J. Pharm. Pharmacol. 1996, 48: 1201–1205
Received September 14, 1995
Accepted March 21, 1996

© 1996 J. Pharm. Pharmacol.

Study of Interaction Effects of Polyacrylic Acid Polymers (Carbopol 940) on Antimicrobial Activity of Methyl Parahydroxybenzoate Against Some Gram-negative, Gram-positive Bacteria and Yeast

MARCELLO SCALZO, CLELIA ORLANDI*, NICOLA SIMONETTI* AND FELICE CERRETO

*Dipartimento di Studi di Chimica e Tecnologia delle Sostanze Biologicamente Attive, and *Istituto di Microbiologia, Facoltà di Farmacia, Università, 'La Sapienza' Roma, Italy*

Abstract

Cosmetic or pharmaceutical formulations containing hydrophilic polymers of natural or synthetic origin, may be more exposed to successful microbial contamination because of a polymer-preservative interaction. The experimental data reported in this paper relate to the possible interference of Carbopol 940 with methyl parahydroxybenzoate.

Results show that this hydrophilic polymer, widely employed in many formulations, exerts, on the contrary, an interesting synergism on microbicidal activity of the preserving agent against *E. coli* and *P. Aeruginosa*.

A reduction in microbicidal activity against *S. aureus* and *C. albicans* is observed for a polymer concentration higher than that needed for anti-Gram-negative synergy.

Many hydrophilic polymeric materials of both synthetic and natural origin are widely employed in pharmaceutical or cosmetic formulations (Pillai et al 1988). In cosmetic products, their presence mainly serves to increase the viscosity of the water phase to improve, for instance, the physical stability of dispersions. In many pharmaceutical formulations these materials are employed as rheological modifiers, suspending or emulsifying agents and excipients. Furthermore, they act as a barrier to drug availability because of their trapping effect and are successfully employed in drug delivery systems. Unfortunately, these polymers, by also exerting their binding effect towards preservative agents present in the formulations (Tillman & Kuramoto 1957; Miyawaky et al 1959; Jurgensen 1967; Jurgensen Eide & Speiser 1967; Bottari et al 1976), may enhance the risk of microbial contamination of finished products especially during their use. In this respect, Kurup et al 1967; (1992), re-evaluating the interactions of preservatives with several natural hydrocolloids, hypothesized that the observed reduction of microbicidal activity could be attributed either to some chemical or physical interaction of preservative with the macromolecule or to the physical protection of microbial cells by the hydrocolloids. These considerations conflict with some experimental data reported by Orth et al (1989) who, testing the preservative efficacy of methyl parahydroxybenzoate (methylparaben) in a set of formulations, showed good anti-*Pseudomonas* activity of methyl parahydroxybenzoate when Carbopol polymers were present. This author supposed the chelating power of carboxy-late ions of the Carbopol molecule to be responsible for the increase in microbicidal activity of methyl parahydroxy-benzoate which is normally poorly active against Gram-negative bacteria. In effect, according to Ferris (1989) and Beveridge (1989), subtracting Ca^{2+} ions from the outer membrane of Gram-negative bacteria decreases the molecular sieving properties of the membrane itself, causing a greater susceptibility of the bacteria to antimicrobial agents. Nevertheless Orth, et al (1989) in their approach which was restricted only to *Pseudomonas* species carried out in a complex model, were unable to demonstrate a consistent relationship with this mechanism of action.

At present the Carbopols, because of their chemical stability and resistance to microbial attack, are the most frequently employed polymers in formulations. Considering that good preservation of many formulations is an unresolved problem, a wider investigation into the synergistic or inhibiting effects of these materials on the antimicrobial activity of preservatives could offer a desirable contribution in this direction. In fact, according to Denyer et al (1985), such synergism, especially for complex formulations, is the preferred route to provide safer preservation. In this paper, we attempt to correlate the effect of different concentrations of Carbopol 940 on the antimicrobial activity of relevant preservatives against microorganisms having substantial differences in cell-wall structure. For this purpose two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), a Gram-positive bacterium (*Staphylococcus aureus*) and a yeast (*Candida albicans*) were tested. Each experiment was carried out in aqueous solution containing only the polymer and the preservative agent; this simple model system being chosen to eliminate any misleading interfering factors.

Materials and Methods

The effect of Carbopol 940 on methyl parahydroxybenzoate activity was investigated by adding the polymer to an aqueous solution containing methylparaben 2.5 mg mL^{-1} to give final concentrations of Carbopol 940 of 1 to 2% w/v. The chemical stability of the preservative was unaffected. Each sample containing the hydrophilic polymer was prepared by sus-

Correspondence: M. Scalzo, Dipartimento di Studi di Chimica e Tecnologia delle Sostanze Biologicamente Attive, Facoltà di Farmacia, Università 'La Sapienza', P.le Aldo Moro 5 00185 Roma, Italy.

pending a suitable amount of Carbopol 940 in water or in an aqueous solution of methyl parahydroxybenzoate, slowly stirring for 24 h and then successively neutralizing (pH 7) with a 50% v/v solution of Triethanolamine in water according to the method of Perotti (1970). These solutions were tested against *E. coli, P. aeruginosa, S. aureus* and *C. albicans*. The pH values were measured with a Metrohm Herisau pH Meter E 512. The Carbopol 940 was purchased from BF Goodrich Company. The methyl parahydroxybenzoate, Triethanolamine (TEA), Na_2EDTA and $CaCl_2$ were from Farmitalia Carlo Erba (Milan, Italy). Sabouraud broth, Tripticase Soy Broth (TSB) and Mueller-Hinton broth were purchased from BBL (Beckton Dickinson Microbiology System, Cockeyville USA).

Microbial strains

Escherichia coli 79 M, *Pseudomonas aeruginosa* 179 TR, *Staphylococcus aureus* 20 and *Candida albicans* 243 were from the collection of the Microbiology Institute, Faculty of Pharmacy University of Rome 'La Sapienza'. All microbial strains were isolated from clinical specimens and identified by standard methods. They were designated according to their collection number. For the inoculum preparation the strains were incubated for 18 h at 37°C: *E. coli* was grown in TSB, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were grown in Mueller-Hinton broth and *C. albicans* was grown in Sabouraud broth. The microbial cells were washed and resuspended in water containing NaCl (0·85%) before use. The cell-suspension concentration was evaluated at 540 on a Perkin-Elmer 552 spectrophotometer. For viable cell counts, the test samples were seeded into the relevant agar prepared from the broth previously reported. Gram-negative bacteria and *S. aureus* were incubated for 24 h at 37°C and *Candida albicans* for 48 h at 37°C. To dilute test samples for viable counting, a solution (solution E) prepared from TSB [Trypticase peptone (1·7 g), phytone peptone (0·3 g), sodium chloride (0·5 g), dipotassium phosphate (0·25 g), dextrose (0·25 g), water (1000 g)] and Tween 20 (50 ml), according to Lucas (1978), was employed.

Time-survivor curves

The microbicidal activity was determined at 25°C. Survival curves were obtained by linear regression of the log number of surviving microorganisms as a function of the time after inoculation into the test system. The D-value (time required to achieve 90% reduction of viable cells) was also calculated (Orth 1979) for each microorganism in each experiment. Experimental points (at least 5 for each experiment) covered at least 3 log cycles of viable counts.

Aqueous solutions. Each 90-mL sample containing methyl parahydroxybenzoate was inoculated with 10 mL of microbial suspension to give a trial cell concentration 10^6–10^7 cells mL^{-1}. Immediately after inoculation and at each sampling time, 1 mL of sample was diluted 1:100 with solution E to arrest the preservative activity. To perform viable counts each sample was decimally diluted and colony-forming units (CFU) were counted, on plates containing 30–300 colonies, after incubation on the appropriate medium (as previously described).

Aqueous solutions containing Carbopol 940. Each 90-mL sample, prepared as previously reported, was inoculated with 10 mL of microbial suspension to obtain a suspension of 10^6–10^7 cells mL^{-1} and carefully and slowly mixed for 2 min with a mechanical stirrer. The whole sample was transferred into a 100-mL glass syringe to ensure accurate dispensing of samples. Immediately after inoculation and at each sampling time, 1 g of sample was expelled from the syringe and diluted 1:100 with solution E to arrest the preservative activity. To perform viable counts each sample was decimally diluted and CFU were counted as for aqueous solutions.

Statistical analysis

To test if the slopes (β) of regression curves derived from individual experiments were significantly different (parallel regression), a *t*-test was used according to Armitage (1975). All statistical analyses were at the 5% level of significance.

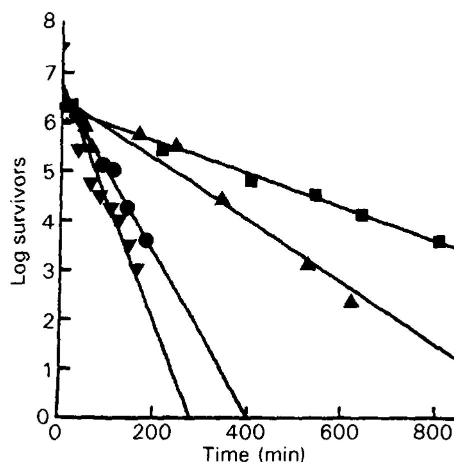

FIG. 1. Survivor curves of *E. coli* in the presence of methyl parahydroxybenzoate (2·5 mg mL^{-1}) and Carbopol 940 concentrations of 1·8% (▼), 1 0% (●), 0·3% (▲), and 0.0% (■).

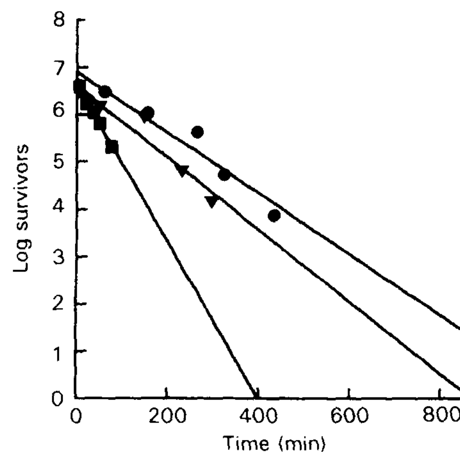

FIG. 2. Survivor curves of *P. aeruginosa* in the presence of methyl parahydroxybenzoate (2·5 mg mL^{-1}) and Carbopol 940 concentrations of 0.0% (●), 0·1% (▼), and 0·2% (■).

Table 1. Synergy of Carbopol 940 on methyl parahydroxybenzoate antimicrobial activity against *E. coli*.

Carbopol (%)	I	β	R^2	s	D
0·0	6·271	−0·0034	0·991	0·11	294
0·3	6·472	−0·0062	0·949	0·40	161
0·6	5·286	−0·0094	0·899	0·39	106
0·8	6·433	−0·0124	0·980	0·21	81
0·9	6·564	−0·0133	0·978	0·28	75
1·0	6·632	−0·0169	0·986	0·16	59
1·4	6·601	−0·0181	0·991	0·35	55
1·8	6·796	−0·0245	0·883	0·56	41

I, intercept of regression curve; β, slope of regression curve; R^2, correlation coefficient from regression; s, standard deviation of regression; D, D-values (min)

Table 2. Synergy of Carbopol 940 on methyl parahydroxybenzoate antimicrobial activity against *P. aeruginosa*.

Carbopol 940 (%)	I	β	R^2	s	D
0·0	6·910	−0·0064	0·940	0·34	156
0·1	6·614	−0·0076	0·924	0·34	132
0·2	6·653	−0·0167	0·976	0·09	60
0·3	*				

I, intercept of regression curve; β, slope of regression curve; R^2, correlation coefficient from regression; s, standard deviation of regression; D, D-values (min); *, rate of death too fast to be measured.

Results

Neutralized aqueous solutions of Carbopol 940 without methyl parahydroxybenzoate exibited no microbicidal activity or growth inhibitory effect. In Tables 1 and 2 and Figs 1 and 2 are reported the survival curves obtained from the experimental data relating to aqueous solutions of methyl parahydroxybenzoate to which different amounts of Carbopol 940 have been added and tested against *E. coli* and *P. aeruginosa*. In Tables 3 and 4 are reported the survivor curves for *S. aureus* and *C. albicans*. Figs 3 and 4 show how the rate of microbial death (β) increases with increasing polymer concentration. Some experiments were carried out against *E. coli* adding $CaCl_2$ to the samples or substituting Na_2EDTA for the polymer. The results reported in Table 5 show the dependence of synergy on the chelating power of Carbopol 940 toward the calcium ion. After the addition of $CaCl_2$ at the specified concentration, the pH of the medium, which was low due to the salt hydrolysis, was raised to neutral (pH 7) using a TEA solution; in these condition Ca^{2+} was unable to precipitate the Carbopol and to affect the methyl parahydroxybenzoate activity. The synergism of Carbopol 940 on methyl parahydroxybenzoate antimicrobial activity against *E. coli* was further tested by determining the minimum inhibitary concentration (MIC) values in a medium devoid of calcium ions (Na_2HPO_4 2·78 g, KH_2PO_4 2·78 g, $(NH_4)_2SO_4$ 1 g, glucose 20 g, $FeSO_4$ 1 mg, H_2O 1000 mL). Identical experiments were repeated, against the same microorganism, but adding calcium ions to the medium. The MIC values of these two sets of experiments are reported in Table 6.

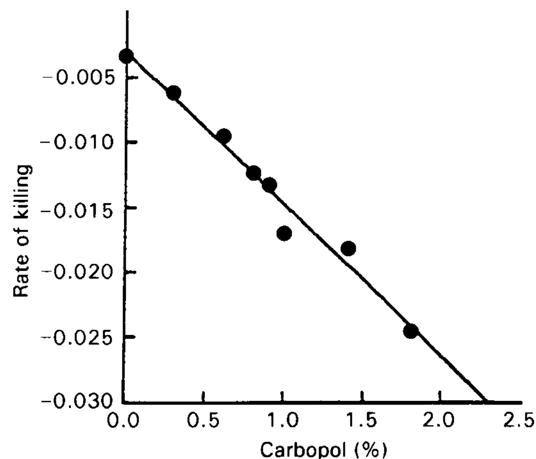

FIG. 3. Rate of killing (β) of *E. coli* vs Carbopol percentage in the presence of methyl parahydroxybenzoate (2.5 mg mL^{-1}).

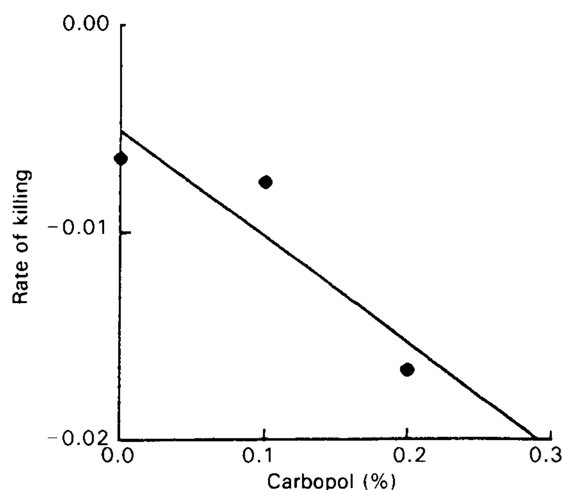

FIG. 4. Rate of killing (β) of *P. aeruginosa* vs Carbopol percentage in the presence of methyl parahydroxybenzoate (2.5 mg mL^{-1}).

Table 3. Inhibiting effect of Carbopol 940 on antimicrobial activity of methyl parahydroxybenzoate against *S. aureus*.

Carbopol 940 (%)	I	β	R^2	s	D
0·0	6·972	−0·0068	0·899	0·66	147
0·1	7·422	−0·0042	0·949	0·61	238
0·3	7·440	−0·0040	0·975	0·19	250
0·5	*				
1·0	*				

I, intercept of regression curve; β, slope of regression curve; R^2, correlation coefficient from regression; s, standard deviation of regression; D, D-values (min); *, the experimental data do not show a significant microbicidal activity.

Discussion

The synergism exerted by Carbopol 940 with methyl parahydroxybenzoate against *E. coli*, plotted in Fig. 3, is clearly demonstrated by good correlation between the rate of death and polymer concentration (β = −0·0117, I = −0·0031, R^2 =

Table 4. Inhibiting effect of Carbopol 940 on antimicrobial activity of methyl parahydroxybenzoate against *C. albicans*.

Carbopol 940 (%)	I	β	R^2	s	D
0·0	7·021	−0·0078	0·903	0·39	128
0·5	6·287	−0·0045	0·932	0·18	222
1·0	5·897	−0·0038	0·922	0·12	263
1·5	*				

I, intercept of regression curve; β, slope of regression curve; R^2, correlation coefficient from regression; s, standard deviation of regression; D, D-values (min); *, the experimental data do not show a significant microbicidal activity.

Table 5. Synergy of Carbopol 940 or Na_2EDTA on methyl parahydroxybenzoate activity against *E. coli*.

Carbopol 940 (%)	I	β	R^2	D
0·0	6·271	−0·0034	0·991	294
1·0	6·632	−0·0169	0·986	59
1·0 (+ $CaCl_2$ 0·1%)	6·881	−0·0060	0·939	166
Na_2EDTA (%)				
0·0	6·271	−0·0034	0·991	294
0·05	7·793	−0·0162	0·995	62
0·01	7·699	−0·0142	0·998	70

I, intercept of regression curve; β, slope of regression curve; R^2, correlation coefficient from regression; D, D-values (min).

Table 6. MIC values of methyl parahydroxybenzoate against *E. coli*.

Carbopol 940 (%)	Ca^{2+} (%)	MIC (mg mL^{-1})
0	0·000	1·250
1	0·000	0·625
1	0·100	1·250
1	0·050	1·250
1	0·025	1·250

0·977). Similarly the anti-*P. aeruginosa* synergism plotted in Fig. 4 ($\beta = -0{\cdot}0515$, $I = -0{\cdot}0051$, $R^2 = 0{\cdot}836$) appears very pronounced, only low percentages of polymer being required to achieve almost immediate kill.

To validate the hypothesis of calcium dependence of synergism against Gram-negative bacteria, the effect of Carbopol 940 and Na_2EDTA were compared, and the chelating effect exerted on Ca^{2+} ions present on the outer membrane of Gram-negative bacteria was confirmed by the data by Table 5. In fact, adding Ca^{2+} ions contributes to restoration of the normal membrane conditions and, as expected, depressed the synergy of Carbopol towards methyl parahydroxybenzoate. Replacing Carbopol with Na_2EDTA, a potent metal chelator able to scavenge calcium ions bound to the outer membrane of Gram-negative bacteria (Ferris 1989), produced a similar synergism. The effect of calcium ions in the test medium employed to evaluate the MIC values reported in Table 6, further confirmed this.

In the case of *S. aureus* and *C. albicans*, microorganisms whose membrane is insensitive to calcium, the effect of Carbopol was similar to that exerted by other hydrocolloids which generally depress the antimicrobial effect of hydroxybenzoates. The inhibitory effect was clearly demonstrated in Tables 3 and 4 for *S. aureus* and *C. albicans* respectively.

Because of its moderate bactericidal activity, use of methyl parahydroxybenzoate as a preservative appears to be a valid system for monitoring the synergistic effect exerted by Carbopol upon microbial killing. In addition, by using species having a membrane structure insensible to the chelating power of Carbopol resin such as *S. aureus* and *C. albicans*, methyl parahydroxybenzoate proves useful in the investigation of any binding effect exerted upon it by Carbopol.

Conclusions

Pharmaceutical and particularly cosmetic formulations offer serious challenges to their preservation. Among the preservatives permitted, the hydroxybenzoates, because of their non-irritant and safe nature (Kabara 1980), are the most frequently employed (Steinberg 1992) even though they are normally recognized as having relatively low activity. In this respect, Carbopol antibacterial synergism appears a simple mechanism to justify the use of hydroxybenzoates, considering that the polymer concentration needed to create synergy (<0.3%), appears insufficient to provoke a strong inhibitory effect against *S. aureus* and *C. albicans*. According to Orth (1979), a D-value of less than 4 h is recommended to assure good preservation against pathogenic microorganisms. The data reported in Tables 3 and 4 show that a significant inhibition for these two species starts only at Carbopol concentrations greater than 0·3–0·5%; this is higher than the concentration of 0·2–0·3% needed to produce a considerable increase in the viscosity and the stability of pharmaceutical and cosmetic formulations (Perotti 1970). The anti-*Pseudomonas* synergism is a valuable effect, particularly useful in formulations that may come into contact with the eye, since *P. aeruginosa* is responsible for serious damage to the cornea and is able to develop resistance to antimicrobial agents (Bowman & Lindstrom 1985).

References

Armitage, P. (1975) Statistica Medica Feltrinelli (ed.) pp 274–282

Beveridge, T. J. (1989) Metal Ions and Bacteria. John Wiley & Sons, pp 15

Bottari, F., Di Colo, G., Nannipieri, E., Saettone, M. F., Serafini, M. F. (1976) Dynamic permeation-note II evaluation of the interaction of pectin with some pharmaceuticals Boll. Chim. Farm. 115: 113–123

Bowman, P. I., Lindstrom, S. M. (1985) Resistence of *Pseudomonas* strains to imidalidinyl urea. J. Soc. Cosm. Chem. 36: 413–423

Denyer, S. P., Hugo, W. B., Harding, V. D. (1985) Synergy in preservative combination. Int. J. Pharm. 25: 245–253

Ferris, F. G. (1989) Metallic ion interactions with the outer membrane of Gram-negative bacteria. In: Beveridge, T. J., Ronald, J. D. (eds) Metal Ions and Bacteria John Wiley & Sons (ed) pp 302–305

Jurgensen-Eide, P., Speiser, P. (1967) Interaction between drugs and nonionic macromolecules. Acta Pharm. Suecica 4: 185

Kabara, J. J. (1980) GRAS antimicrobial agents for cosmetic products. J. Soc. Cosm. Chem. 31: 1–10

Kurup, T. R. R., Wan Lucy, S. C., Chan, L. W. (1992) Interaction of preservatives with macromolecules: Part I–natural hydrocolloids. Pharm. Acta Helv. 67: 301–307

Lucas, J. P. (1978) Microbiological methods for cosmetics. In: Bacteriological Analytical Manual. F&DA, XXIII pp 1–17

Miyawaki, G. M., Patel, N. K., Kostenbauder, H. B. (1959) Interaction

of preservatives with macromolecules III. Parahydroxybenzoic acid esters in the presence of hydrophilic polymers. J. Am. Pharm. Assoc. (Sci. Ed.) 48: 315

Orth, D. S. (1979) Linear regression method for rapid determination of cosmetic preservative efficacy. J. Soc. Cosm. Chem. 30: 321–332

Orth, D. S., Lutes Anderson, C. M., Smith, D. K.,Milstein, S. R. (1989) Synergism of preservative system components: use of survival curve slope method to demonstrate anti-*Pseudomonas* synergy of methyl paraben and acrylic acid homopolymer/copolymers *in vitro* J. Soc. Cosm. Chem. 40: 347–365

Perotti, A. G. (1970) I carbossiviniln polimeri nella Tecnica Farmaceutica. Il Farmaco Ed. Pr. 25: 651–688

Pillai, J. C., Babar, A., Plakogiannis, F. M. (1988) Polymers in Cosmetic and Pharmaceutical Industries. Pharm. Acta Helv. 63: 46–53

Steinberg, D. C. (1992) Cosmetic preservation: current international trends. Cosm. Toilet. 107 (9): 77

Tillman, W. J., Kuramoto, R.(1957) A study of the interaction between methyl cellulose and preservatives. J. Am. Pharm. Assoc. 46: 211–214