Sterility Test Method for Petrolatum-Based Ophthalmic Ointments

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A sensitive sterility testing procedure for the detection of microbial contamination in petrolatum-based ointments is described. The method involves dissolving the ointment in filter-sterilized isopropyl myristate and filtering through a membrane filter. Improved sensitivity is obtained by blending the membrane in Trypticase Soy Broth before incubation. Filter-sterilized isopropyl myristate is shown to be less toxic to microorganisms than heat-sterilized isopropyl myristate. The isopropyl myristate method is more sensitive than the polyethylene glycol-ether method for the detection of microbial contamination.

Microbiological contamination of pharmaceutical preparations has been of increasing concern (5). Techniques described for the testing of non-sterile products without filtration (1, 6, 8) are not ideal in many instances because of the presence of antibiotics or other inhibitory substances. Petrolatum-based ointments present a particular problem since they are difficult to solubilize without adversely affecting contaminants that may be present. Sokolski and Chidester (9) reported a method for detection of microorganisms in petrolatum-based ointments by using isopropyl myristate as the solvent. The method is described in the Code of Federal Regulations as a sterility test for ophthalmic ointments (2). However, the method lacks sensitivity since less than 50% of added cells are recovered.

MATERIALS AND METHODS

Test organisms. The following microorganisms were used to determine recovery and toxicity: Staphylococcus aureus ATCC 6538P; Bacillus subtilis ATCC 6633; Escherichia coli Upjohn Culture Collection (UC) 3114; several clinical isolates of Pseudomonas aeruginosa UC no. 95, 104, 350, 393, 394, 399, 744, and 3363; and Salmonella newington UC 3476. Vegetative cells were grown in Trypticase Soy Broth (TSB; BBL) for 24 hr at 35 C and stored in liquid nitrogen until used. Spores of B. subtilis were obtained from BBL.

Solvent. Isopropyl myristate was obtained from three suppliers: Givaudan-Delawanna, Inc., New York, N.Y. as Deltyl Extra; Goldschmidt Chemical, Division of Wilson Pharmaceutical, New York, N.Y.; and M. W. Parsons-Plymouth, Division of S. B. Penick & Co., New York, N.Y. It was sterilized either by dry heat or by filtration. The dry-heat sterilization was accomplished by heating 50-ml quantities in 125-ml Erlenmeyer flasks at 180 C for 4 hr. Sterilization by filtration was accomplished by filtering through a 0.45-µm membrane filter (Millipore Corp., Bedford, Mass.).

Rinse medium. A 0.5% concentration of Brain Heart Infusion broth (Difco) was prepared and filtered through 0.45-µm membrane filter. A 1-ml amount of Tween 80 (Atlas Chemical Industries, Inc., Wilmington, Del.) was added to 1,000 ml of broth and steamed for 10 min to dissolve the Tween 80. The medium was dispensed in 200-ml quantities and autoclaved for 15 min at 121 C.

Filtration procedure. Approximately 0.5 g of ophthalmic ointment was transferred aseptically onto the blades of a sterile Waring Blender. A 50-ml amount of warm (42 C) isopropyl myristate was added, and the mixture was blended for 20 sec. After blending, the isopropyl myristate-ointment mixture was filtered through a 0.45-µm membrane filter. As soon as the mixture passed through the filter, usually within 30 sec, 200 ml of warm (42 C) rinse medium was added to the filter. Immediately after filtration and rinsing, the membrane filter was aseptically removed and (i) placed on Trypticase Soy Agar (TSA) plates or placed into TSB or (ii) placed in a sterile Waring Blender and blended for 30 sec with 100 ml of TSB. The blended suspension was transferred into a sterile test tube (38 by 200 mm) and incubated for 7 days at 35 C. Sample preparation and filtration should be conducted in a laminar-flow hood to minimize contamination.

Preparation of experimentally contaminated ointment. A sample of ointment was melted by heating to approximately 80 C. The melted ointment was poured into a water-jacketed semimicro Waring Blender cup maintained at 44 C with a Lauda K-2/R circulating water bath. When the temperature of the ointment reached 44 C, 0.1 ml of an aqueous suspension of microorganisms containing 100 to 200

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cells per ml was added. The inoculated ointment was blended vigorously at 44 C for 30 sec to achieve a uniform suspension and then cooled while gently mixing until it congealed.

RESULTS AND DISCUSSION

Toxicity of the solvent. Since the possible presence of microorganisms such as \textit{S. aureus}, \textit{E. coli}, \textit{P. aeruginosa}, and \textit{Salmonella} is of concern in ophthalmic ointments, the test method should permit quantitative recovery of these microorganisms. A 0.1-ml amount of a cell suspension, containing approximately 100 organisms, was added directly to isopropyl myristate. Test samples were filtered at various time intervals. The experiments were repeated at least three times. Figure 1 demonstrates the survival of \textit{E. coli} in heat- and filter-sterilized isopropyl myristate. Since rate of cell death was assumed to follow first-order kinetics, the number of surviving organisms was plotted against time on semi-log graph paper, and a linear regression line was drawn by using the least squares method. \(D\) values (time in minutes required to kill 90\% of the microorganisms) were calculated by using the following formula (10): \(D = U/(\log a - \log b)\), where \(U\) = time in minutes to decrease the number of microorganisms from \(a\) to \(b\) (\(Ub - Ua\)), \(a\) = number of microorganisms at time \(Ua\), and \(b\) = surviving number of microorganisms at time \(Ub\). Figure 2 shows the survival of \textit{P. aeruginosa} in heat- and filter-sterilized isopropyl myristate. It is apparent from these figures that the filter-sterilized isopropyl myristate is significantly less toxic than heat-sterilized isopropyl myristate which was reported by Sokolski et al. (9). \(D\) values also were obtained for other species of organisms that were placed in heat- and filter-sterilized isopropyl myristate (Table 1).

Of the organisms tested, \textit{E. coli} appeared to be the more resistant to filter-sterilized isopropyl myristate. However, when isopropyl myristate was heat-sterilized, \textit{E. coli} was highly sensitive to the solvent.

Since \textit{P. aeruginosa} appeared to be one of the most sensitive organisms to the solvent system, several isolates were tested to verify this observation. Eight different clinical isolates were obtained and exposed to filter-sterilized isopropyl myristate for as long as 10 min. Table 2 shows the

\begin{figure}[h]
    \centering
    \includegraphics[width=\textwidth]{fig1.png}
    \caption{Survival of \textit{Escherichia coli} UC 3114 in heat- and in filter-sterilized isopropyl myristate.}
\end{figure}

\begin{figure}[h]
    \centering
    \includegraphics[width=\textwidth]{fig2.png}
    \caption{Survival of \textit{Pseudomonas aeruginosa} UC 104 in heat- and filter-sterilized isopropyl myristate.}
\end{figure}
TABLE 1. Survival of microorganisms in heat- and filter-sterilized isopropyl myristate

| Organism                        | D value in isopropyl myristate | Heat-sterilized | Filter-sterilized |
|--------------------------------|--------------------------------|-----------------|-------------------|
| Bacillus subtilis spores        | 45.1<sup>a</sup>               | 100.3           |
| Staphylococcus aureus           | 11.0<sup>b</sup>               | 188.0           |
| Escherichia coli                | 4.5<sup>b</sup>                | 329.0           |
| Salmonella newington            | 1.7<sup>b</sup>                | 18.0            |
| Pseudomonas aeruginosa          | 1.9<sup>b</sup>                | 19.0            |

<sup>a</sup> Indicates a significant difference from filter-sterilized isopropyl myristate at the 95% level.
<sup>b</sup> Indicates a significant difference from filter-sterilized isopropyl myristate at the 99% level.

D values obtained for each of the isolates. Even though there was considerable variation in the D values for the eight isolates, these values did not prove to be statistically different. P. aeruginosa UC 104 was inoculated into isopropyl myristate obtained from three suppliers. The D values in filter-sterilized solvent were: Givaudan-Delawanna, 19.2; Goldschmidt, 10.8; M. W. Parsons-Plymouth, 13.3. These D-value differences were not significant at the 95% level.

The following experiment was performed to investigate the cause of the increased toxicity observed with heat-sterilized isopropyl myristate. A 5-ml amount of water was added to 100 ml of heat-sterilized (4 and 18 hr at 180°C) and filter-sterilized isopropyl myristate. The mixtures were shaken vigorously for 1 hr. The pH of the water extract was then determined, and the titrable acidity of the water extract was determined by neutralizing 5 ml of water extract with 0.01 N NaOH. The results obtained were as follows. With filter-sterilized isopropyl myristate, the pH of the water extract was 5.0, and 0.058 ml of 0.01 N NaOH was needed for neutralization. With heat-sterilized (4 hr) isopropyl myristate, the pH of the water extract was 3.3, and 0.600 ml of 0.01 N NaOH was needed for neutralization.

TABLE 2. Survival of various isolates of Pseudomonas aeruginosa in filter-sterilized isopropyl myristate

| Isolate | D value |
|---------|---------|
| UC 95   | 27.2    |
| UC 104  | 19.2    |
| UC 350  | 44.6    |
| UC 393  | 64.2    |
| UC 394  | 60.0    |
| UC 399  | 24.6    |
| UC 744  | 19.4    |
| UC 3363 | 23.0    |

TABLE 3. Comparative study of microbial recovery from an inoculated petrolatum-based ophthalmic ointment by the filter-sterilized isopropyl myristate method and by the polyeethylene glycol-ether method of the Millipore Corp.

| Determination | Viable count | Isopropyl myristate method | Polyeethylene glycol-ether method |
|---------------|--------------|----------------------------|----------------------------------|
| Escherichia coli | Day 1         | 26 ± 5                     | 12 ± 6                           |
|                | Day 2         | 25 ± 2                     | 13 ± 7                           |
|                | Avg per cent recovery | 5 ± 2                      | 4 ± 1                           |
| Pseudomonas aeruginosa | Day 1       | 53 ± 6                     | 44 ± 2                           |
|                | Day 2         | 35 ± 2                     | 26 ± 9                           |
|                | Avg per cent recovery | 8 ± 4                      | 8 ± 1                           |

TABLE 4. Comparison of recoveries of microorganisms by plate count, with blended and nonblended membrane filter methods

| Microorganism    | No. of replicates | No. of positive tubes/number of tests with membrane filter in TSA<sup>a</sup> | Blended | Non-blended |
|------------------|--------------------|--------------------------------------------------------------------------------|---------|-------------|
| Escherichia coli | 4                  | 2.5                                                                           | 4/4     | 3/4         |
| Pseudomonas aeruginosa | 6            | 0.5                                                                           | 6/6     | 0/6         |

<sup>a</sup> Abbreviations: TSA, Trypticase Soy Agar; TSB, Trypticase Soy Broth.
<sup>b</sup> Number of cells added was 2.1/0.5 g of ointment.
<sup>c</sup> Number of cells added was 1.8/0.5 g of ointment.

With heat-sterilized (18 hr) isopropyl myristate, the pH of the water extract was 2.7, and 1.220 ml of 0.01 N NaOH was needed for neutralization. The increase in toxicity of the heat-sterilized isopropyl myristate may be due to the increase in acidity. Gas chromatography and mass spectrometry were used to attempt to identify the nature of the acid produced. Results were inconclusive.

**Microbial recovery.** The efficiency of recovery of microorganisms by the isopropyl myristate method and the polyeethylene glycol-ether method of the Millipore Corp. (7) was compared by studying a purposely contaminated ophthalmic ointment (Table 3). The Millipore method recovered 23% of the inoculated E. coli and only 8% P. aeruginosa. The isopropyl myristate method made possible recovery of twice as many E. coli cells and
10 times more *P. aeruginosa* than the Millipore method. The inefficiency of the Millipore method may be due to its inherent difficulty of transfer of microorganisms from an oil layer to an aqueous layer. The apparently low recovery of the isopropyl myristate method, although better than the Millipore method, may be that microorganisms on a membrane filter are still encased in an oil film and thus are denied access to nutrients required for multiplication. If this hypothesis is correct, then the efficiency of the isopropyl myristate method might be improved by removing the oil film from the microorganisms, e.g., by blending the membrane filter in a nutrient medium.

The following experiment was designed to evaluate such a blending procedure. Ophthalmic ointment was purposely contaminated with approximately four cells per g of either *E. coli* or *P. aeruginosa*. Samples (0.5 g) of the contaminated ointments were dissolved in isopropyl myristate and filtered by the procedure described above. The filter pads were (i) placed directly on TSA plates, (ii) placed into tubes containing 100 ml of sterile TSB, or (iii) blended with 100 ml of TSB in a sterile Waring Blender jar. The plates, tubes, and blender jars were incubated for 48 hr at 37 C, and any resulting growth was confirmed as *E. coli* or *P. aeruginosa* by streaking on EMGB Agar (BBL) or Pseudosel Agar (BBL), respectively (Table 4). When the filter pads were blended, growth of *E. coli* was obtained in each of the four replicates tested, and *P. aeruginosa* growth was obtained from each of the six replicates tested. In contrast to the efficiency of the blended method, only three positives of four replicates for *E. coli* and zero positives of six replicates for *P. aeruginosa* were obtained when the filter pads were not blended but placed directly into the TSB medium.

The benefit obtained from blending the filter pad supports the hypothesis that, in the nonblended test, microbial cells remain coated with an oil film or cells are trapped within the oily filter membrane structure (3), or both occur, and thus are denied access to nutrients required for multiplication. Thus, the filter-sterilized isopropyl myristate method when coupled with the blending of the membrane filter would be a suitable sterility test for petrolatum-based ophthalmic ointments.

**Sterility testing of petrolatum-based ophthalmic ointments.** Ten different ophthalmic ointments, manufactured by The Upjohn Co., were tested for sterility. Filter-sterilized isopropyl myristate was used, and the membrane filters were blended in TSB medium. Table 5 shows the results of this study. Although several positive tests were obtained, none of the organisms isolated was a pathogen. Assuming even distribution of microorganisms in ointments, the most-probable number of contaminating microorganisms per gram of ointment was calculated by using the following formula (4): \( \bar{X} = \frac{2,302 \log n}{q} \), where: \( \bar{X} \) = the most-probable number of microorganisms per sample, \( n \) = total number of replicate tubes, \( q \) = number of negative tubes. The level of contamination in these products is very low.

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**Table 5. Sterility test of petrolatum-based ophthalmic ointments**

| Product* | No. of positive in 6 replicate ointment tubes | Most-probable no. of microorganisms per g of ointment |
|----------|---------------------------------------------|-----------------------------------------------------|
| Baciguent | 5                                           | 3.6                                                 |
| Myciguent | 4                                           | 2.2                                                 |
| Neo-Cortef 0.5% | 1                                         | 0.2                                                 |
| Neo-Cortef 1.5% | 2                                         | 0.7                                                 |
| Neo-Cortef w/Tetracaine | 1                                         | 0.2                                                 |
| Neo-Delta-Cortef | 1                                         | 0.2                                                 |
| Neo-Medrol | 2                                           | 0.7                                                 |
| Neo-Predif | 1                                           | 0.2                                                 |
| Neo-Predif w/Tetracaine | 2                                         | 0.7                                                 |
| Neosone   | 0                                           | 0                                                   |

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