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Effect of ethanol, propylene glycol and glycerol on the interaction of methyl and propyl *p*-hydroxybenzoate with *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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Abstract

Co-solvents ethanol, propylene glycol and glycerol were used to increase the aqueous concentrations of methyl and propyl *p*-hydroxybenzoate above their saturation solubility. The increased aqueous concentration of parabens in co-solvent solutions was associated with increased antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, but the extent of this effect increased with increasing hydrophobicity of the co-solvent. Addition of co-solvents to aqueous solutions of parabens caused a reduction in their octanol/water partitioning and a reduction in the uptake of the parabens by the cells, the extent of the reduction increasing as the hydrophobicity of the co-solvent increased. The addition of co-solvents also potentiated cell membrane damage as evidenced by leakage of radio-labelled phosphate from parabens-treated cells, but in this situation the extent of the damage was correlated with increasing hydrophobicity of the co-solvent. The results indicate that the increase in antibacterial activity of parabens preservatives in co-solvent solutions can largely be accounted for by their combined effects on the integrity of the cell membrane. © 1997 Elsevier Science B.V.

Keywords: Parabens; Co-solvent; Preservation; Membrane damage

1. Introduction

The alkyl esters of *p*-hydroxybenzoic acid, commonly known as the parabens, constitute one of the most important groups of chemical agents used as preservatives in pharmaceutical, cosmetic and other products. Although various studies of the homologous series of parabens esters has shown that their activity increases with increasing ester chain length (Hansch et al., 1972; Eklund, 1985; Russell et al., 1985; Russell and Gould,

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1988), in practice the usefulness of the higher homologues is limited by their decreasing water solubility which in turn limits their bioavailablity within the system. In previous studies (Darwish, 1992; Darwish and Bloomfield, 1995) we have shown that pharmaceutical co-solvents such as ethanol, propylene glycol and glycerol can be used to increase the aqueous solubility of methyl and propyl parabens (MHB and PHB) preservatives, thereby increasing their antibacterial action against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, but the extent of this effect was different for different co-solvents. The object of this investigation was to determine the mechanisms by which the different co-solvents exert their effect on the antibacterial activity of the parabens esters.

2. Material and methods

2.1. Bacterial test suspensions

Staphylococcus aureus NCTC 10788 and *Pseudomonas aeruginosa* NCTC 06750 were grown overnight at 37°C on nutrient agar containing (g l^{-1}) Lab-lemco, 1; yeast extract, 2.0; peptone 5.0; NaCl, 5.0; agar No. 3, 15.0; pH 7.4. Organisms were harvested, washed twice with peptone water, containing (g l^{-1}) peptone, 1; NaCl, 5; pH 7.2, and diluted to contain the required number of colony forming units per ml (cfu ml^{-1}). Media bases were obtained from Oxoid, Basingstoke, UK.

2.2. Preparation of test solutions

Solutions of co-solvents were prepared by adding weighed quantities of ethanol (EtOH) (Hayman, Essex, UK), propylene glycol (PG) (Fisons, Loughborough, UK) and glycerol BP (Gly) (MacCarthy, Romford, UK) to sterile distilled water. To prepare solutions of parabens, weighed quantities of methyl and propyl *p*-hydroxybenzoate (BDH Chemicals, Dorset, UK) were added to sterile distilled water or co-solvent solution, and dissolved using sonication and/or gentle heat when necessary. By using heat or sonication, solutions containing parabens concentrations higher than their saturation solubility (as determined by standard methods) could be prepared, which were stable over at least 48 h. Using solutions prepared in this way it was possible to compare the effects of the three co-solvents on the activity of MHB and PHB at concentrations up to 0.36% and 0.1%, respectively.

2.3. Preparation of solutions of [^{32}P]

[^{32}P]*Ortho*-phosphate in acid solution (Amersham, UK; specific activity 6.06 MCi/ml) was diluted in 0.05 mM phosphate carrier and the pH adjusted to 7.0.

2.4. Evaluation of bactericidal activity

Test solutions and bacterial test suspensions were placed in a water bath at 25°C to equilibrate. At zero time a sample of the bacterial test suspension was added to each test solution to give an inoculum size of 10^6–10^8 cfu ml^{-1} in the test mixture. Samples were withdrawn at predetermined time intervals (t), neutralised by dilution in quarter strength Ringer's solution (1 in 100), and viable counts estimated by plating (in duplicate) 50-μl aliquots onto nutrient agar plates using a Model D spiral plater (Don Whitley, Shipley, UK). Plates were incubated at 37°C for 24 h. Colonies were counted using the laser counter (Model 500 Colony Counter, Don Whitley, Shipley, UK) and the log number of cfu ml^{-1} determined. The microbicidal effect (ME value) was calculated as log number of cfu ml^{-1} at time t minus the log number of cfu ml^{-1} at time zero. Where samples gave no detectable survivors (NDS) the actual number of survivors in the final dilutions is $< 2 \times 10^3$ cfu ml^{-1} (i.e. < 1 cfu in 50 ml). Preliminary experiments showed that dilution of 1 ml of the reaction mixtures with 99 ml of quarter strength Ringer's solution was sufficient to neutralise the effect of the parabens.

2.5. The effect of co-solvents on the partitioning of parabens between water and octanol

Accurately weighed quantities of parabens were dissolved in 1-octanol by sonication. The same

volumes of distilled water or co-solvent solutions (3 M) saturated with 1-octanol were added. Tubes were stoppered and inverted 20 times per min for 48 h at room temperature. The mixtures were centrifuged (1000 × *g*, 15 min) and the concentration of parabens in the aqueous layer determined by spectrophotometric analysis at 255 nm. A partition coefficient was calculated as the ratio of the parabens concentration in the oil phase:parabens concentration in the aqueous phase.

2.6. The effect of co-solvents on the adsorption of parabens by the bacterial cells

Different concentrations of parabens were dissolved in distilled water and co-solvent solutions using sonication or gentle heat when necessary. Bacterial test suspension was added to give a cell concentration of 6×10^9 cfu ml^{-1} and the test mixture maintained at 25°C for 1 h. Cells were removed by centrifugation (1000 × *g*, 15 min) and the parabens concentration of the supernatant determined by spectrophotometric analysis at 255 nm.

2.7. The effect of parabens and co-solvents on the bacterial membrane

Bacterial suspensions of cell density 1×10^{10} cfu ml^{-1} were incubated in 0.1% peptone water with 1 ml of 0.05 mM [^{32}P]phosphate for 2 h at 37°C. Cells were harvested by centrifugation (3000 × *g*, 10 min) washed and resuspended in quarter strength Ringer's solution. Quantities of preloaded cells were added to the test solutions (parabens, co-solvents, parabens/co-solvents solutions) to give a cell density of 10^9 cfu ml^{-1} in the test mixture. Controls were set up as described above using sterile distilled water instead of test solutions. To determine the initial [^{32}P] content of the cells, one of the control tubes was heated at 100°C for 10–15 min. Samples of test mixture were withdrawn at predetermined time intervals, filtered through membrane filters (millipore, pore size 0.2 μm). The membranes were added to 5 ml optiphase 'Hisafe' liquid scintillant (Amersham, UK) and the [^{32}P] content of the cells determined using an LKB Wallac 1209 Liquid Scintillation Counter (Wallac, Milton Keynes, UK). Results were expressed as a percentage of the initial [^{32}P] content of the cells.

2.8. Replication of results

All results were confirmed by not less than two replicate experiments.

3. Results and discussion

Previous investigations (Darwish, 1992; Darwish and Bloomfield, 1995) showed that pharmaceutical co-solvents can be used to increase the aqueous solubility of methyl and propyl parabens preservatives and thereby increase their antibacterial activity against *S. aureus* and *Ps. aeruginosa*. The least polar agent EtOH at 3 M, was the most effective co-solvent giving a saturation solubility of 0.44% and 0.09% for MHB and PHB, respectively, and producing the most significant increase in activity. By contrast, the most polar co-solvent Gly (3 M) was the least effective giving solubilities of 0.13% and 0.035% for MHB and PHB, respectively, with relatively less increase in activity at equivalent parabens concentrations. Saturation solubility determinations agree with the findings of Yalkowsky (1981) who showed that the solubility of a drug depends on its polarity with respect to the co-solvent, the best solvent for a particular solute being the one which matches its polarity.

Mechanism of action studies (Freese et al., 1973; Freese and Levin, 1978; Eklund, 1980, 1985) suggest that the parabens act mainly by causing disorganisation of the microbial cell membrane; at low (bacteriostatic) concentrations parabens appear to cause energy uncoupling which inhibits the uptake of metabolites, whilst at higher (bactericidal) loss of the membrane semipermeability occurs. From studies of the effects of hydrophilic/hydrophobic balance on antibacterial activity Hansch et al. (1972) concluded that the activity of the membrane active antibacterials such as the parabens depends on their ability to move freely in the aqueous phase, and yet be lipophilic enough to partition through the microbial outer cell envelope (where present) and the cytoplasmic

membrane. Studies of various alcohol and glycol compounds (Hugo and Russell, 1982; Russell and Chopra, 1996) show that these agents also have antimicrobial activity which is associated with damage to the cytoplasmic membrane.

From the various studies described above a number of possible explanations for the observed effects of the co-solvents on the activity of the parabens can be proposed. On one hand the co-solvents may interact with the parabens in the aqueous phase thereby altering the hydrophilic/lipophilic balance and affecting their uptake and/or interaction with the cell. On the other hand, since the co-solvents themselves cause damage to the cell membrane, potentiation of antimicrobial action may result from a direct effect on the microbial outer cell envelope (where present) or cytoplasmic membrane. To evaluate these possibilities adsorption isotherms, octanol o/w partition coefficients and cytoplasmic membrane damage were determined for MHB and PHB in aqueous solution and co-solvent systems against *S. aureus* and *P. aeruginosa*, and the results compared with the effects of the co-solvents on the antibacterial activity of the parabens assessed under comparable conditions.

Results in Fig. 1 show the bactericidal activity of combinations of concentrations of PHB from 0.04% to 0.1% in 3 M co-solvent solutions, compared with that of the 3 M co-solvent solutions alone and 0.04% PHB alone. Aqueous solutions of PHB at saturation solubility (0.04%) had little effect on *S. aureus* but produced a 2–3 log reduction against *Ps. aeruginosa* in 2–4 h. PG and Gly at 3 M had little or no activity against both *S. aureus* and *Ps. aeruginosa*, over 24 h but 3 M EtOH produced total kill (i.e. no detectable survivors) within 2 h. For all the PHB/co-solvent solutions an increase in PHB concentration above the aqueous saturation solubility was associated with increased antibacterial activity but, for a given increase in parabens concentration, the increase in activity over and above that of the co-solvent alone was generally greater for PHB in EtOH compared with PHB in PG which in turn was greater than that observed with PHB in Gly. With PHB in Gly ME values of solutions up to 0.1% against *Ps. aeruginosa* were actually less than that of 0.04% saturated solution of PHB alone. Results with MHB which similarly also show that, for a given increase in concentration, the increase in activity was generally greater for MHB solutions in EtOH compared with PG and Gly solutions have been previously described (Darwish, 1992; Darwish and Bloomfield, 1995).

Evidence from the literature (Higuchi and Kuramoto, 1954; Guttman and Higuchi, 1957; Patel and Kostenbauder, 1958; Van Doorne et al., 1988) indicates the tendency of some formulation components used in pharmaceuticals and cosmetics to form molecular complexes with parabens preservatives. Van Doorne et al. (1988) demonstrated that, although β-cyclodextrins could be used to increase the aqueous solubility of parabens esters, the nature of the complexation was such that there was a decrease in antibacterial activity. Patel and Kostenbauder (1958) demonstrated a similar effect for solutions of parabens in Tween-80. To evaluate the possibility of interaction between EtOH, PG and Gly co-solvents and parabens, UV spectra of MHB and PHB solutions in water and 3 M co-solvent solutions were determined and compared (results not shown). From the chemical structure of the parabens and the co-solvents it would be expected that the interaction between these molecules would be largely hydrophobic. This correlates with the fact that there was no apparent change in the UV spectrum of parabens following addition of co-solvents indicating no complex formation involving charge transfer.

By contrast, results in Table 1 indicate that addition of co-solvents to solutions of MHB and PHB was associated with a decrease in the partitioning of parabens into octanol. Results indicate that in general the reduction in partitioning was greatest for the least polar co-solvent EtOH and least for the most polar co-solvent Gly, although there was some discrepancy in the results for MHB in EtOH and PG. It must be borne in mind that the octanol/water partitioning of EtOH, PG and Gly was not determined and may have had some effect on the parabens partitioning.

The relative effects of co-solvents on the octanol/water partitioning of PHB is in agreement with their effects on the uptake of PHB by cells of

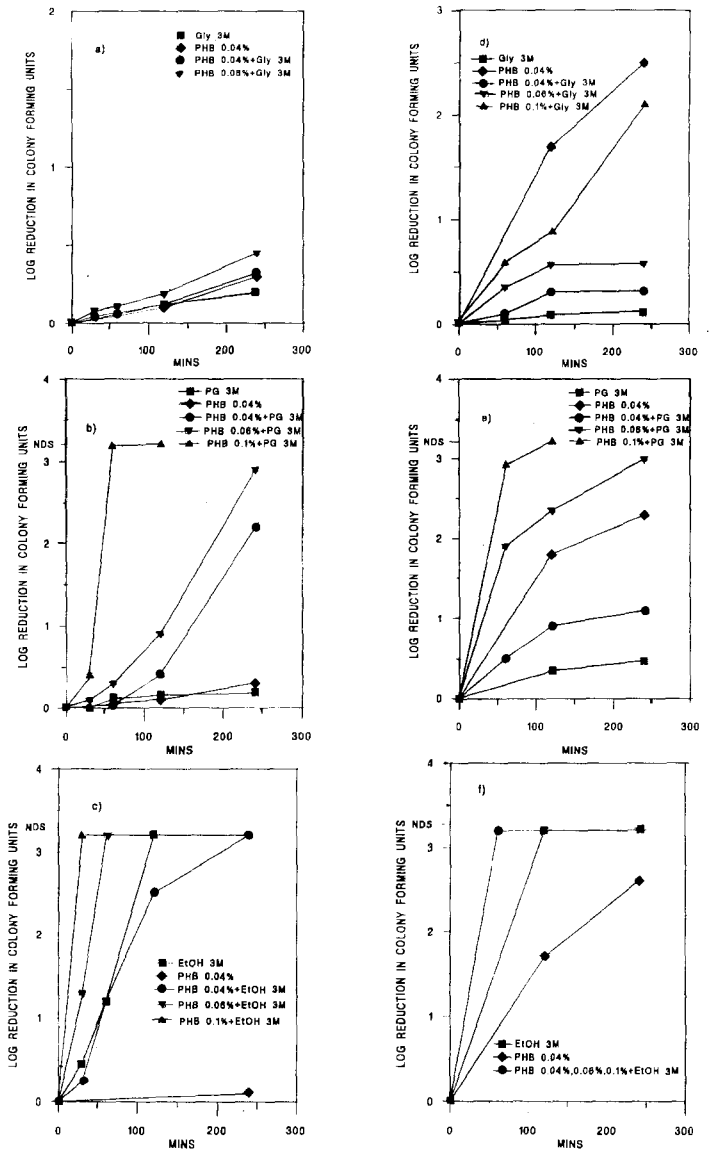

Fig. 1. Log reduction in viable counts of (a), (b), (c) *Staphylococcus aureus* and (d), (e), (f) *Pseudomonas aeruginosa* in 3 M co-solvents glycerol (Gly), propylene glycol (PG) and ethanol (EtOH), propyl *p*-hydroxybenzoate (PHB) 0.04%, and combinations of PHB/co-solvents, (NDS, no detectable survivors).

S. aureus and *Ps. aeruginosa*. Results in Fig. 2 show that, although the amount of PHB adsorbed by the bacterial cell increased with increasing concentration of parabens, the uptake of PHB from solutions of PHB in Gly was slightly greater than for PHB in PG, which in turn was greater than for PHB in EtOH. The results imply that, since the parabens are less soluble in Gly, which is the most polar co-solvent, than in EtOH which is the least polar co-solvent used, the tendency of parabens to leave the Gly containing system and adsorb onto the cells is greater than the tendency to leave the EtOH containing system. However, the order of the increase in the uptake and partitioning is in contradiction with the order of activity, PHB in EtOH being more active than PHB in

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