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Antimicrobial Activity of Parabens in Submicron Emulsions Stabilized with Lecithin

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Antimicrobial efficacy of methyl and propylparaben combination as potential preservatives for submicron emulsions, and the effect of oil and lecithin concentration on the microbial growth were investigated. Parabens were ineffective in standard or doubled concentrations as per pharmacopoeial criteria. Poor growth inhibition and multiplication of reference strains point to protective and growth properties of submicron emulsions. No correlation was observed between oil/lecithin ratio and efficacy of parabens; partitioning of the latter into the oily phase and lipophilic domains could be the reason for such effect. Further studies are necessary to establish a stable and safe composition of such formulations.

Keywords submicron emulsion; paraben; lecithin; antimicrobial preservation; microbial growth kinetics

INTRODUCTION

Submicron emulsions are oil-in-water dispersions stabilized usually with lecithin. Mean droplet size ranging from 200 to 500 nm as well as biocompatible emulsifying agent enable intravenous administration of such formulations. This type of emulsions has not only been used for nearly 50 years as parenteral nutrition agent, but has also been recently introduced as an intravenous carrier for lipophilic drugs such as propofol, diazepam, and fat-soluble vitamins. Moreover, attempts have been made to use submicron emulsions for nonparenteral applications such as ocular, dermal, or rectal (Sznitowska, Gajewska, Janicki, Radwanska, & Lukowski, 2001; Tamilvanan & Benita, 2004; Youenang Piemi, Korner, Benita, & Marty, 1999).

Pharmaceutical products, when distributed in multidose containers, should be properly preserved against microbial contamination and proliferation during storage under normal conditions and proper use. In particular, home use products are of the greatest hazard of infection, because they are applied by an untrained patient. Another risk factor is that substances of natural origin, used as components (e.g., lecithin), not only promote microbial growth but are usually contaminated themselves. Lack of studies on efficacy of antimicrobial agents in modern dispersed systems containing lecithin (submicron emulsions or liposomes) is one of the reasons why these formulations are not being used on a larger scale. Sznitowska, Janicki, Dabrowska, and Gajewska (2002) and, recently, Han and Washington (2005) screened several antimicrobial agents for their best compatibility with submicron emulsions, and found that the most promising preservatives were parabens. They did not demonstrate compatibility problems, except very little creaming of the emulsion after 2 years of storage.

Parabens are alkyl esters of *p*-hydroxybenzoic acid, and their mechanism of action is based on penetration/partition into the phospholipid bilayer of the cytoplasmic membrane. As a consequence, there is a change of interactions between polar groups of fatty acids, so that the movement of acyl chains is limited (especially in unsaturated fatty acids). Accumulation of such compounds in the membrane leads to its "swelling" with consecutive disruption of integrity and increase in its fluidity and permeability, which results in transmembrane transport disruption and leakage, and uncoupling of metabolic processes (Denyer & Stewart, 1998; Mrozik et al., 2002; Rieger, 2000a, 2000b).

Parabens are active against microorganisms over a wide pH range (4–8); however, their activity decreases with increase in pH, because phenolate anion is formed during dissociation; above pH 8.4 (which is the pK_a value of parabens), this forms dominates (Rieger, 2000a; Schmidt & Franck, 1993). When the molecule is charged, its partitioning to the lipophilic membrane is limited, thereby reducing its antimicrobial activity. Besides, in emulsions, the effectiveness of preservatives depends on their concentration in the aqueous phase, where microorganisms are present. Thus partitioning from the aqueous phase to the oily phase, which depends on lipophilicity of antimicrobial agents, is

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another factor that reduces the activity. Among parabens, the partitioning to the oily phase increases in the following order: methyl- < ethyl- < propyl- < butylhydroxybenzoic acid.

On the basis of the mechanism of paraben action discussed above, another factor may be considered to influence the activity of parabens in submicron emulsions. It is suspected that the presence of phospholipids of egg lecithin may inhibit parabens activity against microorganisms owing to an easy incorporation of parabens into phospholipid-rich domains present in the emulsion. This hypothesis can be also supported by the fact that egg yolk lecithin is one of the substances recommended by the European Pharmacopoeia (Ph. Eur. 5.0, 2004) to neutralize phenolics when tests for sterility or total aerobic count are performed.

This study was designed to answer the question: how efficient are parabens in submicron emulsions, and whether it is possible to obtain sufficiently protected emulsions by increasing their concentrations above the level usually allowed for parenteral or ocular administration? Moreover, the effect of oil and lecithin concentration in the paraben-preserved emulsions on the kinetics of the microbial growth was studied.

MATERIALS AND METHODS

Materials

Reference strains: *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Candida albicans* ATCC 10231, and *Aspergillus niger* ATCC 16404 were used. The strains were obtained from the American Type Culture Collection (Manassas, VA, USA).

Culture media and solutions (according to Ph. Eur. 5.0): Casein soya bean digest agar and Sabouraud 4% glucose agar without antibiotics were purchased from Merck-VWR International SAS (Fontenay Sous Bois, France). Buffered sodium chloride–peptone solution (BSP, pH 7.0) was purchased from Scharlau Chemie (Barcelona, Spain); buffered sodium chloride–peptone solution (pH 7.0) with 1–2 g/L polysorbate 80 (BSP-T); sodium chloride, 9 g/L solution (0.9% NaCl), and sodium chloride (9 g/L solution) with polysorbate 80 (0.5 g/L; 0.9% NaCl-T) were prepared from chemicals.

Chemicals: Soya bean oil and egg yolk lecithin (Lipoid E 80) were purchased from Lipoid (Ludwigshafen, Germany); methyl-*p*-hydroxybenzoate (paraben M), propyl-*p*-hydroxybenzoate (paraben P), and polysorbate 80 (Tween 80) were from Fluka Chemie (Steinheim, Germany); glycerol was from Pollena-Strem (Dabrowa Gornicza, Poland); and sodium chloride was from POCh (Gliwice, Poland).

Preparation of Emulsions

Three types of emulsions (A, B, and C) were prepared using a standard method of preparation (Benita & Levy, 1993), and their composition is given in Table 1. Briefly, egg lecithin was dispersed in a mixture of glycerol and water, and the oily phase containing parabens was added to the aqueous phase at 70°C. Parabens were added by dissolving them in the oily phase. The pH was adjusted to 8.0 with 0.1 mol/L NaOH solution. The emulsion was filtered through Durapore 0.45-μm filter (Millipore, Bedford, MA, USA), packed in sterile glass vials under nitrogen, and sterilized in an autoclave at 121°C for 15 min. The preparations were stored at 4°C, in dark. Two batches of the emulsion A (A1 and A2) were prepared and tested. Methods of physicochemical analysis were published earlier by Watrobska-Swietlikowska and Sznitowska (2006), and results are presented in Table 1. Droplet size was measured using laser diffractometer (Mastersizer E; Malvern Instruments, Malvern, UK).

TABLE 1
Composition (%, wt/wt) and Characteristics of the Investigated Submicron Emulsions

	A1	A2	B	C
Composition				
Soya bean oil	10.0		20.0	10.0
Egg yolk lecithin	1.2		1.2	1.2
Glycerol	2.33		2.33	2.33
Methyl-*p*-hydroxybenzoate (paraben M)	0.18	0.02	0.18	0.36
Propyl-*p*-hydroxybenzoate (paraben P)			0.02	0.04
Analysis				
pH	7.8	6.8	7.8	8.0
Mean diameter of oily droplets (μm)	0.35	0.31	0.41	0.31
Maximum droplet size (μm)	0.81	0.95	0.99	0.95

Inoculum Preparation and Determination of Antimicrobial Activity

The strains were subcultured on a casein soya bean digest agar (bacteria) or Sabouraud 4% glucose agar (fungi), and incubated at 30–35ºC for 18–24 h (bacteria), or at 20–25ºC for 2–3 days (yeasts), or until good sporulation was obtained (moulds). The suspensions of bacterial strains and *C. albicans* were prepared in 0.9% NaCl and those of *A. niger* in 0.9% NaCl-T. Suspensions were standardized by the measurement of optical density. The number of cells or spores per milliliter of each stock suspension was determined using a surface-spread method (in triplicate).

Emulsions in vials (20 mL) were inoculated under aseptic conditions with 100 μL of the stock microbial suspension. Each sample was mixed on a vortex and incubated at the controlled temperature of 20–25ºC for 28 days. At time intervals of 0, 6, 24 h and 2, 7, 14, 28 days, aliquot quantities of the

inoculated emulsion were withdrawn to determine viable aerobic count using the membrane filtration method. Mixed cellulose ester filters (0.45 μm; Millipore, Molsheim, France)—white for bacteria and black for fungi—were used.

The emulsions were diluted 10-fold in BSP, and 1,000 μL of each dilution was transferred into 100 mL of BSP, mixed gently, and filtered immediately. Membranes were washed three times with 100 mL of BSP. For undiluted emulsion, as well as for 10- and 100-fold dilutions, the use of BSP-T followed by rinsing three times with 100 mL of BSP enabled better removal of the residual emulsion film from the membrane. Membranes were transferred onto the agar plates and incubated at 30–35ºC for bacteria and 20–25ºC for fungi for up to 7 days. The colonies were counted, and log reduction was calculated against the initial inoculum.

The tests were performed in duplicate for emulsions A1, B, and C and in one replication for emulsion A2.

Appropriate control tests were performed to confirm the validity of the method used in the study. To show that recovery of microorganisms was not inhibited by the sample or by the neutralization method, that is, recovery of each reference strain after neutralization, without and with 1 mL of an emulsion, was compared with the introduced inoculum (10–100 colony forming units [cfu]). A recovery level of not less than 70% was the acceptance criterion (according to USP 28).

RESULTS

The investigated submicron emulsions contain lecithin as an emulsifying agent, and isotonicity is achieved using glycerol. Combination of two parabens was used for preservation of the emulsions A and B—concentrations commonly used in pharmaceutical preparations (1.8 mg/mL of paraben M and 0.2 mg/mL of paraben P) and doubled concentrations (3.6 mg/mL of paraben M and 0.4 mg/mL of paraben P) in emulsion C. In this study, three bacterial and two fungal reference strains as well as methods recommended by the Ph. Eur. 5.0 to test antimicrobial preservation in dosage forms were used.

The control tests confirmed that neutralizing method was sufficient to recover viable organisms from the inoculated emulsions, which was in agreement with the findings presented earlier (Denyer & Stewart, 1998; Russel, 1998).

Figure 1 presents survivor graphs of the reference strains in the emulsions. Relative standard deviations (RSD) of raw data at each point, calculated as percentage of the mean count, are presented in Table 2 and were generally less than 25%. Lower reproducibility of the counts was noted, for example, for *P. aeruginosa* in emulsions B and C, *S. aureus* in emulsions B, *A. niger* in emulsion A, *C. albicans* in emulsions A and C, and *E. coli* in emulsion C.

Two types of emulsions containing standard concentrations of parabens were tested. They contained 10%, wt/wt (two batches of emulsion A: A1 and A2) or 20%, wt/wt (emulsion B) of oil, whereas the concentration of lecithin was constant (1.2%, wt/wt). Owing to different lecithin/oil ratio, different phase distribution and activity of parabens in emulsions A and B can be expected. For both types of emulsions, no decrease in the microbial growth was observed within the first 2 days, although according to Ph. Eur. in the case of parenteral and ophthalmic drugs, 2 $\log_{10}$ and 3 $\log_{10}$ reduction is required for bacteria as early as 6 and 24 h, respectively (Table 3). Surprisingly, after 24 h an increase in the number of *E. coli* and *S. aureus* cells was noted; for emulsion B the number of *E. coli* cells remained at an increased level for 7–14 days. The standard concentration of parabens was neither effective for *C. albicans*, which demonstrated growth up to 0.6–0.8 $\log_{10}$ between 7th and 28th day of incubation.

A constant but slow decrease in growth was observed only for *A. niger* and *S. aureus*, but this occurred with the same, above mentioned, delay of 2 days. Unlike Gram-positive *S. aureus*, the behavior of Gram-negative *P. aeruginosa*, similar to *E. coli* (also Gram-negative rod), was much more complicated as increases and decreases in $\log_{10}$ reduction were observed throughout the test period (Figure 1). In the case of these two strains (i.e., *P. aeruginosa* and *E. coli*), the reproducibility of the growth observed in two batches of emulsion A is less satisfying than for the strains where reductions of growth occurred (*S. aureus* and *A. niger*). Very low reproducibility was observed, especially for *P. aeruginosa*: for example, 3 $\log_{10}$ and 0.6 $\log_{10}$ reduction against initial inoculum was noted after 7 days for emulsions A1 and A2, respectively.

The main difference between both emulsions, A1 and A2, is their pH value, which is lower for emulsion A2 by more than 1 unit. As explained in the Introduction section, lower pH values result in a larger ratio of undissociated paraben molecules, and this leads to the increased antimicrobial activity of parabens (Haag & Loncrini, 1984; Parker & Hodges, 2002; Rieger, 2000a, 2000b). However, this effect was not observed in emulsion A2. The complex structure of nanodispersed phases and partitioning of parabens to different regions (not only oily phase but also micellar and interregional phase) may be one of the reasons that the relationship between pH and activity was not demonstrated; moreover, the reproducibility of the test for two batches is lower than that expected for solutions.

Similar growth kinetic profiles were observed for 10 and 20% emulsions (A vs. B) containing standard paraben concentrations, and the relationship between the oily phase content or lecithin/oil ratio and the activity of parabens in the system was not observed.

Increased concentration of parabens in 10% emulsion resulted, as expected, in more effective antimicrobial protection of the system. However, similar to emulsions A and B containing lower paraben concentrations, no significant decrease in *P. aeruginosa*, *S. aureus*, *A. niger*, and *C. albicans* cell numbers was observed within first 2 days after inoculation of emulsion C. Only the number of *E. coli* cells decreased during this time by 0.7 $\log_{10}$. Between the 2nd and the 7th day, fast

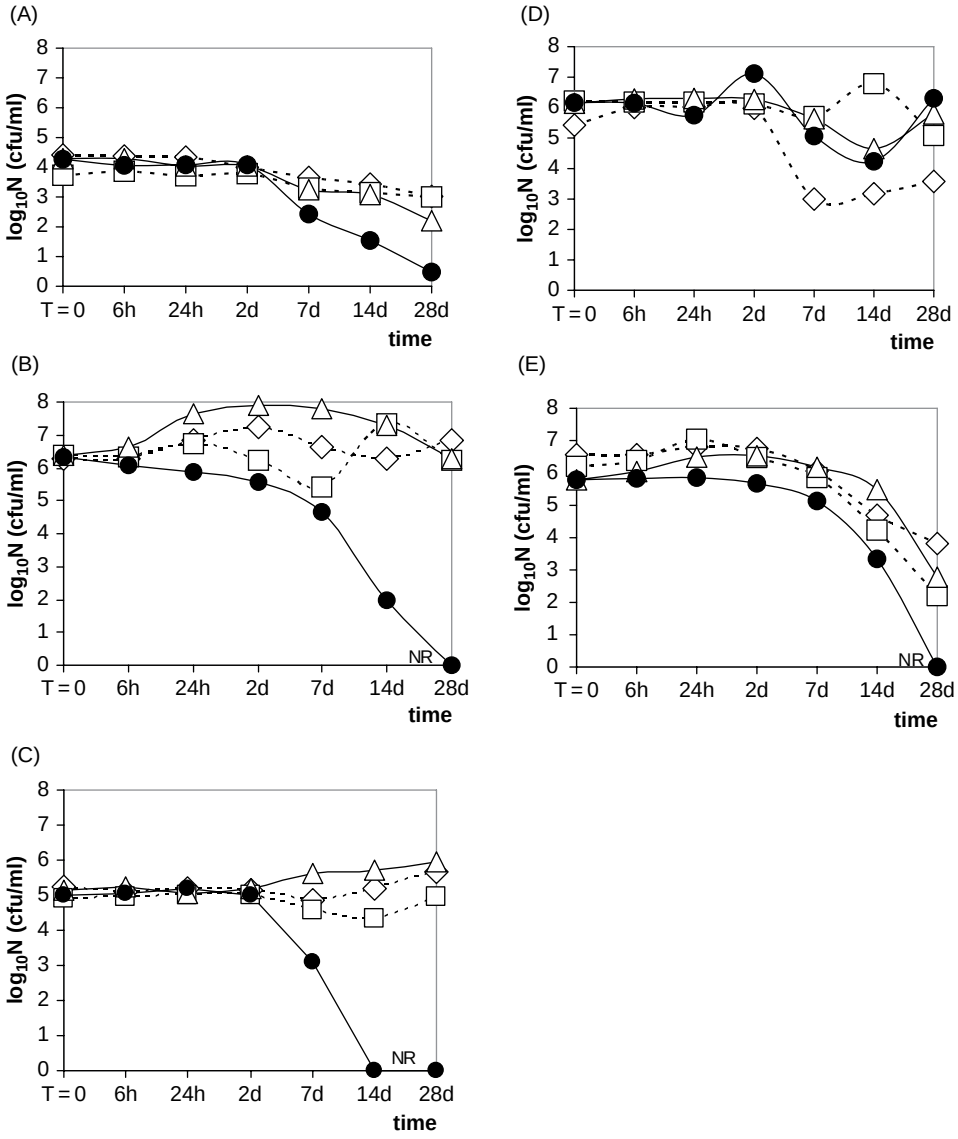

FIGURE 1. Survivor of the reference strains in the submicron O/W emulsions during the incubation period. (A) *Aspergillus niger* ATCC 16404; (B) *Escherichia coli* ATCC 8739; (C) *Candida albicans* ATCC 10231; (D) *Pseudomonas aeruginosa* ATCC 9027; (E) *Staphylococcus aureus* ATCC 6538; *N*, of viable microorganisms in 1 mL of an emulsion; cfu, colony-forming unit; h, hours; d, days; NR, no recovery. Emulsion type (refer to Table 1): A1 ◇– – – ; A2 □ – – –; B △—— ; C ●——.

and constant reduction in all strains except *P. aeruginosa* occurred. Finally, no recovery of *S. aureus*, *E. coli* (after 28 days), and *C. albicans* (after 14 days) was achieved; however, this was not the case for *A. niger.* For moulds, only 3.9 $\log_{10}$ reduction was observed after 28 days. *P. aeruginosa* was most resistant to doubled concentrations of parabens: the growth profile of this Gram-negative rod in emulsion C was similar to that observed for emulsions A and B, with no decrease in the cell number at the end of the incubation period.

Table 3 presents the results obtained for emulsion C, in comparison with the Ph. Eur. 5.0 requirements regarding efficacy of preservation for different drug formulations. For parenteral and ophthalmic drugs, 2 $\log_{10}$ and 3 $\log_{10}$ reduction of bacteria after 6 and 24 h, respectively, and "no recovery" after 28 days are the basic criteria that should be fulfilled, as well as 2 $\log_{10}$ reduction of fungi after 7 days and "no increase" in the viable count after 28 days. Moreover, emulsion C, despite doubling the concentration of parabens, did not pass the test for antimicrobial preservation of sterile drugs. For drugs that could not fulfill the above criteria, Ph. Eur. allows (when justified) that criterion "II" be fulfilled (Table 3); however, this was also not fulfilled, even when topical or oral preparations were considered. For emulsion C, criterion "I" required for topical drugs was fulfilled only in the case of

TABLE 2
Relative Standard Deviations (RSD) for the Results Obtained in the Antimicrobial Efficacy Tests in the Investigated Emulsions

	Emulsion														
	Pseudomonas aeruginosa			*Staphylococcus aureus*			*Escherichia coli*			*Aspergillus niger*			*Candida albicans*		
Incubation Time *T*	A1	B	C	A1	B	C	A1	B	C	A1	B	C	A1	B	C
0	61.4	30.3	14.6	26.8	1.2	10.4	0.0	22.6	40.9	10.9	3.6	3.8	20.2	10.1	0.0
6 h	0.0	14.9	30.3	5.8	12.9	5.2	19.1	11.4	82.5	11.8	10.9	18.5	10.9	23.6	18.9
24 h	20.2	21.2	13.8	10.0	9.1	3.9	5.2	4.7	13.5	19.3	25.7	11.8	13.7	15.7	53.6
2 days	7.4	34.4	18.4	5.0	8.3	10.4	13.7	3.8	7.9	42.4	12.9	11.8	47.1	12.9	31.4
7 days	NR	9.9	104.5	6.2	75.4	5.2	4.8	11.2	29.6	27.7	20.2	24.0	6.6	23.0	23.6
14 days	0.0	15.7	0.0	14.4	11.6	6.4	29.8	25.4	32.8	15.7	23.6	10.3	4.6	6.7	NR
28 days	1.9	6.8	0.0	9.7	10.5	0.0	14.8	7.4	NR	6.7	0.0	47.1	13.1	18.1	NR

NR, no recovery.

TABLE 3
Acceptance Criteria for the Efficacy of Antimicrobial Preservation According to Ph. Eur. 5.0 and Results Obtained for Bacterial and Fungal Reference Strains in Submicron Emulsion (Type C) Preserved with Methyl and Propyl Paraben (3.6 and 0.4 mg/mL, respectively) and $\log_{10}$ Reduction in the Number of Viable Microorganisms Against the Value Obtained for the Inoculum

	Results			Ph. Eur. 5.0 Requirements				
	Bacteria			Cat.1 Criterion		Cat.2 Criterion		Cat.3 Criterion
Time	*Pseudomonas aeruginosa* ATCC 9027	*Staphylococcus aureus* ATCC 6538	*Escherichia coli* ATCC 8739	I	II	I	II	Cat.3
6 h	–0.01	0.13	0.16	2	–	–	–	–
24 h	0.39	0.1	0.34	3	1	–	–	–
2 days	–0.98	0.28	0.68	–	–	2	–	–
7 days	1.08	0.83	1.6	–	3	3	–	–
14 days	1.91	2.62	4.26	–	–	–	3	3
28 days	–0.16	NR (≥ 5.96)	NR (≥ 6.24)	NR	NI	NI	NI	NI
	Fungi							
	Aspergillus niger ATCC 16404	*Candida albicans* ATCC 10231						
6 h	0.27	0.25		–	–	–	–	–
24 h	0.25	0.12		–	–	–	–	–
2 days	0.25	0.29		–	–	–	–	–
7 days	1.91	2.21		2	–	–	–	–
14 days	2.79	NR (≥ 5.29)		–	1	2	1	1
28 days	3.85	NR (≥ 5.29)		NI	NI	NI	NI	NI

Note. Cat.1—preparations required to be sterile, e.g., parenteral and ophthalmic products; cat.2—topically used products; cat.3—products for oral and rectal administration. Criterion I—recommended efficacy to be achieved; criterion II—satisfactory in justified cases, when criterion I cannot be attained; NR—no recovery; NI—no increase.

fungi, whereas criterion "II" for topical or oral drugs was fulfilled for *E. coli*. It is worth noting that the preservation of submicron emulsions with parabens was also insufficient when less restrictive requirements of US Pharmacopoeia (USP 28, 2005) were considered.

DISCUSSION

Many studies indicate that parabens are good preservative agents for different dosage forms (Doron, Friedman, Falach, Sadovnic, & Hirschfeld, 2001; Haag & Loncrini, 1984; Steinberg et al., 1999; Zani, Minutello, Maggi, Santi, & Mazza, 1997). The concentration of preservatives should be properly chosen with regard to drug application routes and their own toxicity; however, standard concentrations of 1.8 and 0.2 mg/mL of paraben M and paraben P, respectively, are usually sufficient (Haag & Loncrini, 1984; McEvoy, Edwards, & Snowden, 1996; Rieger, 2000a, 2000b; Schmidt & Franck, 1993). Parabens are widely used in food, drugs, and cosmetics, which are multiphase systems (e.g., mayonnaise, dermatologic creams, or skin care emulsions), where they may interact with surfactants or other constituents leading to their lower efficacy. It is well known that partitioning phenomenon occurring in such systems requires higher concentrations of antimicrobial agents, and combination of low and more lipophilic preservatives, for example, paraben M and P is also desired.

Improper preservation could be expected for parabens alone; however, additive effect of combined paraben M and P was successfully reported (Darwish & Bloomfield, 1995; Rieger, 2000a, 2000b). For parabens used independently, Darwish and Bloomfield (1995) observed 0.60–0.82 $\log_{10}$ reduction after 4 h for 0.18% paraben M and 0.20–0.30 $\log_{10}$ reduction after 2 h for 0.04% paraben P in case of *S. aureus*, and 2.19–2.84 $\log_{10}$ reduction after 4 min and 2 $\log_{10}$ reduction after 2 h in the case of *P. aeruginosa*, respectively. The mixture of 0.18% paraben M and 0.04% paraben P after 2 h gave more than 2.72–2.85 $\log_{10}$ reduction for *S. aureus* and 2.80–2.90 $\log_{10}$ reduction for *P. aeruginosa*. In our studies, when similar concentrations of parabens were used, no reduction of the microbial growth was observed within 2 days either for bacteria or for fungi. Partitioning of the parabens into the oily phase or other internal lipophilic domains (micelles, interphase) can be the reason for such highly negative preservation effect observed.

The results obtained by Kurup, Wan, and Chan (1992) for emulsions indicated that 0.4% of paraben M alone is not sufficient to protect this dosage form against the growth of *P. aeruginosa* ATCC 19429. Nearly 1 $\log_{10}$ reduction was achieved after 24 h only when 0.8% concentration of paraben M was used. The minimal inhibitory concentrations (MIC) of methyl paraben for *P. aeruginosa* ATCC 9027, *S. aureus* ATCC 6538P, *E. coli* ATCC 8739, *C. albicans* ATCC 10231, and *A. niger* ATCC 10254 are respectively 4,000, 2,000, 1,000, 2,000, and 1,000 μg/mL, whereas for propylparaben the respective MIC values are more than 1,000, 500, 500, 250, and 200 μg/mL (Rieger, 2000a, 2000b).

Concerning MIC values, it may be concluded that increased concentration of paraben M (3,600 μg/mL) in emulsion C is effective against all strains except *P. aeruginosa.* Similarly, paraben P concentration of 400 μg/mL is effective against fungi but not against bacteria. This may be even more certain because we have tested these preservatives in combination. Our results confirmed that submicron emulsion preserved with double concentration of parabens was unprotected against *P. aeruginosa* contamination, but preservation against the other strains, although much better, was not sufficient. Because of partitioning to the oily phase and other internal nanostructures of the emulsions, availability of free paraben molecules in the aqueous phase is limited. It was determined experimentally in a separate partitioning study (Watrobska-Swietlikowska & Sznitowska, 2006) that the concentrations of free methyl and propylparaben in the aqueous phase of emulsion C were 1,220 and 24.1 μg/mL, respectively. These corrected values indicate that at least for *E. coli* and *A. niger* the efficacy of parabens is sufficient, whereas for *S. aureus* and *C. albicans* it may be low. Our studies revealed, however, that *A. niger* was the most resistant among four strains, not confirming the relationship between the available MIC values and efficacy of the parabens in the emulsions. Besides, it is difficult to explain an observed 2-day delay in the inhibition of microbial growth in the presence of concentrations of methyl paraben in the aqueous phase, which theoretically should have sufficient antibacterial and antifungal activity against *E. coli* and *A. niger*, respectively. Probably, it is a time necessary for adaptation to new environment because the cells originally grown on solid nutrient medium after being transferred to the emulsion change the activity of their metabolic systems to be able to utilize nutrient components from the fluid environment. It is probable that the rate of this process is decreased in the presence of antimicrobials and their acquisition from the emulsion may influence transmembrane transport and metabolic processes, suppressing the growth of cells.

Improper preservation of submicron emulsions reported in our study was probably due to low concentration of parabens in the aqueous phase on the one hand, and the presence of a specific component, that is lecithin, on the other. Lecithin is capable of forming micelles or other nanostructures, which can reduce availability of the preservatives in the free unbound form, serving, for example, as competitive target sites for parabens, as it is observed in the cellular membranes of microorganisms as mentioned in the Introduction section (Denyer & Stewart, 1998; Shimamoto, Ogawa, & Ohkura, 1973). Unfortunately, published data on the efficacy of combination of methyl and propylparaben against pharmacopoeial reference strains in traditional emulsions are not available; thus, comparison of emulsions stabilized with lecithin and other surfactants is not possible. The hypothesis on the importance of lecithin for the observed effects can be supported by the fact that no evident

difference between microbial growth kinetics was observed in emulsions containing 10 or 20% oil (emulsion A vs. B), although increased partitioning of parabens to the oily phase in emulsion B was expected.

Neutral or slightly alkaline pH is important for physicochemical stability of submicron emulsions, and this is another factor that could be responsible for insufficient antimicrobial protection of this type of formulation, because only undissociated molecules of a preservative are active. However, we have not observed any difference in the microbial growth in the pH range 6.8–8.0, as mentioned earlier.

Our findings and that of Shimamoto, Ogawa, & Ohkura (1973) demonstrate that the amount of parabens available in the aqueous phase was too low to exert biocidal effect. At least 2-day delay in the inhibition of the bacterial growth observed for all tested strains in emulsion C or lack of antimicrobial effect in emulsions A and B suggests that the emulsions provide a very good environment for the microbial survival and growth; moreover, in this case lecithin may be considered as a component responsible for such property. Pseudomonads, for example, are known as ubiquitous bacteria, easily adapting to different environments and acquiring cross-resistance to different antibiotics and biocides (Chapman, 1998; Ramos-González, Godoy, Alaminos, Ben-Bassat, & Ramos, 2001; Tattawasart, Maillard, Furr, & Russell, 2000;). *P. aeruginosa* in the tested emulsions demonstrated increases and decreases in cell count throughout the test period. Such kinetics is probably a result of cell adaptation to the ingredients in emulsion and the selection of resistant individuals, a process taking time and requiring sublethal concentrations of toxic chemicals (e.g., parabens).

As mentioned earlier (Rieger, 2000a, 2000b), better activity of parabens against yeasts and moulds than against bacteria was not confirmed in our study for typical concentration of these agents. Especially in the case of *S. aureus* and *C. albicans* rather bactericidal than fungicidal efficacy was observed; however, we confirmed better efficacy against Gram-positive than Gram-negative microorganisms.

CONCLUSION

Methylparaben in combination with propylparaben is not effective as sole preservative agents for submicron O/W emulsions stabilized with lecithin. Even increased concentration of parabens does not protect such formulations against microbial contamination and proliferation. The parabens poorly partition to the aqueous phase; in addition, lecithin is probably responsible for their micellization and inactivation. Hence, they are available in too low concentrations for microorganisms that can contaminate the product during manufacturing or use. Gram-negative microorganisms are adapting quickly to low concentrations of *p*-hydroxybenzoic esters, probably because of membrane changes and/or other mechanisms of resistance (Poole, 2005; Russel, 1995); however, the role of lecithin in this phenomenon may be also important.

Further studies on preservation of such formulations are necessary to establish a stable composition and to achieve a proper antimicrobial efficacy. According to guidance of EMEA (2003) on excipients, antioxidants, and antimicrobial preservatives, parabens should be excluded in any dosage form for parenteral use, so their possible use in combination with other antimicrobial agents is limited to ophthalmic, topical, and oral drugs in multidose containers.

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