APPLIED MICROBIOLOGY, Jan. 1973, p. 139-145
Vol. 25, No. 1

Printed in U.S.A.

Microbial Toxicity of Isopropyl Myristate Used for Sterility Testing of Petrolatum-Based Ophthalmic Ointments

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Received for publication 2 October 1972

The toxicity of isopropyl myristate (IPM) to microorganisms varies from lot to lot and between manufacturers. The toxicity is not directly proportional to the number and level of IPM homologues present nor to the small quantities of fatty acids, alcohols, aldehydes, or ketones found in both filter- and heat-sterilized IPM. The toxicity of IPM, both filter- and heat-sterilized, has no correlation with the usual physical and chemical characteristics of IPM but shows excellent correlation (correlation coefficient, r = 0.88) with the *p*H of a water extract of IPM. The toxic compounds can be removed and the toxicity of both filter- and heat-sterilized IPM can be reduced by basic alumina treatment. These findings may indicate that trace amounts of acidic catalysts remaining after production of IPM are responsible for the toxic effect.

In 1964, eight cases of severe eye disorder were reported in Sweden in workmen treated with an ophthalmic ointment (4). As a result of the infection, one patient lost an eye and a second suffered reduced vision. *Pseudomonas aeruginosa* was isolated both from the infected eyes and from the ointment used in the treatment. After this unfortunate incident, Scandinavian countries have placed a sterility requirement on ophthalmic ointments (5). Since then, several European countries are requiring sterility of ophthalmic ointments (1, 2), and an announcement appeared in the Third Interim Revision of the USP XVIII which would require all ophthalmic ointments marketed in the United States to be sterile after 1 June 1973 (8).

The bacteriological retentative membrane filtration method in which isopropyl myristate (IPM) is used as a solubilizing agent has been adopted by USP XVIII for sterility testing of ophthalmic ointments (7). Because it was shown that filter-sterilized IPM makes it possible to recover organisms that were not found when heat-sterilized IPM was used (6), the possibility appeared to exist that differences in manufacturing methods and subsequent purification steps of IPM may cause differences in toxicity of filter-sterilized IPM from lot to lot or between manufacturers. Therefore, it appeared desirable to establish guidelines for selecting IPM suitable for sterility tests. Although the D value (time in minutes required to kill 90% of the test microorganisms) of IPM is indicative of the solvent's toxicity, the determination is lengthy and cumbersome. The purpose of this study is to elucidate the toxicity of the filter- and the heat-sterilized IPM in order to develop a test which measures the toxicity of IPM for microorganisms.

MATERIALS AND METHODS

Test microorganism. IPM was shown by Tsuji et al. (6) to be more toxic to gram-negative than to gram-positive microorganisms, and *Pseudomonas aeruginosa* was the most sensitive to IPM among the gram-negative microorganisms tested. A clinical isolate of *P. aeruginosa* UC104 (ATCC 10145) found to be the most sensitive to IPM among eight cultures tested (6) was used in this study. The microorganism was grown on Trypticase soy agar (TSA) for 24 hr, and cells grown at 35 C were harvested in Trypticase soy broth. The cell suspension (approximately 10^9 cells per ml) was dispensed into ampoules, 1.2 ml per ampoule, and rapidly frozen by immersion into liquid nitrogen. The frozen cultures were then kept in a liquid nitrogen atmosphere (-160 C) until used. Prior to use, the frozen culture was quickly thawed while shaking vigorously in a 47 C water bath. The thawed culture was diluted with sterile water to contain approximately 10^3 cells per ml.

Isopropyl myristate. IPM was obtained from five suppliers: Givaudan Corporation, Clifton, N.J., as Deltyl Extra; Emery Industries, Inc., Cincinnati,

Ohio; EGA-Chemie K.G., Albuch, W. Germany; Fluka A.G., Buchs, Switzerland; and Schuchardt, Munchen, W. Germany. The IPM was filter-sterilized through a presterilized 0.22-μm GS filter (Millipore Corp., Bedford, Mass.) before use. The solvent was then dispensed in 50-ml quantities into 250-ml presterilized round-bottom flasks. The flasks, capped with 24/40 ground-glass stoppers, contained two glass beads of 6-mm diameter.

Rinse medium. A 0.5% brain heart infusion broth was prepared and filtered through a 0.22-μm membrane filter. A 1-ml amount of Tween 80 (Atlas Chemical Industries, Wilmington, Del.) was added to 1 liter of the broth and steamed for 10 min to dissolve the Tween 80. The medium was then dispensed in 400-ml quantities and autoclaved for 20 min at 121 C.

D value determination. An appropriate number of flasks (six to eight) containing IPM were placed on an Eberbach reciprocating shaker (Eberbach Corp., Ann Arbor, Mich.) and inoculated with 0.1 ml of the *P. aeruginosa* suspension (10^2 cells/0.1 ml) by using an Eppendorf 100-μliter micropipette with a presterilized disposable tip (distributed by Brinkmann Instruments, Inc., Westbury, N.Y.). Plate counts were used to determine the initial concentration of *P. aeruginosa*. Flasks were then shaken vigorously (250 cycles/min) for given time intervals. At the end of each time interval, a flask was removed from the shaker, and immediately the contents were filtered through a presterilized 0.45-μm HA filter under 100-μm Hg vacuum. The filter was then rinsed immediately with 200 ml of the rinse medium. After filtration was completed, the filter was removed and aseptically transferred to a prepoured TSA plate and incubated at 32 C for 2 days and then an additional 5 days at room temperature. After incubation for 2 and 7 days, colonies were counted, and higher counts were used to calculate D values.

The D value of IPM tested was calculated by using the following formula: D = U/(log a − log b), where: D = time in minutes required to kill 90% of the microorganisms; U = time in minutes required to decrease the number of microorganisms from a to b; a = number of microorganisms at time Ua; b = number of microorganisms at time Ub.

Basic alumina wash. A glass column (20 mm inner diameter) was packed with basic aluminum oxide, activity grade I (M. Woelm, Eschwege, W. Germany) to a height of 15 cm. A 500-ml portion of IPM was chromatographed under a slight pressure, and the eluent was collected.

Chemical determinations. (i) Homologues of IPM occurring as impurities were determined with an F and M model 400 gas chromatograph by using a flame ionization detector. Gas flow rates were: hydrogen, 40 ml/min; air, 600 ml/min; and helium, 100 ml/min. Chart speed was 0.25 inch/min, and oven temperature was 170 C. A glass column, 3 by 1,850 mm (6 ft), was packed with 10% OV-1 on Gas Chrom Q, 100–120 mesh (Applied Science Laboratories, Inc., State College, Pa). A 0.5-μliter amount of IPM was directly injected into the column. The compounds eluted were quantitated with a Hewlett Packard Electronic Integrator, model 3370A, and were identified with an LKB 9000 gas chromatograph-mass spectrometer.

(ii) For the determination of isopropanol, acetone, propionaldehyde and other heat-catalyzed decomposition products in heat-sterilized IPM, an F and M model 400 gas chromatograph with a 3- by 1,850-mm glass column packed with Porapak Q (Water Associates, Inc., Framingham, Mass.) was used at 150 C. IPM was injected directly onto the column and the peaks eluted were quantitated by peak height and identified with the LKB 9000 gas chromatograph-mass specrometer.

(iii) The *p*H and titratable acidity of the water extract were determined from 100 ml of IPM poured into a 250-ml centrifuge bottle containing 10 ml of distilled water. The ratio of IPM to water must be maintained at 10:1 to obtain reliable data. The bottle was sealed tightly with a neoprene rubber stopper and placed in a position horizontal to the direction of movement of an Eberbach shaker and shaken vigorously at 250 strokes/min for 1 hr. The shaking time may be shortened to 5 min when shaken at 250 strokes/min. After shaking, the bottle was centrifuged at 1,800 rev/min for 20 min. The IPM layer was aspirated off, and 5 ml of the remaining water extract was pipetted into a beaker for the determination of *p*H and titratable acidity with a Beckman Expandomatic SS-2 *p*H meter. Titrations were made with 0.1, 0.01, and 0.001 N NaOH dispensed from a Hamilton microsyringe.

(iv) Acid, saponification, iodine, and ester values were determined by following methods described in USP XVIII (7).

RESULTS

Toxicity of IPM from various suppliers. The microbial toxicity of filter-sterilized IPM differed greatly from lot to lot and from manufacturer to manufacturer (Table 1). The IPM manufactured by Company B showed the highest D value (222 min), and that of Company D showed the lowest D value (26 min) among the five manufacturers. I. Dony of Belgium also experienced high bactericidal action of IPM manufactured by Company D.

TABLE 1. *Toxicity of IPM from various sources against Pseudomonas aeruginosa UC104*

IPM source	D value[a]	*p*H[a]	Titratable acidity[b]
Company A			
lot 1	72.3; 83.2	4.55	262
lot 2	64.6; 42.7	3.75	1,126
Company B	257.4; 185.1	5.95	82
Company C	76.8; 65.1	4.95	200
Company D	38.4; 12.6	3.95	1,400
Company E	91.7	5.25	260

[a] Correlation coefficient between *p*H and D value = 0.88.

[b] Expressed as microliters of 0.001 N NaOH.

Toxicity and purity of IPM. Since the source of myristic acid for the production of IPM is coconut oil, various chain-length fatty acid esters may be expected in it. Figure 1 shows that IPM is composed of various homologues and only 78% is isopropyl myristate. The major impurities found were isopropyl palmitate, 11%; isopropyl laurate, 4%; isopropyl caprylate, 3.4%; and minor impurities: isopropyl caprate, isopropyl pentadecanoate, isopropyl heptadecanoate, isopropyl stearate, *n*-propyl laurate, *n*-propyl myristate, *n*-propyl pentadecanoate, and *n*-propyl palmitate.

To examine the toxicity of the homologues of IPM, (i) isopropyl palmitate, isopropyl laurate, isopropyl isostearate, and *n*-propyl myristate were obtained from Emery, and (ii) IPM was distilled by Burdick Jackson, Muskegon, Mich., into nine different fractions. The D values of *n*-propyl myristate, isopropyl palmitate, isopropyl laurate, and isopropyl isostearate were 43.6, 52.8, 48.6, and 85.2 min, respectively.

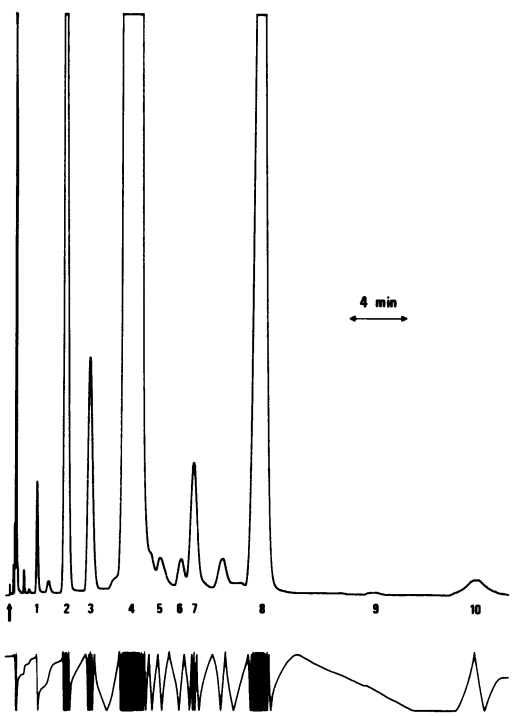

FIG. 1. *Chromatogram indicating purity of isopropyl myristate of a commercial source (isothermally chromatogrammed at 190 C by using an OV-1 column). 1, isopropyl caprylate; 2, isopropyl caprate; 3, isopropyl laurate; 4, isopropyl myristate; 5, n-propyl myristate; 6, isopropyl pentadecanoate; 7, n-propyl pentadecanoate; 8, isopropyl palmitate; 9, isopropyl heptadecanoate; 10, isopropyl stearate.*

The chromatograms show (Fig. 2) that fraction F contains the highest amount of myristate whereas fractions A and H are devoid of isopropyl myristate. The D values in Table 2 were determined on 1 ml; hence variations are considerable. Fractions A, B (boiling points ranges of 110–117 C, 117–149 C, respectively), and the fraction H (pot residue) had low D values. Fractions C to G, which contained varying amounts of isopropyl myristate, had comparable D values whereas fraction E, which is less pure than fraction F, had the highest D value.

These data indicate that the microbial toxicity of filter-sterilized IPM is not directly proportional to the number and level of isopropyl myristate homologues present. It is thus possible that the nontoxic homologues of IPM may be used as solvents for the sterility test of ophthalmic ointments. Saponification, iodine, and ester values of IPM showed no correlation with its toxicity.

Toxicity of compounds produced by heat sterilization. The results from the gas chromatographic analysis of IPM with the Porapak Q column indicated that the amounts of methanol, ethanol, isopropanol, butanol, isobutanol, propionaldehyde, and acetone increased with an increase in heat sterilization time (Fig. 3 and Table 3). Various quantities of isopropanol, acetone, propionaldehyde, and myristic acid which corresponded to the levels found in 6-, 12-, and 36-hr heat-sterilized IPM at 180 C were added either singularly or in combination into IPM supplied by Company B, and their effect on the D value was examined (Table 4). Although some decreases in D value were noted by the addition of compounds, the resulting D values were not as low as that seen with 6-hr heat-sterilized IPM (D = 0.8 min). Also, the quantity of isopropanol in filter-sterilized IPM did not significantly correlate with D values observed.

The data also indicated that myristic acid even at the concentration of 20.6 mM showed no toxicity for *P. aeruginosa*. Galbraith et al. found that *Pseudomonas phaseolicola* was insensitive to 1.2 mM myristic acid whereas *Bacillus megaterium* was inhibited at 0.15 mM (3). Therefore, even though myristic acid was nontoxic to *Pseudomonas*, it would be toxic to gram-positive microorganisms; hence myristic acid would be an important factor to consider in the isolation of microorganisms from ointments. Efforts are being made to identify the unknown gas chromatographic peaks in heat-sterilized IPM to determine their toxicity to microorganisms.

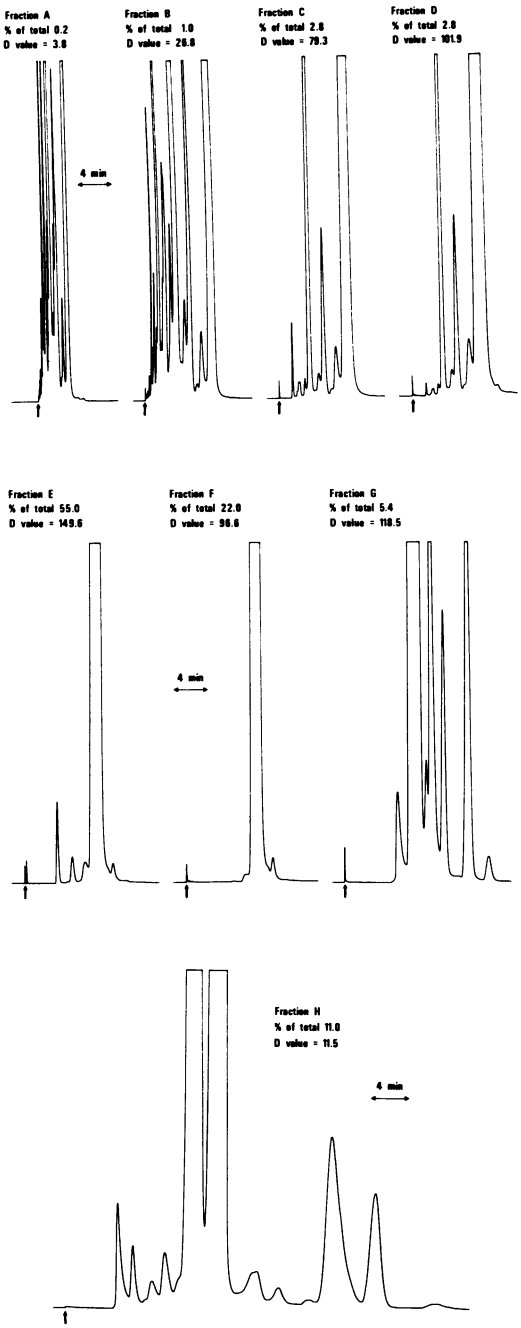

FIG. 2. *Chromatograms of distillation fractions (A, B, C, D, E, F, G, and H) of IPM. Isothermally chromatogrammed at 190 C by using an OV-1 column.*

The IPM kept at 50 C for 9 days showed a D value of 54.7 min. The D value prior to the treatment was 77.8 min. Since change in D value is minimal, IPM may be kept at 50 C for 1 day during testing for sterility without significantly affecting its toxicity.

Toxicity of the acid catalyst. One or more acid catalysts are used for the production of IPM to catalyze esterification of myristic acid with isopropanol. Most of these catalysts are removed by a subsequent water wash. However, since up to 2% of acid catalysts are added,

TABLE 2. *Toxicity of IPM fractions against Pseudomonas aeruginosa UC104*

IPM fraction	Proportion in IPM (%)	D value[a, b]	pH[a, c]	Titratable acidity[d]
A	0.2	3.8		
B	1.0	28.0; 25.5	3.00	740
C	2.8	88.3; 70.3	3.72	80
D	2.8	71.3; 132.5	4.05	58
E	55.0	95.9; 135.3	4.50	15
F	22.0	84.2; 109.0	3.75	95
G	5.4	119.3; 117.6	3.50	120
H	11.0	8.2; 14.8	2.90	1,390

[a] Correlation coefficient between pH and D value = 0.80.
[b] Determined by using 1 ml.
[c] Determined by using 2 ml of fraction added to 20 ml of water.
[d] Expressed as microliters of 0.01 N NaOH.

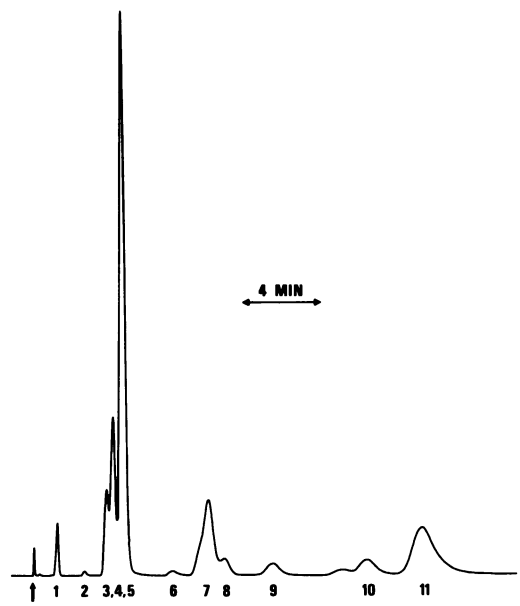

FIG. 3. *Chromatogram of heat-sterilized IPM at 180 C for 36 hr indicating production of hydrolyzed products by heat. Isothermally chromatogrammed at 180 C using Porapak Q. 1, Methanol; 2, ethanol; 3, propionaldehyde; 4, acetone; 5, isopropanol; 6, n-propanol; 7, unknown; 8, butanol; 9, isobutanol; 10 and 11, unknown.*

TABLE 3. *Amount of isopropanol, acetone, propionaldehyde, and myristic acid in IPM heat-sterilized at 180 C for 6, 12, and 36 hr*

Compound	Filter-sterilized IPM (mM)	Heat-sterilized IPM at 180 C (mM)		
		6 Hr	12 Hr	36 Hr
Isopropanol	0.236	0.785	2.22	8.64
Acetone	Less than 0.05	0.204	0.478	1.71
Propionaldehyde	Less than 0.05	0.065	0.262	1.18
Myristic acid	0.016	2.06	6.15	20.6

TABLE 4. *Survival of Pseudomonas aeruginosa UC104 by the quantitative addition of isopropanol, acetone, propionaldehyde, and myristic acid*

Materials added	D value[a]		
	6 Hr	12 Hr	36 Hr
Isopropanol	136	64.2	45.0
Acetone	104	64.3	73.5
Propionaldehyde	65.2	57.0	84.2
Combination of isopropanol, acetone, and propionaldehyde	61.4	53.8	17.8
Myristic acid	126	124	132

[a] Original IPM D value = 185. Materials were added at concentrations equivalent to those seen in IPM heated at 180 C for the hours shown.

trace amounts may remain in the IPM which may differ from lot to lot and from manufacturer to manufacturer. To determine whether residual quantities of acid catalysts contribute to the toxicity of filter-sterilized IPM, the catalyst p-toluene sulfonic acid was added to IPM at 20 and 2 μliters/liter, and the D values were determined (Table 5). Reduction of D values by trace amounts of p-toluene sulfonic acid was dramatic. Therefore, it is possible that the toxicity differences seen with various sources of IPM and from lot to lot may be due to residual amounts of acid catalysts.

Toxicity of water extract. In our previous work it was indicated that the *p*H and titratable acidity of water extracts of heat-sterilized IPM correlated with the D values (6). Therefore, *p*H and titratable acidity of water extracts were again examined (Tables 1, 2, 5). The *p*H showed excellent correlation with D values (r = 0.88), whereas the correlation between D value and the titratable acidity was smaller (r = 0.69). Therefore, the *p*H of a water extract of IPM may be used to select suitable lots for sterility testing of ointments.

Since the *p*H of the water extract correlated very well with the toxicity of IPM, the presence of toxic compounds in the extract was examined with the cylinder cup agar diffusion method by using *P. aeruginosa* as a test microorganism. A zone of inhibition from a water extract of heat-sterilized IPM was detected, indicating that the toxic compounds are extracted in water. However, the agar diffusion method was not sensitive enough to detect minor differences in toxicity of filter-sterilized IPM. The possibility of developing a turbidimetric assay method by using the water extract is being investigated. Gas chromatographic analyses of water extracts were performed but were inconclusive other than for alcohols, aldehydes, and ketones.

TABLE 5. *Effect of p-toluene sulfonic acid in IPM and basic alumina treatment on the survival of Pseudomonas aeruginosa UC104*

Treatment	D value	*p*H
IPM alone	185	5.95
IPM with p-toluene sulfonic acid (2 μliters/liter)	22.8	3.95
IPM with p-toluene sulfonic acid (20 μliters/liter)	2.1	2.60
Basic alumina treatment of IPM with 20 ppm p-toluene sulfonic acid	97.8	6.60

Neither the USP acid value method for fats and oils (7) nor *p*H commonly determined by adding 76 ml of 3A alcohol and 4 ml of water to 20 ml of IPM showed any correlation with the D value.

Removal of toxicity. A glass column packed with basic alumina was used to determine whether the toxic compounds in IPM could be adsorbed on basic alumina and thus improve the survival of *P. aeruginosa*.

(i) IPM manufactured by Company D, with the lowest D value (25.5 min) among the lots examined, was treated with basic alumina, and the D value was determined (Fig. 4). The D value was considerably improved (181 min) and became comparable to the IPM from Company B which showed the highest D values.

(ii) The IPM which showed considerable reduction of D values by the addition of 2 μliters of p-toluene sulfonic acid per liter (D = 2 min) was treated with basic alumina (Table 5). Considerable improvement in D value was experienced with this treatment (D = 98 min).

(iii) Survival of *P. aeruginosa* in heat-sterilized IPM was improved by basic alumina treatment. As was reported previously (6), heat sterilization significantly decreased D values (from 83 to 0.8 min). The basic alumina treatment of the heat-sterilized IPM considerably improved the D value to 82 min (Fig. 5).

The basic alumina treatment did not change the ointment - solubilizing characteristics;

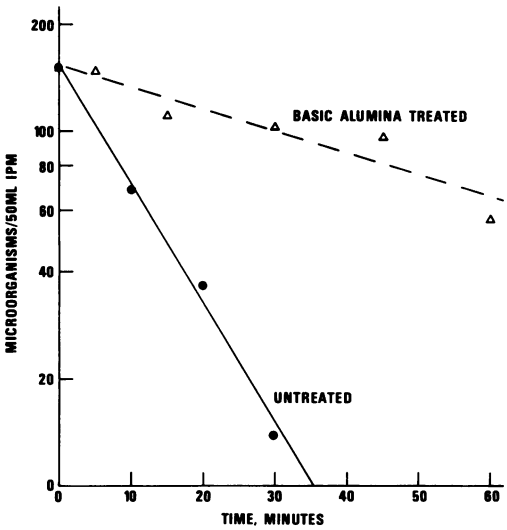

FIG. 4. *Improvement of filter-sterilized isopropyl myristate from Company D by basic alumina treatment for the survival of Pseudomonas aeruginosa UC104.*

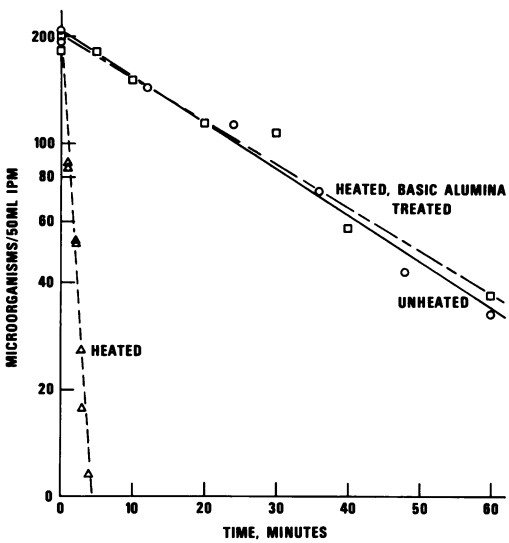

FIG. 5. *Increase of toxicity of isopropyl myristate by heat sterilization (6 hr at 180 C) and improvement of the survival of Pseudomonas aeruginosa UC104 in heat-sterilized isopropyl myristate by the basic alumina treatment.*

therefore, it should be helpful in removing the toxicity of IPM for the sterility test of ointments. A water wash of IPM was also tried but did not significantly change the *p*H of water extract, and an alkaline water rinse created an emulsion which was difficult to break.

Theoretically, a 0.22-μm filter should recover as many or a higher number of *P. aeruginosa* than a 0.45-μm filter. However, the D value was less than 1.0 with a 0.22-μm filter, whereas the D value was 185 with a 0.45-μm filter by using the same lot of IPM. This may be due to the increase in filtration time required by the 0.22-μm filter. The vacuum level used for filtration also affects the speed of filtration and hence the D value. Under a vacuum of 100 μm of Hg, a D value of 83.2 min was obtained, whereas under 580 mm of Hg the D value was 22.0 min with the same lot of IPM.

DISCUSSION

The experiments established that the varying degrees of toxicity experienced in filter-sterilized IPM supplied by various manufacturers may be due to a trace amount of catalyst remaining in the IPM. However, the amount remaining is small and the types, kind, and number of catalysts used in the production of IPM vary and are unpublished information. Therefore, it would not be advisable to choose one or two chemical tests which are specific for one type of catalyst to express the degree of toxicity of IPM.

The IPM having a D value of 60 min is expected to decrease 10% of the *P. aeruginosa* population in 3 min, and it usually takes 3 min to dissolve an ointment sample and to complete filtration (6). Therefore, the IPM used for sterility test should have a D value of at least 60 min. Since the *p*H of the water extract at *p*H 5.0 corresponds to a D value of approximately 100 min, *p*H 5.0 may be used with some safety to select IPM for sterility use.

Basic alumina treatment may be used when IPM does not meet the specification of *p*H 5.0 or above or a D value of 60 min or above.

ACKNOWLEDGMENTS

Emery Industries, Inc. is acknowledged for the supply of n-propyl myristate, isopropyl palmitate, isopropyl laurate, and isopropyl isostearate. Burdick Jackson is acknowledged for the fractionated distillation of IPM. J. B. Kirsch of Upjohn-Belgium is acknowledged for the supply of IPM of EGA, Fluka and Schuchardt. P. B. Bowman is acknowledged for the mass spectrometric analysis and A. R. Lewis for statistical analysis.

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