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Short communication

Investigation of fatty acid esters to replace isopropyl myristate in the sterility test for ophthalmic ointments

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Abstract

Several pharmacopoeias recommend the membrane filtration method for the sterility test of ophthalmic ointments. Isopropyl myristate, a fatty acid ester that exhibits high toxicity mainly against Gram-negative microorganisms, is indicated as a solvent for ointments. In this study, six fatty acid esters (diethyl adipate, diisopropyl adipate, ethyl laurate, ethyl myristate, methyl caprylate and isopropyl palmitate) were evaluated as solvents to replace isopropyl myristate in the sterility test for ophthalmic ointments. The logarithm of the partition coefficient (log *P*) of the fatty acid esters was calculated from the sum of the substituent hydrophobicity constants (π) of the functional groups present in their molecules. The ability of the solvents to dissolve an ophthalmic ointment base was investigated. The *D*-value method was used to assess the antimicrobial activity of isopropyl palmitate, ethyl myristate, ethyl laurate and isopropyl myristate against *Pseudomonas aeruginosa*. Isopropyl palmitate was the least toxic solvent to this microorganism, since it had the highest *D*-value (171.1 min). No significant difference was observed between the *D*-values of ethyl myristate (89.4 min) and isopropyl myristate (92.5 min). Ethyl laurate exhibited the lowest *D*-value (27.2 min). Using gas chromatography coupled to mass spectrometry, other fatty acid esters were detected as the predominant impurities in the solvents, as well as acid contaminants in low or insignificant amounts.

Keywords: Sterility test; Isopropyl myristate; *D*-Value; Isopropyl palmitate; Ethyl laurate; Ethyl myristate; Antimicrobial activity

1. Introduction

The presence of antimicrobial agents and water immiscible excipients is a complicating factor in the detection of microbial contaminants in semi-solid preparations. Sokolski and Chidester reported the use of isopropyl myristate as a solvent for ophthalmic ointments in the sterility test by the membrane filtration method in 1964 [1]. The publication of the United States Pharmacopoeia officialized this method in 1970 [2]. Since then, several authors have reported the sensitivity of microorganisms to isopropyl myristate [3–6], particularly *Pseudomonas aeruginosa*, the bacterium most sensitive to this fatty acid ester [7]. Thus, the use of isopropyl myristate in the sterility test of ointments can produce false-negative results and cause health problems for some users of such a pharmaceutical preparation. However, the latest editions of the pharmacopoeias still recommend the usage of isopropyl myristate as a solvent for ointments in sterility tests [8–10].

The aim of the present study was to replace isopropyl myristate, used in the sterility test for ointments by the membrane filtration method, by a fatty acid ester without antimicrobial activity. The theoretical and experimental capacity to dissolve the ophthalmic ointment base and the antimicrobial activity against *P. aeruginosa* of fatty acid esters (isopropyl myristate, diethyl adipate, diisopropyl adipate, ethyl laurate, ethyl myristate, methyl caprylate and isopropyl palmitate) were investigated.

The fatty acid esters were analyzed by gas chromatography coupled to mass spectrometry (GC/MS) to verify a possible correlation between the presence of impurities and microbial toxicity. The efficiency of the flow of the solvents through an aluminum oxide column, which is preconized by the pharma-

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copoeias to increase the pH value of isopropyl myristate, was also evaluated.

2. Experimental

2.1. Fatty acid esters

Isopropyl myristate (Vetec, RJ, Brazil); ethyl laurate (Spectrum, New Brunswick, NJ); ethyl myristate (Spectrum, New Brunswick, NJ); isopropyl palmitate (Spectrum, New Brunswick, NJ); diethyl adipate (Aldrich, Milwaukee, USA); methyl caprylate (Aldrich, Milwaukee, USA) and diisopropyl adipate (Isp Van Dyrk, Belleville, NJ) were used. The pH value of the fatty acid esters was measured by the addition of ultra-purified water (1 mL) to 10 mL of the fatty acid esters. The resulting system was magnetically stirred for 2 h and centrifuged for 20 min, and the pH of the aqueous layer was measured. Those potential solvents that showed pH values below 5.5 were filtered through an alkaline-treated aluminum oxide column. All the solvents were sterilized by filtration through a GS 0.22 μm membrane filter (Millipore, SP, Brazil).

2.2. Determination of the capacity of the fatty acid esters to dissolve the ointment base

To theoretically predict the log *P* (partition coefficient) of the fatty acid esters, the calculations were performed by the summation of the substituent hydrophobicity constants (π) of the functional groups of each molecule [11]. To evaluate the capacity of all the fatty acid esters to dissolve the ointment base, visual and nephelometric (at 580 nm) analyses were performed. The ointment base (0.2 g) was added to 10 mL of a potential solvent at 44 °C. For visual evaluation of the solubility of the base in different fatty acid esters, the degree of dissolution of the ointment in isopropyl myristate was taken as a reference. The mixture ointment/solvent that exhibited the same aspect as the solvent alone was considered as "very soluble". When the system (ointment/solvent) had an aspect almost equal to that described above, it was considered to be "soluble" and when it showed a milky aspect, it was considered to be "slightly soluble".

2.3. Obtaining the ophthalmic ointment base

A simple base of an ophthalmic ointment was prepared by the melting method using wool fat (100.0 g), soft yellow paraffin (800.0 g) and liquid paraffin (100.0 g) [10]. After preparation, the base was sterilized for 1 h at 150 °C.

2.4. Microorganism and culture media

The test microorganism was *P. aeruginosa* ATCC 9027 supplied by the Instituto Nacional de Controle de Qualidade em Saúde (INCQS, RJ, Brazil). The microorganism was cultured in antibiotic agar number 1 (Merck, Darmstadt, Germany). Sabouraud-dextrose agar (Difco, Sparks, USA) and trypticase soy agar TSA (Difco, Sparks, USA) were used to perform the sterility test of the membrane washing fluid and also for control of the environment.

2.5. Inoculum standardization

The stock suspension of *P. aeruginosa* was prepared in sterile saline solution from a 24-h culture (transmittance value of $33.0 \pm 2\%$ at 580 nm). Then it was diluted from 10^{-1} to 10^{-6} in sterile saline solution and an aliquot (130 μL) of the last dilution was used to prepare the inoculum containing 100–200 cells.

2.6. Antimicrobial activity of the solvents: determination of D-value

Only the fatty acid esters that dissolved the ointment base to a degree similar to that achieved with isopropyl myristate were submitted to the test for antimicrobial activity. To determine the *D*-value, two independent experiments were performed for each solvent, in triplicate, for each contact time of the solvent with the microorganism.

Each solvent (3 mL), previously sterilized by filtration, was inoculated with *P. aeruginosa* (100–200 cells) and was maintained for a specific contact time (0, 1, 10, 20 min) with stirring. The "zero time" was defined as the contact time of 10 s. For ethyl laurate, the test for the contact time of 20 min could not be performed because of an insufficient quantity of solvent.

At each contact time, the inoculated solvent was filtered through a GS 0.45 μm membrane filter (Millipore, SP, Brazil). The filter was washed with three 100-mL portions of washing fluid (0.9% sodium chloride and 0.5% polysorbate 80) and placed in a Petri plate containing TSA. The plates were incubated for 24 h at 36 ± 1 °C.

After incubation, the number of cells on the membrane was determined. The same procedure was performed for the determination of the number of cells initially added to the solvent (blank), using sterile saline solution (0.9%, w/v sodium chloride solution, SS) and homogenization for 10 s.

The *D*-value, defined as the time necessary for a 90% decrease in the inoculum initially added, was calculated for each solvent using the straight-line equation ($y = a + bx$) [12]; where $y = \log N_t/N_0$; x = contact time of the microorganism with the solvent in min; N_t = average number of surviving cells at time t; N_0 = initial average number of cells (blank). From the slope (b), the *D*-value was calculated as follows: $D = -1/b$.

2.7. Investigation of solvent impurities

The samples were analyzed before and after passing through an alkaline-treated aluminum oxide column (pH ≥5.5) to obtain a profile of the chemical contaminants found in the solvents. A gas chromatograph (Hewllet Packard 6890, Agilent Technologies, Palo Alto, USA) coupled to a mass spectrometer (Hewllet Packard 5989A, Agilent Technologies, Palo Alto, USA), with electronic impact of 70 eV, a column of 25 m × 0.25 mm (Hewllet Packard, Agilent Technologies, Palo Alto, USA) and helium as the carrier gas were used. The samples were dissolved in dichloromethane (Vetec, SP, Brazil) in a 1:10 ratio.

Table 1
Partition coefficient (log *P*) values calculated for the solvents and the visual and turbidity evaluation of the solvent/base system

Solvent	log *P*	Visual evaluation	Turbidity evaluation (transmittance %)[a]
Isopropyl myristate	7.3	Very soluble	99.0
Ethyl myristate	6.8	Soluble	99.0
Ethyl laurate	5.8	Soluble	98.0
Diisopropyl adipate	3.6	Slightly soluble	57.0
Methyl caprylate	3.3	Soluble	99.0
Diethyl adipate	2.6	Slightly soluble	48.0
Isopropyl palmitate	8.3	Soluble	99.0

[a] Determined at 580 nm.

2.8. Statistical analysis

D-Values are estimated from data using a simple linear regression. Two curves were obtained for each solvent and were statistically compared by regression analysis in groups. The *D*-values found for each solvent were also evaluated by analysis of variance (ANOVA). If a significant difference was detected, Tukey's test [13] was applied. Statistical analysis was performed with a level of significance set at 0.05.

3. Results and discussion

3.1. Selection and treatment of solvents

The selection of the solvents that would be submitted to the antimicrobial test was made considering the capacity of the solvent to dissolve the ointment base. Then, the partition coefficient (log *P*) of the solvents was calculated [11]. The values are listed in Table 1. Ethyl laurate, ethyl myristate and isopropyl palmitate, which presented log *P* values similar to that of isopropyl myristate, were considered as potential solvents for the ointment base.

To confirm the theoretically calculated solvating potential, visual and spectrophotometric analyses of the ointment base dissolved in each of the solvents were performed. The results (Table 1) revealed that methyl caprylate, ethyl laurate, ethyl myristate and isopropyl palmitate were suitable solvents. The experimental results matched those for the calculated log *P*, except for methyl caprylate, which presented a log *P* value much lower than isopropyl myristate but dissolved the ointment base.

The fatty acid esters selected for determination of the antimicrobial activity were ethyl laurate, ethyl myristate and isopropyl palmitate. These esters furnished the most suitable results in both theoretical and experimental determination of their capacities to dissolve the ophthalmic ointment base.

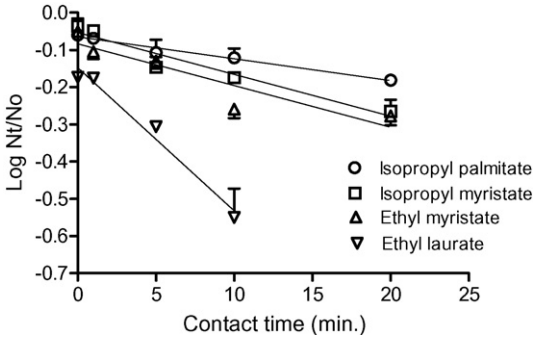

Fig. 1. Survival curves of *Pseudomonas aeruginosa* in the solvents (isopropyl myristate, isopropyl palmitate, ethyl myristate and ethyl laurate). Each point represents mean ($n = 6$) ± S.E.

Only ethyl laurate, which had a pH value of the aqueous extract higher than the recommended minimum (5.5), was not passed through the aluminum oxide column. Ethyl myristate, isopropyl palmitate and isopropyl myristate presented pH values lower than 5.5 and were treated by passage through the alkaline-treated aluminum oxide column to increase the pH values to the range of 6–7.

3.2. Antimicrobial activity of the solvents

The selected solvents (ethyl laurate, ethyl myristate and isopropyl palmitate) and isopropyl myristate showed a linear relationship between the number of surviving microorganisms and the contact time ($r > 0.90$). There was no significant difference between the slopes obtained from two independent experiments for each solvent ($p < 0.05$), which resulted in a single straight line. There was a decrease in the number of microorganisms as a function of the contact time for all the solvents investigated (Fig. 1).

The *D*-values of the solvents tested (isopropyl myristate, ethyl laurate, ethyl myristate and isopropyl palmitate) are summarized in Table 2. There was a statistically significant difference among the *D*-values of isopropyl palmitate (171.1 min), ethyl laurate (27.2 min) and the other esters. Ethyl laurate, with the lowest *D*-value, was the solvent with the highest toxicity against *P. aeruginosa*, whereas isopropyl palmitate demonstrated the highest *D*-value and lowest toxicity. No significant difference was found between the *D*-values of ethyl myristate (92.5 min) and isopropyl myristate (89.4 min). Consequently, isopropyl

Table 2
D-Value straight-line equation and correlation coefficient for the antimicrobial activities of the solvents against *Pseudomonas aeruginosa*

Solvents	*D*-Value (min)	Straight-line equation	Correlation coefficient (*r*)
Isopropyl myristate	92.5 c ± 22.9	$y = 0.052 - 0.0111x$	0.9688
Ethyl myristate	89.4 c ± 9.6	$y = 0.084 - 0.0113x$	0.9185
Ethyl laurate	27.2 a ± 7.6	$y = 0.1484 - 0.0382x$	0.9880
Isopropyl palmitate	171.1 b ± 6.2	$y = 0.0652 - 0.0059x$	0.9894

Results are expressed as means ($n = 6$) ± S.E. Different letters indicate significant differences between *D*-values and identical letters indicate that there is no significant difference between *D*-values at $p \leq 0.05$.

myristate and ethyl myristate showed similar antimicrobial activities.

3.3. Analysis of the solvent impurities

It was verified from the GC/MS analysis that the contaminants in the solvents were fatty acids and fatty acid esters. A typical chromatogram (isopropyl myristate) is shown (Fig. 2).

In isopropyl myristate, the main contaminant detected was isopropyl dodecanoate with a retention time of 6.28 min and relative percentages of 1.28 and 0.49 before and after its passage through the column, respectively. According to Klaffenbach and Kronenfeld [14], isopropyl dodecanoate is one of the prevailing impurities found in this solvent. Ethyl myristate and isopropyl palmitate are frequent contaminants encountered in isopropyl myristate as well, but they were not detected in the batch used.

Ethyl myristate had non-anoic acid ethyl ester, hexadecanoic acid methyl ester, and octanoic acid ethyl ester as contaminants, with retention times of 5.69, 7.97 and 12.95 min, respectively. No reduction in these impurities after passing the sample through the aluminum oxide column was observed. The presence of an unidentified contaminant with a retention time of 6.28 was detected in ethyl laurate, the only solvent that was not treated by passage through the aluminum oxide column.

Isopropyl palmitate was contaminated with isopropyl myristate with a retention time of 10.06 min, and acid impurities such as dodecanoic acid (6.38 min) and octadecanoic acid (17.23 min) were detected before passage through the column. After passing the solvent through column, dodecanoic acid was no longer detected and octadecanoic acid was reduced. The presence of impurities such as isopropyl myristate and acids in isopropyl palmitate may have contributed to its antimicrobial activity. The purity of the solvents was suitable (purity over 98%, considering the percentage of area) with the exception of isopropyl palmitate that presented a purity of approximately 94%.

Some considerations can be made upon comparing the data obtained before and after passing the solvents through the column. As expected, the column does not retain non-acid contaminants, but in all the cases investigated, it was effective in increasing the pH values of the solvents.

In a study by Tsuji and Robertson [6], the toxic activity of isopropyl myristate towards microorganisms was associated with the presence of acid impurities. According to the data presented in this study, the antimicrobial activity of this solvent could not be attributed only to the action of acid contaminants, since the toxic activity towards *P. aeruginosa* could still be observed after passing the solvent through the aluminum oxide column with the consequent increase in the pH range to values

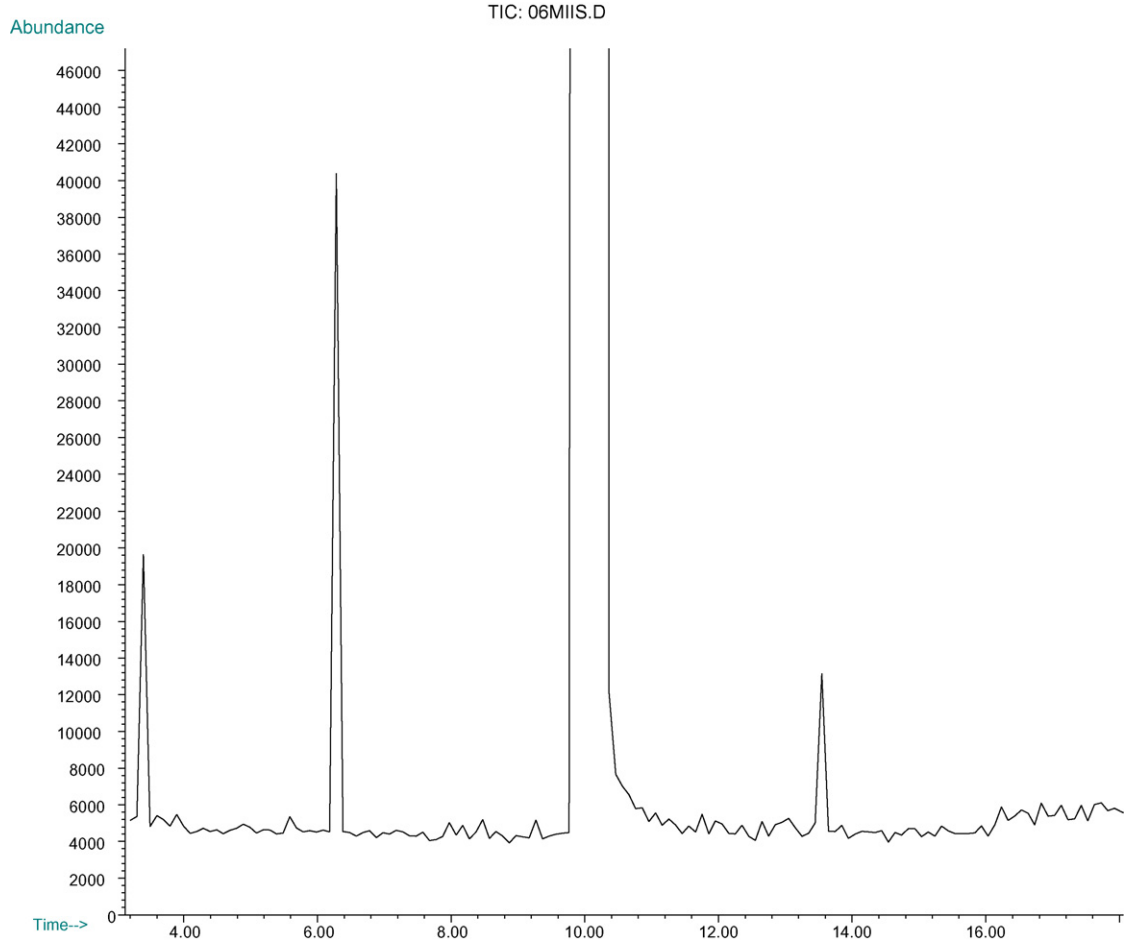

Fig. 2. Isopropyl myristate chromatogram obtained after its passage through the aluminum oxide column.

recommended by the pharmacopoeias [8–10]. The toxic activity of ethyl laurate, a solvent that did not exhibit detectable acid contaminants, was also observed. One could assume that the antimicrobial activity observed in this study for the fatty acid esters may be due to this kind of chemical and low pH values, as occurs for parabens, which are weakly acid esters [15,16].

The results indicate that isopropyl palmitate may be a promising substitute for isopropyl myristate as a solubilizing agent for ophthalmic ointments in the sterility test by the membrane filtration method. Among the fatty acid esters investigated, isopropyl palmitate demonstrated the lowest antimicrobial activity despite the presence of acid impurities and isopropyl myristate as contaminants. Isopropyl palmitate presented the lowest *D*-value (171.1 min) and is about one-half as toxic to the microorganism as isopropyl myristate and showed the same solvating ability. One could assume that further purification could decrease its antimicrobial activity even more.

4. Conclusions

The solvents ethyl laurate, ethyl myristate and isopropyl palmitate were able to dissolve the ophthalmic ointment base. The antimicrobial activity of these solvents and of isopropyl myristate against *P. aeruginosa* was revealed by the *D*-values. Isopropyl palmitate exhibited the lowest antimicrobial activity, followed by isopropyl myristate, ethyl myristate, and ethyl laurate. Since it exhibits a lower toxicity and an equal capacity for dissolving the ointment base as isopropyl myristate, isopropyl palmitate could replace isopropyl myristate in the sterility test for ophthalmic ointment.

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