation of Nasal Sprays:** Twenty five mL of each nasal spray and a water control were added to sterile sample jars. Microbial cultures were added to the sample jars for a final concentration of 10^5 to 10^6 cfu/mL. At times 0, 4, 8, 24 and 48 hours post-inoculation, 0.1 mL of sample was added to 9.9 mL of PB and mixed. Two 3 mL aliquots of each sample were loaded on the RBD 3000 and analyzed for Biomass and Dead Cell counts. Also at each time point, the samples were diluted in PBT and spread-plated in duplicate on TSA to yield 30-300 cfu/plate. Plates were incubated at $37^\circ\text{C} \pm 2^\circ\text{C}$ ($30^\circ\text{C} \pm 2^\circ\text{C}$ for *C. albicans*) for 18-24 hours. By plotting the percent dead cells versus time, the relative effectiveness of the preservative was determined (Table 2).

RESULTS

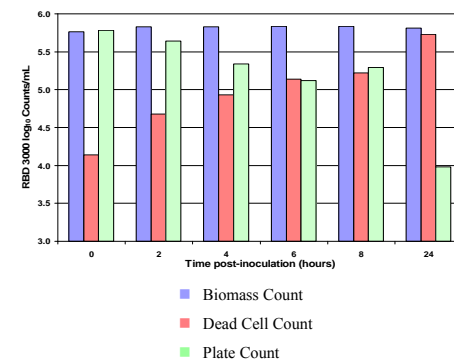
Table 1: Decimal reduction times (D-value) for 0.01% Benzoic Acid

Preservative	D-value (hours)	Correlation coefficient
Benzoic Acid + <i>Ps. aeruginosa</i>	31.7	0.983
Water + <i>Ps. aeruginosa</i>	143.6	0.981

Table 2: Time to reach 90% Dead Cells Count in challenged Nasal Sprays

	<i>Ps. aeruginosa</i>	<i>S. aureus</i>	<i>C. albicans</i>
Brand A	24 hr	24 hr	4 hr
Brand B	4 hr	4 hr	4 hr
Brand C	>48 hr	>48 hr	4 hr
Water Control	>48 hr	>48 hr	>48 hr

Figure 1: 0.01% Benzoic Acid Challenged with *Ps. aeruginosa*



DISCUSSION/CONCLUSIONS

- Tracking RBD 3000 percent dead cell counts over time determines the amount of time it will take to reach the D-value
- Biomass and Dead Cell Test Kits, in conjunction with the RBD 3000, are an effective way to test for percent dead and track preservatives effectiveness
- Testing procedure using the RBD 3000 provides a rapid method for screening candidate preservative systems
- This testing procedure generated reproducible results

