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In vitro modification of antimicrobial efficacy by protamine

P. Boussard, M.J. Devleeschouwer and J. Dony

Laboratoire de Microbiologie et d'hygiène, CP 205/2, Institut de Pharmacie, Université Libre de Bruxelles, Campus Plaine, Boulevard du Triomphe, 1050 Bruxelles (Belgium)

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Summary

The influence of serum and protamine on the in vitro effectiveness of some preservatives has been studied. The products tested were formaldehyde, benzalkonium chloride, esters of parahydroxybenzoïc acid, benzoic and sorbic acids. Serum only strongly depleted the activity of benzalkonium chloride. In the case of protamine, this basic protein markedly enhanced the bactericidal effect of benzoic and sorbic acids and allowed a reduction in the concentration by a factor of four while still maintaining the same bactericidal power on *Pseudomonas aeruginosa*.

Introduction

The aim of the preservation of multidose pharmaceuticals, as prescribed by the pharmacopoeias, is to maintain the microbiological purity during the entire life of the preparation. In addition to the microbiostatic action of these preservatives, assumed by their role as stabilizers of the preparation it is imperative that they kill the organisms accidentally introduced in order that the user should not be infected during the next application of the drug. The preservatives must then be microbicidal (Dony, 1984). This requirement of a microbicidal action (a bactericidal and fungistatic effect) led to the use of preservative concentrations which in order to be effective especially as fungistatics are close to concentrations providing some toxic effects.

It is thus important to study these products which could have a synergistic action with the antimicrobial preservatives in order to lower their effective concentrations and concomitantly their toxicity.

In previous work on disinfectants and antibiotics, we studied the sensitivity of *Pseudomonas aeruginosa* to these products according to the growth conditions and interfering substances. (Boussard et al., 1986a, b; Devleeschouwer et al., 1986; Boussard and Dony, 1988). It appeared that protamine, a basic protein, could markedly influence the antimicrobial power of some antibiotics and disinfectants. It therefore seemed interesting to complete our study by investigating the action of protamine on the antibacterial effective-

Correspondence: M.J. Devleeschouwer, Laboratoire de Microbiologie et d'hygiène, CP 205/2, Institut de Pharmacie, Université Libre de Bruxelles, Campus Plaine, Boulevard du Triomphe, 1050 Bruxelles, Belgium.

ness of various preservatives. For a first screening, only *P. aeruginosa* was retained as the test organism, since it is one of the more resistant organisms when looking for antimicrobial effectiveness.

Material and Methods

The strain tested was *Pseudomonas aeruginosa* ATCC 15442.

Antimicrobial preservatives (*J. Off. Commun. Eur.*, 1982; Kabara, 1984; *J. Off France*, 1987) were: formaldehyde 0.05% (maximum authorized 0.2%); benzalkonium chloride 0.01% (max. authorized 0.5%); nipagine 0.07% and nipasol 0.03%; benzoic acid 0.25%, 0.1%. 0.05%; 0.025%; 0.01% (max. authorized 0.5%); sorbic acid: 0.1%, 0.05%, 0.025%, 0.01%. Protamine (salmine sulfate) was obtained from Serva (no. 33740).

Methodology

For this in vitro study, we adopted two different approaches. The first consisted of adding the protamine directly to the mixture of the antibacterial and the suspension of *P. aeruginosa*. In the results this approach is designated by the symbol *. The second consisted of initially mixing the bacteria and the protamine for 10 min at 37 °C and thereafter adding the antibacterial. This is referred to in the results by the symbol #.

The method used to evaluate the bactericidal effect of the preservatives is adapted from a method used in disinfectant testing (Dony and Devleeschouwer, 1978).

The inoculum is prepared by incubation of the strain in tryptic soy broth (Difco) for 16 h at 37 °C after three successive transfers on tryptic soy agar (Difco) slants. The inoculum is then homogenized on glass balls and calibrated at about 10^9 organisms/ml. The preservatives are diluted in hard water ($MgCl_2 \cdot 6H_2O$ 139 mg, $CaCl_2 \cdot 2H_2O$ 402 mg/l water) to a suitable concentration.

To 10 ml of this preservative solution supplemented or not with 10% horse serum (Eco bio) and an appropriate amount of protamine, 0.4 ml of inoculum are added. After 10 min, 1, 2, 4, 5 and 6 h, 1 day, 1 week of contact, 1 ml of the suspension is transferred into 9 ml of inactivating solution (peptamine, 10 g; beef extract, 5 g; lecithin, 3 g; Tween 80, 30 g; L-histidine, 1 g; sodium thiosulfate, 5 g; NaCl, 5 g; 1 l water). The number of surviving bacteria is then counted by the plate count technique using tryptic soy agar (Difco). The results are then interpreted as the decrease in the number of organisms expressed as the logarithm over time.

TABLE 1

Influence of protamine (100 μg/ml) on the in vitro antibacterial efficacy of formaldehyde (0.05%) on Pseudomonas aeruginosa ATCC 15442: logarithmic decrease in number of bacteria vs time

Time	Without protamine		With protamine			
	WS	S	*		#	
			WS	S	WS	S
1/2 h	1.6	1.9	1.5	2.1	1.5	1.2
1 h	2.0	1.7	2.3	1.6	2.1	1.6
2 h	3.8	2.8	4.0	3.1	2.7	2.2
4 h	> 8	3.8	> 8	4.1	4.7	4.4
6 h	> 8	4.1	> 8	> 8	> 8	> 8
1 day	> 8	> 8	> 8	> 8	> 8	> 8
1 week	> 8	> 8	> 8	> 8	> 8	> 8

WS, without serum; S, with serum.

Results

In Table 1, it appears that protamine in our experimental conditions did not influence the antibacterial effectiveness of formaldehyde at 0.05%. Moreover, the presence of serum did not interfere with the bactericidal power of formaldehyde.

Table 2 illustrates the fact that the action of benzalkonium chloride at 0.05% is not influenced by the addition of protamine. Here, however, there is a drastic influence of serum which completely depletes the antibacterial activity of the benzalkonium chloride.

In contrast, the mixture of nipagine and nipasol exhibited poor antibacterial effectiveness against *P. aeruginosa*. Neither protamine nor serum had any influence on this antibacterial ineffectiveness (Table 3).

When analyzing the antibacterial activity of benzoic acid (Table 4), the results obtained without

TABLE 2

Influence of protamine (100 μg/ml) on the in vitro antibacterial efficacy of benzalkonium chloride (0.01%) on Pseudomonas aeruginosa ATCC 15442: logarithmic decrease in number of bacteria vs time

Time	Without protamine		With protamine			
			*		#	
	WS	S	WS	S	WS	S
10 min	1.9	0.1	0.3	0.4	2.5	0.4
1/2 h	2.4	0.1	1.7	0.3	5.5	0.5
1 h	2.5	0	2.9	0	> 7	0.8
2 h	6.9	0	> 7	0	> 7	0.7
4 h	> 7	0	> 7	0	> 7	1.2
6 h	> 7	0	> 7	0	> 7	0.8
1 day	> 7	0	> 7	0	> 7	0.4
1 week	> 7	0	> 7	0	> 7	0

WS, without serum; S, with serum.

serum showed an immediate decrease in the number of organisms. In this condition it was impossible to evaluate the influence of protamine. This influence of protamine appeared in the experiments supplemented with serum where it nearly abolished the negative influence of serum on the antibacterial capacity.

We then studied the influence of 100 μg/ml of protamine on the antibacterial power of decreasing concentrations of benzoic acid (Table 5). At a concentration of 0.1% benzoic acid the addition of protamine markedly enhanced (3 to 4 log units) the bactericidal effect especially during the first 2 h of contact. Moreover, by addition of protamine it is possible to decrease the concentration of benzoic acid from 0.1% to 0.025% and still obtain the same bactericidal power. It should be noted that after a contact time of 6 h the decrease in number of organisms is the same among all the experiments.

Identical results were obtained with sorbic acid (Table 6). The presence of protamine allows the concentration to decrease from 0.1% to 0.01% while maintaining the antibacterial activity. However, a difference appeared at the end point of the experiments as this antibacterial never killed all

TABLE 3

Influence of protamine (100 μg/ml) on the in vitro antibacterial efficacy of nip-nip mixture (0.1%) on Pseudomonas aeruginosa ATCC 15442: logarithmic decrease in number of bacteria vs time

Time	Without protamine		With protamine			
			*		#	
	WS	S	WS	S	WS	S
10 min	0.6	0.9	0.8	0.9	−0.3	0
1 h	0.6	1.0	0	0.8	0.4	0.3
2 h	0.6	1.1	1.1	0.9	−0.4	0.7
4 h	0	0	0.7	0	−0.4	0.7
6 h	0.4	0	1.3	1.1	0.6	0.9
1 day	0.6	0.2	1.3	0.3	0	−0.3

WS, without serum; S, with serum.

TABLE 4

Influence of protamine (100 μg/ml) on the in vitro antibacterial efficacy of benzoic acid (0.25%) on Pseudomonas aeruginosa ATCC 15442: logarithmic decrease in number of bacteria vs time

Time	Without protamine		With protamine			
			*		#	
	WS	S	WS	S	WS	S
1 h	> 8	1.6	> 8	> 8	> 8	2.5
2 h	> 8	3.6	> 8	> 8	> 8	> 8
4 h	> 8	> 8	> 8	> 8	> 8	> 8
6 h	> 8	> 8	> 8	> 8	> 8	> 8
1 day	> 8	> 8	> 8	> 8	> 8	> 8
1 week	> 8	> 8	> 8	> 8	> 8	> 8

WS, without serum; S, with serum.

TABLE 5

Influence of protamine (100 μg/ml) on the in vitro antibacterial efficacy of benzoic acid at 0.1, 0.05, 0.025 and 0.01% on Pseudomonas aeruginosa ATCC 15442: mean values of the logarithmic decrease in organisms for three assays

Time	Protamine (μg/ml):	0	100	100	100	100
	Benzoic acid (%):	0.1	0.1	0.05	0.025	0.01
1/2 h		1.6	6.4	5.8	5.2	4.3
1 h		3.3	7.4	7.0	5.8	4.9
2 h		6.0	> 8	7.2	7.2	3.8
4 h		> 8	> 8	7.3	7.1	6.0
6 h		> 8	> 8	> 8	> 8	6.6
1 day		> 8	> 8	> 8	> 8	> 8
1 week		> 8	> 8	> 8	> 8	> 8

TABLE 6

Influence of protamine (100 μg/ml) on the in vitro antibacterial efficacy of sorbic acid at 0.1, 0.05, 0.025 and 0.01% on Pseudomonas aeruginosa ATCC 15442: mean values of the logarithmic decrease in organisms for three assays

Time	Protamine (μg/ml):	0	100	100	100	100
	Sorbic acid (%):	0.1	0.1	0.05	0.025	0.01
1/2 h		0.4	5.9	4.7	4.3	3.3
1 h		1.2	6.6	5.6	4.7	4.3
2 h		1.9	6.9	6.5	7.0	4.2
4 h		2.9	7.0	6.7	6.4	6.3
6 h		4.5	6.8	6.3	5.4	> 7
1 day		> 7	> 7	> 7	6.4	> 7
1 week		6.3	6.6	6.8	6.5	7.0

the organisms in the assay. In some cases regrowth of some surviving bacteria was even possible.

Discussion

It appears from the results that all the preservatives tested did not exhibit the same antibacterial power on *P. aeruginosa*. The esters tested had no activity even after 24 h of contact. It is likely that these preservatives are destroyed by the enzymes of *P. aeruginosa* and used as carbon sources (Close and Nielsen, 1976). In contrast, the solutions of formaldehyde at 0.05, benzoic acid at 0.25%, sorbic acid at 0.1% and benzalkonium chloride at 0.01% are capable, under our experimental conditions of producing a logarithmic decrease in the number of bacteria of more than 7 units after 24 h contact.

All these products span the outer membrane through the porins due to the fact that formaldehyde is a small molecule, benzalkonium chloride is positively charged and the two acids are of hydrophilic character.

The addition of serum had no influence on the in vitro antibacterial capacity of formaldehyde, benzoic and sorbic acids and the esters of *p*-hydroxybenzoic acid. However, the antibacterial effectiveness of benzalkonium chloride was abolished by the proteins of the horse serum as a result of association that prevented the molecule from binding to the outer membrane of *P. aeruginosa*. By interfering with this step, the preservative is not able to penetrate into the bacteria (Sakagami et al., 1989).

The influence of protamine was studied either by first mixing the protamine with the bacteria 10 min before contact with the preservative or by immediately mixing the three components together. The results show that both procedures gave similar results.

Nevertheless, the preservative's effectiveness was not affected in the same manner. No modification of activity appeared with the *p*-aminobenzoic acid esters, benzalkonium chloride or formaldehyde. The in vitro efficacy of benzoic acid and sorbic acid was enhanced by protamine, but this effect disappeared in the presence of serum.

This enhancement was also previously demonstrated for chloroxylenol and phenol, and some β-lactamines (Boussard et al., 1986a; Boussard and Dony, 1988).

Small molecules and hydrophilic species traverse the outer membrane through the porins (Caulcott et al., 1984; Hancock, 1984, 1986; Nikaido and Hancock, 1986; Woodruff et al., 1986; Hancock and Woodruff, 1988; Yoshihara and Nakae, 1989). *P. aeruginosa* harbours several types of porins among which the most important are the porins P and F. These protein channels have their own specificity. Porins P have positively charged fixation sites localized outside the bacteria and will thus attract negatively charged compounds. In contrast, porins F are preferentially passed by cations.

Antibacterials, such as β-lactamines, chloroxylenol, phenol, benzoic acid and sorbic acid with activity which is enhanced by protamine, should pass through the P porins. Protamine by fixation to an outer membrane structure could promote the opening of the P porins and so enhance the passage of various compounds.

Finally, we measured the influence of protamine on decreasing concentrations of benzoic and sorbic acids. For these two preservatives, the protamine allows a reduction of the concentrations from 0.1% to 0.025% while still maintaining the same bactericidal power on *P. aeruginosa*.

This approach could be useful for lowering the concentrations of these products used as preserva-

tives in certain protein free pharmaceutical and cosmetics products.

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