Microcount Method for Petrolatum-Based Topical Ointments Containing Waxes

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Received for publication 9 July 1970

A practical solvent system for the detection of microorganisms in topical ointments has been developed. The method involves dissolving 0.5 g of topical ointment in 50 ml of a solvent mixture (92 parts isopropyl myristate, 6 parts carbon disulfide, and 2 parts xylene) and filtering it through a 0.45-μm membrane filter. Residual solvent is then washed from the filter pad with 200 ml of sterile 0.5% Brain Heart Infusion broth containing 0.1% Tween 80. The filter pad is then removed and placed on a petri plate containing Trypticase Soy Agar medium. The petri plates thus prepared are then incubated at 37°C for 7 days, and the colonies produced are then counted. The toxicity of the solvent mixture was determined against Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella newington, and spores of Bacillus subtilis and was found to be less toxic than the heat-sterilized isopropyl myristate and comparable to the filter-sterilized isopropyl myristate.

Although a method with isopropyl myristate (1, 2) is applicable for microbial detection in ophthalmic ointments, it is not applicable to topical ointments since it does not dissolve all of the ingredients in topical ointments, especially waxes, thereby preventing filtration through a membrane filter. The method described in this paper fulfills four of the major requirements in microbial detection by filtration. (i) It dissolves all of the ingredients of the ointment. (ii) It is compatible with the membrane filter. (iii) There is limited but controlled toxicity to the microorganisms during the extraction period. (iv) It is capable of recovering chance contaminants.

MATERIALS AND METHODS

Test organisms. The test microorganisms used were Staphylococcus aureus ATCC 6538P, Escherichia coli UC 3114, Pseudomonas aeruginosa ATCC 10145, Salmonella newington UC 3476, and spores of Bacillus subtilis ATCC 6633. S. aureus, E. coli, P. aeruginosa, and S. newington were grown in Trypticase Soy Broth (BBL) for 24 hr at 35°C and stored frozen in liquid nitrogen until used. Spores of B. subtilis were obtained from BBL. Spores were dried overnight under vacuum over silica gel at room temperature.

Solvent mixture. The solvent system consists of isopropyl myristate (obtained from Givaunden-Delawanna, Inc., New York, N.Y., as Delthyl Extra), carbon disulfide, and xylene (A. R., Mallinckrodt Chemical Works). The three solvents are essential for filtration of all of the ingredients in petrolatum-based topical ointments manufactured by The Upjohn Co. The three solvents are combined at a ratio of 92 parts isopropyl myristate, 6 parts carbon disulfide, and 2 parts xylene. They are then sterilized by passage through a 0.45-μm membrane filter (sterilized; Millipore Corp., Bedford, Mass.).

Rinse medium. A 0.5% concentration of Brain Heart Infusion broth (Difco) was prepared and filtered through a 0.45-μm membrane filter to remove excipient matter. A 1-ml amount of Tween 80 (Atlas Chemical Industries, Inc., Wilmington, Del.) was added to 1,000 ml of the broth which was then steamed for 10 min to dissolve Tween 80. The medium was dispensed in 200-ml quantities and autoclaved for 20 min at 120°C.

Sample preparation. By using aseptic techniques, approximately 0.5 g of topical ointment was weighed on an acetate weighing paper which had been sterilized with ethylene oxide. The ointment was transferred into a 100-ml sterile, round-bottom flask (equipped with a ground-glass stopper) which contained five sterile 6-mm glass beads. The ointment was then spread in a thin film on the inside surface of the flask with a sterile spatula.

Inoculation of microorganisms for recovery study. Inoculation into the ointment was accomplished by melting 50 g of ointment at 80°C. This ointment was then transferred into a jacketed semimicro Waring Blender at 44°C regulated by a Lauda K-2/R circulating water bath (Brinkman Instruments, New York, N.Y.). When the ointment temperature equilibrated to 44°C, 0.5 ml of Tween 85 (Atlas Chemical) was added and blended at low speed for 30 sec. A 0.1-ml amount of inoculum was then added to the ointment-Tween 85 mixture and blended at high speed for 30 sec. The inoculated ointment was then aseptically transferred into a sterile container and placed in an ice bath and mixed by hand until congealed.
Filtration procedure. A 50-ml amount of the solvent mixture was added to the sample flask, and the mixture was shaken vigorously for 1 min or until the ointment dissolved. Most of the topical ointments tested dissolved in 40 to 50 sec. The resulting solution was poured onto a sterile 0.45-μm membrane filter. As soon as the solution completely passed through the filter, usually in 20 to 30 sec, 200 ml of the rinse medium which was kept at 40 C was gradually added to the filter. The solvent was stored and used at 40 C. Variability in filtration time with different lots of the Millipore filters was sometimes observed. Selection of a suitable filter lot is important to minimize assay variation. When the filtration process was completed, the filter pad was aseptically removed and placed on a Trypticase Soy Agar (TSA) medium in a disposable petri plate. The plates were incubated at 37 C for 7 days and colonies were counted. The method developed was evaluated by comparison to the isopropyl myristate method (1, 2). All of the experiments described are the average of at least four separate determinations.

RESULTS AND DISCUSSION

Toxicity of the solvent mixture. Since S. aureus, E. coli, P. aeruginosa, and Salmonella are a major cause of concern in pharmaceutical preparations, the toxicity of the solvent mixture to these microorganisms was determined. A 0.1-ml amount of a microorganism suspension that contained approximately 100 cells was added directly into 50 ml of the solvent mixture. Samples were filtered at various time intervals. Table 1 indicates D values (time in minutes required to reduce 90% of the microorganism) of various microorganisms tested. The results indicate that the solvent mixture exhibited far less toxicity to the five test organisms than did heat-sterilized isopropyl myristate (2). The D values obtained for S. aureus, spores of B. subtilis, and E. coli were significantly lower (at 95% level) than those obtained with filter-sterilized isopropyl myristate; however, there were no significant differences in D values of P. aeruginosa and S. newington (2).

| Microorganism               | D value |
|----------------------------|---------|
| Bacillus subtilis spore     | 54.5    |
| Staphylococcus aureus       | 72.1    |
| Escherichia coli            | 52.8    |
| Salmonella newington        | 17.6    |
| Pseudomonas aeruginosa      | 10.2    |

* D values = time in minutes required to reduce 90% of the microorganisms (2).

Table 2. Detection of microorganisms from topical ointments

| Product*               | Time to dissolve 0.5 g of ointment | Time to complete filtration process | Viable count per 0.5 g |
|------------------------|------------------------------------|--------------------------------------|------------------------|
| Myciguent              | 26 sec                             | 4 min, 45 sec                        | 1                      |
| Baciguent              | 27 sec                             | 2 min, 10 sec                        | 0                      |
| Mycitracin             | 42 sec                             | 2 min, 4 sec                         | 7                      |
| Neo-Oxyline            | 44 sec                             | 2 min, 47 sec                        | 2                      |
| Neo-Cortef             | 47 sec                             | 2 min, 10 sec                        | 0                      |
| Neo-Delta-Cortef       | 44 sec                             | 3 min, 31 sec                        | 0                      |

* Registered trademark of The Upjohn Co., Kalamazoo, Mich.
ments. Two lots each of six different topical ointments were checked for microbial contamination by the solvent system. As may be seen from the results in Table 2, most of the ointments dissolved quickly and were completely filtered in 3 to 6 min. One to seven microorganisms were isolated from four ointment samples (Table 2); none of the isolated organisms was a pathogen.

ACKNOWLEDGMENT
Technical assistance of S. C. Edwards is acknowledged.

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