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FINAL REPORT

USP ANTIMICROBIAL PRESERVATIVE EFFECTIVENESS TEST

PROCEDURE NO. STP0131 REV 02
PROTOCOL DETAIL SHEET NO. 200803363 REV 01

LABORATORY NO. 452815

PREPARED FOR:

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QAU AUDIT STATEMENT

[X] USFDA (21 CFR PART 58) [ ] USEPA (40 CFR PART 160)

USP ANTIMICROBIAL PRESERVATIVE EFFECTIVENESS TEST

LABORATORY NO. 452815

1. The test was conducted in accordance with the USFDA or USEPA Regulations as noted above.

2. In accordance with the Good Laboratory Practice Regulations, the Plate Count phase(s) of this study was inspected by the Quality Assurance Unit on: 29 Dec 2008. The findings of the inspection(s) were reported to the Study Director on: 02 Feb 2009 and to Management on: 04 Feb 2009.

3. The Quality Assurance Unit has reviewed this report and has determined that the methods and standard testing procedures are accurately described, and that the reported results accurately reflect the raw data.

4. The name of the study director, the names of other scientists or professionals, and the names of all supervisory personnel, involved in the study:

Michael Neilson
Peter Croci
Dr. Jerry Nelson
Jeff Hills

QUALITY ASSURANCE: _____ DATE: 09 Feb 2009

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USP ANTIMICROBIAL PRESERVATIVE EFFECTIVENESS TEST

LABORATORY NUMBER:	452815
PROCEDURE NUMBER:	STP0131 REV 02
PROTOCOL DETAIL SHEET NUMBER:	200803363 REV 01
SAMPLE SOURCE:	EnzySurge
SAMPLE IDENTIFICATION:	Refer to Tables 2-4 P.O. #1008
DEVIATIONS:	None
PROTOCOL APPROVAL DATE:	09 Dec 2008
SAMPLE RECEIVED DATE:	26 Nov 2008
LAB PHASE START DATE:	09 Dec 2008
LAB PHASE COMPLETION DATE:	05 Feb 2009
REPORT ISSUE DATE:	06 Feb 2009

INTRODUCTION:

This study was performed to determine the survival rate of various organisms in the test product. The test employed methods designed to determine antimicrobial effectiveness described in the United States Pharmacopeia (USP).

The samples of the product were inoculated, with five standard test organisms. The inoculated samples were then incubated for a total of 28 days at 20-25°C. Aliquots from the samples were immediately removed and assayed for surviving organisms at 0 hour, 7 day, 14 day, and 28 day time intervals. The log reduction in the level of the test organisms was calculated for each time interval.

ACCEPTANCE CRITERIA:

According to USP, products are placed into categories according to product type. The product category will determine the acceptance criteria for the test. The following table provides a description of the current USP categories.

CATEGORY	PRODUCT DESCRIPTION
1	Injections, other parenterals including emulsions, otic products, sterile nasal products, and ophthalmic products made with aqueous bases or vehicles.
2	Topically used products made with aqueous bases or vehicles, non-sterile nasal products, and emulsions, including those applied to mucous membranes.
3	Oral products other than antacids, made with aqueous bases or vehicles.
4	Antacids made with an aqueous base.

The acceptance criteria for the USP Antimicrobial Effectiveness Test is located in Table 1. No increase is defined as not more than a 0.5 $\log_{10}$ unit higher than the previous value measured.

NEUTRALIZER PREPARATION:

4 L of 0.1 N Sodium Thiosulfate was mixed with 43.29 g of Sodium Thioglycolate. After mixing, 320 mL of NLI water was added. The mixture was filtered through a 0.2 micron filter. The resulting ~1.46% Sodium Thiosulfate and 1% Sodim Thioglycolate solution was used as the neutralizer for this study.

PROCEDURE:

The following organisms were tested:

1) *Staphylococcus aureus* ATCC #6538
   [Bacteria, Gram (+) cocci]
2) *Pseudomonas aeruginosa* ATCC #9027
   [Bacteria, Gram (-) bacillus]
3) *Escherichia coli* ATCC #8739
   [Bacteria, Gram (-) bacillus]
4) *Candida albicans* ATCC #10231
   [Yeast]
5) *Aspergillus niger* ATCC #16404
   [Mold]

The bacteria were transferred to soybean casein digest agar (SCDA) and incubated at 30-35°C for 18-24 hours. The *C. albicans* and *A. niger* were transferred to Sabouraud dextrose agar (SDEX). The *C. albicans* was incubated at 20-25°C for 44-52 hours and the *A. niger* was incubated at 20-25°C for 6-10 days.

The bacteria and *C. albicans* cultures were harvested with the $NaNO^3$ solution provided by the sponsor. The plates were flooded and a glass rod was used to suspend growth in the solution. Bacterial and yeast suspensions were used within 24 hours of harvest.

After harvesting, the cultures were centrifuged. The supernatant as aspirated and washed in 10 mL of $NaNO^3$ solution. The wash was repeated two additional times.

A. niger cultures were harvested in a similar manner using the $NaNO^3$ solution provided by the sponsor. After vortexing, the culture was centrifuged. The supernatant was aspirated and washed in 10 mL of NaNO3 solution. The wash was repeated two additional times.

These results represent duplicate analysis plated in triplicate. Individual samples of the test product were prepared for each challenge organism. The tubes containing the sample were inoculated with the test organisms using a calibrated micropipettor. The volume of the inoculum was between 0.5% and 1.0% of the volume of the product. The final concentration of the test preparation was approximately 10^5 - 10^6 CFU/mL of product. The samples were well mixed.

Positive control tubes were prepared for each organism using sterile water. The volume used for the positive control was equivalent to that used in the samples. Negative controls were also prepared. The positive controls were then inoculated in the same manner as the test samples. All test samples were stored at 20-25°C for a total of 28 days.

Control tubes and test vials were assayed immediately to determine the initial concentration of organisms in each tube. The test suspensions were assayed at the following intervals: 0 hour, 7, 14, and 28 days.

Sample aliquots at each interval were diluted in the neutralizer and plated on SCDA for bacteria and SDEX for *C. albicans and A. niger*. The bacteria plates were incubated at 30-35°C for 3-5 days. The *C. albicans* plates were incubated at 20-25°C for 3-5 days and the *A. niger* plates were incubated at 20-25°C for 3-7 days.

Additional controls were performed to ensure neutralization. This was performed by making a 1:10 ratio of sample to neutralizer. This simulates the highest concentrations of the product plated, and represents the worst case for neutralization. The tubes were then inoculated with an organism suspension to reach a concentration of 2-200 CFU/mL. These were then plated in

mL aliquots on SCDA and SDEX. Control tubes of ~1.46% sodium thiosulfate and 1% sodium thioglycolate, using the same volume as the neutralizer and sample mix, were inoculated concurrently with the same cultures and plated. Neutralization is demonstrated when the number of colonies on the sample plates demonstrates at least 70% recovery when compared to the control plates.

RESULTS:

The results for the samples are reported in Tables 2-3. The greater than (>) values represent the detectable limits of the test where zero CFU were observed on the plates. The approximate (~) symbol is applied to results where plate counts fell outside of the statistically accurate range of 25-250 CFU for bacteria and yeast and 8-80 CFU for mold.

The neutralization data is found in Table 4. The neutralization testing showed adequate neutralization of all products tested at a 1:10 dilution when challenged against the standard test organisms.

All negative controls showed no growth. Testing met the acceptance criteria stated in this report.

CONCLUSION:

Interpretation of the data is the responsibility of the sponsor and no conclusion can be made by Nelson Laboratories, Inc. (NLI).

DATA DISPOSITION:

The raw data and final report from this study are archived at NLI or an approved off-site location.

EnzySurge
Lab Number 452815

USP Antimicrobial Preservative Effectiveness Test

STATEMENT OF UNCERTAINTY:

If applicable, the statement of uncertainty is available to sponsors upon request.

Peter Croci, B.A.
Study Director

09 Feb 2009
Study Completion Date

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TABLE 1. USP Antimicrobial Effectiveness Acceptance Criteria

Category 1 Products	
Bacteria:	Not less than 1.0 log reduction from the initial calculated count at 7 days, not less than 3.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast & Molds:	No increase from the initial calculated count at 7, 14, and 28 days.
Category 2 Products	
Bacteria:	Not less than 2.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast & Molds:	No increase from the initial calculated count at 14 and 28 days.
Category 3 Products	
Bacteria:	Not less than 1.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast & Molds:	No increase from the initial calculated count at 14 and 28 days.
Category 4 Products	
Bacteria, Yeast,& Molds:	No increase from the initial calculated count at 14 and 28 days.

TABLE 2. Inoculum Control Results
Sample Identification: Silver Stream Lot #SIL-001271008
Silver Buffered Solution 250ml
Active ingrediant: Each ml contains Silver Nitrate 0.1mg

ORGANISM	CHALLENGE TITER AT 0 HOUR (CFU / mL)
S. aureus	3.5×10^5
P. aeruginosa	6.6×10^5
E. coli	2.7×10^5
C. albicans	3.4×10^5
A. niger	2.5×10^6

TABLE 3. Summary of Log Reduction Results
Sample Identification: Silver Stream Lot #SIL-001271008
Silver Buffered Solution 250ml
Active ingrediant: Each ml contains Silver Nitrate 0.1mg

ORGANISM	TIME INTERVAL			
	0 HOUR	7 DAY	14 DAY	28 DAY
S. aureus	0.04	>4.25	>4.25	>4.25
P. aeruginosa	>3.52	>4.52	>4.52	>4.52
E. coli	-0.09	>4.14	>4.14	>4.14
C. albicans	~2.55	>4.24	>4.24	>4.24
A. niger	0.19	~5.04	>5.10	>5.10

TABLE 4. Neutralization Data

SAMPLE IDENTIFICATION	ORGANISM	CONTROL	SAMPLE	PERCENT OF CONTROL (%)
Silver Stream Lot #SIL-001271008 Silver Buffered Solution 250ml Active ingrediant: Each ml contains Silver Nitrate 0.1mg	*S. aureus*	28	22	79
	P. aeruginosa	11	22	200
	E. coli	12	15	125
	C. albicans	18	17	94
	A. niger	43	46	107