ANTIBACTERIAL ACTION OF BRONOPOL ON VARIOUS BACTERIA, ESPECIALLY ON *PSEUDOMONAS AERUGINOSA*

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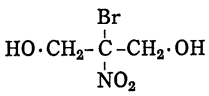
Bronopol is a new antimicrobial agent in a series of aliphatic halogeno-nitro compounds developed by CROSHAW, GROVES and LESSEL[1] of Boots Pure Drug Co., Ltd. in England, and has the following chemical structure :



They reported that bronopol has an antibacterial activity against *Pseudomonas aeruginosa* to the same degree as against other Gram-negative bacteria as well as Gram-positive bacteria and to a lesser extent against fungi unlike various antibacterial agents.

The present observation has been made to compare bronopol with chlorhexidine[2], one of the most excellent ordinary disinfectants for their *in vitro* antibacterial effect on various bacteria, especially on *P. aeruginosa*.

Materials and methods

The bacteriostatic activity of bronopol (bronosol®, Midori-juji Co., Ltd., Osaka) and chlorhexidine digluconate (5% hibitane® solution, Sumitomo Chemical Industry Co., Ltd., Osaka) was determined according to the Standard Methods published by the Japan Society of Chemotherapy[3]. To prepare the plates for inhibitory activity determination, 1 ml of diluted distilled solution of each drug was added to 9 ml of various media (see Table 1) to give a final concentration of 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39 and 0.2 mcg/ml. *Corynebacterium*

Table 1 Antimicrobial spectra of bronopol

Gram	Strains	MIC(μg/ml)		Medium used for MIC determination
		Bronopol	Chlorhexidine	
Positive	*Staphylococcus aureus* 209P-JCI	12.5	0.78	Nutrient agar
	Staphylococcus epidermidis SP-al-1	12.5	0.78	
	Streptococcus haemolyticus Showa Univ.	25	1.56	Brain heart infusion agar
	Corynebacterium diphtheriae G-18	25	0.39	
	Bacillus subtilis PCI-219	12.5	1.56	Nutrient agar
	Candida albicans Nagoya 7491	25	6.25	SABOURAUD glucose agar
	Candida albicans ATCC 10259	50	50	
	Candida albicans IFO 0579	50	100	
Negative	*Escherichia coli* NIHJ-JCZ	12.5	3.13	Nutrient agar
	Klebsiella pneumoniae ST-101	12.5	6.25	
	Proteus vulgaris P-2	12.5	6.25	
	Pseudomonas aeruginosa Ikaken	12.5	50	
	Salmonella typhosa H901W	12.5	1.56	
	Shigella flexneri 2a EW-10	6.25	0.78	
	Shigella sonnei 1 EW-33	6.25	1.56	
	Vibrio parahaemolyticus K-18	6.25	50	3% Sod. chloride nutrient agar

diphtheriae and *Streptococcus haemolyticus* was cultured at 37°C for 24 hours in brain heart infusion broth, *Candida albicans* for 48 hours in SABOURAUD glucose broth and other strains of bacteria for 15 hours in heart infusion broth (containing 3% NaCl in *Vibrio parahaemolyticus*). A loopful of each of the resulting culture media was streaked 2 cm in length on the plate containing a drug. After cultivation was made at 37°C for 48 hours in *C. albicans* and for 24 hours in other bacterial strains, the minimal inhibitory concentration (MIC) of each drug was read. If not specially mentioned hereafter, the antibacterial activity of a drug was determined by the abovementioned procedure with the necessary modification.

In the test with heart infusion broth to 9 ml broth, 1 ml of diluted distilled water solution of a drug was added to give the same final concentration as that of nutrient agar. A loopful of bacteria was inoculated and incubated at 37°C for 24 hours followed by a recording of the presence or absence of bacterial growth.

The bactericidal effect of bronopol and chlorhexidine on *P. aeruginosa* was investigated; *P. aeruginosa* Ikaken strain was cultured for 15 hours in heart infusion broth and the resulting medium was diluted with the same broth to give a final concentration of 27.8×10^5 living cells/ml followed by an incubation period of 3 hours at 37°C. After the living cells were counted, 10 ml of the culture medium thus obtained were taken into each of the 12 sterilized test tubes. They were divided into 3 groups, each containing 4 test tubes. To each test tube of Group I 1,250 μg/ml bronopol solution was added; to those of Group II 1,250 μg/ml chlorhexidine solution was added: And to those of Group III, which was settled as control, distilled water was added at a rate of one-hundredth volume (0.1 ml). They were incubated at 37°C and every 3, 6, 9 and 24 hours, a test tube was taken out to determine the number of living cells. Cell count was carried out as follows: a culture medium was diluted 10 fold serially with sterilized distilled water, and 1 ml of each resulting solution was mixed with 15 ml nutrient agar and cultured for 24 hours at 37°C followed by a counting of colonies.

Using *Salmonella typhosa* H 901 W strain and *P. aeruginosa* Ikaken strain, the phenol coefficients of bronopol and chlorhexidine were examined by the method published in the Nippon Koshueisei Zasshi[4] with the necessary modification.

Results

1. Bacteriostatic activity against various bacteria

The results obtained are summarized in Table 1. Bronopol had the strongest antibacterial action (MIC=6.25 μg/ml) against *Shigella dysenteriae* and *V. parahaemolyticus* and on the contrary the weakest one (MIC=25~50 μg/ml) against *C. albicans*, *S. haemolyticus* and *C. diphtheriae*. All the bacteria tested except for those were supressed to grow at a concentration of 12.5 μg/ml. On the other hand, as for the antibacterial action of chlorhexidine, its MIC was 50 μg/ml, which was lower than that of bronopol, against *P. aeruginosa* and *V. parahaemolyticus*. However, the former was more or less more antibacterial than the latter against the other bacteria.

2. Distribution of *P. aeruginosa* for sensitivity to bronopol

Fifty-six strains of *P. aeruginosa* were distributed for their sensitivity to bronopol and chlorhexidine as shown in Table 2. All strains were susceptible to 12.5 μg/ml of bronopol. By contrast, only 2 were susceptible to the same concentration of chlorhexidine, 4 to 25 μg/ml and the majority or 39 (69.6%) to 50 μg/ml. A large number [11 (19.6%)] of strains were completely supressed to grow at a concentration as high as 100 μg/ml.

3. Bacteriostatic activity of bronopol and pH

Table 2 Distribution of minimal inhibitory concentrations of bronopol and chlorhexidine against *Pseudomonas aeruginosa*

Antimicrobial agent	Number of strains	MIC (μg/ml)					
		3.13	6 25	12.5	25	50	100
Bronopol	56			56			
Chlorhexidine	56			2	4	39	11

of medium

Bronopol was examined for its antibacterial effect on 34 strains of *P. aeruginosa* using nutrient agars kept at pH 6.0, 7.0, 8.0 and 9.0.

As indicated in Table 3, this drug manifested the strongest antibacterial effect at pH 7.0 : its MIC was 12.5 μg/ml against all strains. It was 25 μg/ml 11 (33.4%) at pH 6.0. When the medium was kept at pH 8.0 or 9.0, bronopol had a far weaker antibacterial action : its MIC was 25 μg/ml in the former case and 50 μg/ml in the latter.

4. Bacteriostatic activity and kinds of media

The antibacterial effects of bronopol and chlorhexidine on 6 strains of *P. aeruginosa* ware examined using nutrient agar and heart infusion broth. The results obtained are shown in Table 4. The MIC of bronopol was 12.5 μg/ml in nutrient agar and 12.5～25 μg/ml in heart infusion broth : no significant difference was found between them. In contrast, the antibacterial effect of chlorhexidine was weaker in the nutrient agar than in the heart infusion broth : its MIC was 50～100 μg/ml in the former and 3.13～12.5 μg/ml in the latter.

Table 3 Influence of medium pH on activity of bronopol against *Pseudomonas aeruginosa* (34 strains)

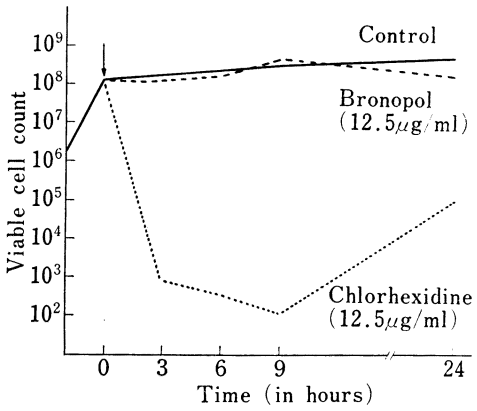
pH	MIC (μg/ml)				
	3.13	6.25	12.5	25	50
6.0			23	11	
7.0			34		
8.0				34	
9.0					34

Table 4 Antibacterial activity of bronopol and chlorhexidine in nutrient agar and heart infusion broth against *Pseudomonas aeruginosa*

Strains	Bronopol (μg/ml)		Chlorhexidine (μg/ml)	
	NA*	HIB**	NA	HIB
Stool	12.5	25	100	12.5
Tamura	12.5	12.5	50	6.25
Kageyama	12.5	12.5	50	3.13
Yamamoto	12.5	25	50	6.25
Nakao	12.5	25	100	12.5
Ikaken	12.5	12.5	50	12.5

* NA : nutrient agar ** HIB : heart infusion broth

Fig. 1. Bactericidal effects of bronopol and chlorhexidine against *Pseudomonas aeruginosa* Ikaken strain



5. Bactericidal effect on *P. aeruginosa*

As presented in Fig. 1, the number of living cells was slightly lower, though not signifiacntly, in the bronopol group than in the control, but continued to increase gradually in the course of time. By contrast, in the chlorhexidine group the number of living cells decreased markedly after 3 hours incubation as compared with that before the addition of the drug and further decreased in the course of time. However, it tended to increase 24 hours after incubation.

6. Influence of protein on bacteriostatic activity of bronopol

The bacteriostatic activity of bronopol against *P. aeruginosa* Ikaken strain was examined using nutrient agar containing 10 per cent human serum and that without serum to examine the influence of protein on it.

Though not shown in the Table, MIC of bronopol was 12.5 μg/ml when the medium was without serum as compared with 25 μg/ml when the serum was added to the medium. Serum protein had little influence on MIC of bronopol.

7. Phenol coefficient

The results obtained are shown in Table 5 (1)～(4). The phenol coefficient of bronopol was 2.5 against *P. aeruginosa* and 2.4 against *S. typhosa*. That of chlorhexidine was 471 against *P. aeruginosa* and 833 against *S. typhosa*, which were far larger than the

Table 5 Phenol coefficient of bronopol
(1) *Pseudomonas aeruginosa* Ikaken strain

Disinfectant	Dilution	Time in minutes 2.5	5	10	15
Bronopol	1 : 200	+	−	−	−
	1 : 225	+	+	+	−
	1 : 250	+	+	+	+
	1 : 275	+	+	+	+
	1 : 300	+	+	+	+
Phenol	1 : 70	−	−	−	−
	1 : 80	+	−	−	−
	1 : 90	+	+	+	−
	1 : 100	+	+	+	+
	1 : 110	+	+	+	+

phenol coefficient = 212.5/85=2.5

Table 5 Phenol coefficient of bronopol
(2) *Salmonella typhosa* H901W strain

Disinfectant	Dilution	Time in minutes 2.5	5	10	15
Bronopol	1 : 200	+	−	−	−
	1 : 225	+	+	+	−
	1 : 250	+	+	+	+
	1 : 275	+	+	+	+
	1 : 300	+	+	+	+
Phenol	1 : 70	−	−	−	−
	1 : 80	−	−	−	−
	1 : 90	+	+	−	−
	1 : 100	+	+	+	+
	1 : 110	+	+	+	+

phenol coefficient=212.5/90=2.36=2.4

corresponding values of bronopol.

Discussion

Recently it has become well known that Gram-negative bacteria frequently causes more markedly general infections than Gram-positive cocci in various clinical fields. SHIBATA *et al.*[5] reported that 20.4 per cent of postoperative infections was due to Gram-positive cocci, 77.2 per cent due to Gram-negative bacteria (*P. aeruginosa*, 31.1%; *Escherichia coli*, 17.4%; *Klebsiella pneumoniae*, 6.0%; *Proteus*, 5.4% and others, 17.3%) and 2.4 per cent due to unknown causes, during the past 10 years from 1961 to 1970 at the First Surgical Departement of Nagoya-City Medical College. In addition, 2,270 strains of Gram-negative bacteria isolated from various diseases materials for 4 years from 1965 to 1968 by the Research Group of Gram-Negative Bacterial Infections[6] were occupied mainly by 47.9 per cent of *E. coli*, 22.1 per cent of *P. aeruginosa*, 12.8 per cent of *Proteus*, and 11.1 per cent of *K. pneumoniae* and a small portion of *Aeromonas aeruginosa*, *Cloaca*, *Citrobacter*, *Arizona*,

Table 5 Phenol coefficient of chlorhexidine
(3) *Pseudomonas aeruginosa* Ikaken strain

Disinfectant	Dilution	Time in minutes 2.5	5	10	15
Chlorhexidine	1 : 30,000	+	−	−	−
	1 : 35,000	+	−	−	−
	1 : 40,000	+	+	−	−
	1 : 45,000	+	+	+	−
	1 : 50,000	+	+	+	+
Phenol	1 : 70	−	−	−	−
	1 : 80	+	−	−	−
	1 : 90	+	+	+	−
	1 : 100	+	+	+	+
	1 : 110	+	+	+	+

phenol coefficient=40,000/85=470.5=471

Table 5 Phenol coefficient of chlorhexidine
(4) *Salmonella typhosa* H901W strain

Disinfectant	Dilution	Time in minutes 2.5	5	10	15
Chlorhexidine	1 : 60,000	−	−	−	−
	1 : 65,000	+	−	−	−
	1 : 70,000	+	−	−	−
	1 : 75,000	+	+	−	−
	1 : 80,000	+	+	+	−
Phenol	1 : 70	−	−	−	−
	1 : 80	−	−	−	−
	1 : 90	+	+	−	−
	1 : 100	+	+	+	+
	1 : 110	+	+	+	+

phenol coefficient=75,000/90=833.3=833

Serratia, *Hafnia* and *B. anitratum*.

In *P. aeruginosa* of the above-mentioned, 4 the main phlogogenous organisms of Gram-negative bacterial infections in hospital-acquired infection is stressed as a spreading pathway. The cases supposed to be infected with the organism from contaminated medical apparatuses, instruments[7~10] or therapeutic drugs[11~14] and those considered to be attached by cross infection from patients carrying or releasing *P. aeruginosa* have been reported[15,16].

Though the infection of a postoperative wound is decreasing in incidence with development of disinfectants and antibiotics, it still occurs with a certain frequency. *P. aeruginosa* is reported to be somewhat more resistant to usual disinfectants than other Gram-negative bacteria. KUWAHARA[17] reported that though *P. aeruginosa* did not vary so from *S. typhosa* and *Staphylococcus aureus* in sensitivity to phenol and cresol, it showed far stronger resistance, differing markedly from strain to strain, to mercurials, inverted soap, and anionic soap than the others.

This fact suggests that inadvertedly, soap used

habitually may rather select *P. aeruginosa* and promote the spread of infections caused by the organism.

CROSHAW *et al.*[1] developed 2-bromo-2-nitropropane-1, 3-diol (bronopol), which is the most stable antimicrobial agent of a series of aliphatic halogeno-nitro compounds in aqueous media. According to their observation this agent is superior to chlorhexidine digluconate, hexachlorophane, cetrimide, domiphen bromide, phenoxyethanol and dequalinium chloride in bacteriostatic activity against *P. aeruginosa*. OGURA *et al.*[18] compared 17 kinds of widely used bacteriostatic and bactericidal agents in addition to 3 new drugs for their bacteriostatic activity and concluded from their wide range of activity mainly against *P. aeruginosa* that chlorhexidine, bronopol and thimerosal were most excellent. They reported that MIC of chlorhexidine was 2.0 to 6.3 μg/ml, that of bronopol 3.15 to 10 μg/ml, and that of thimerosal 3.15 to 10 μg/ml. Thimerosal contains mercury and so a serious consideration should be paid to its use, because toxicity of mercury has become a social problem at present.

Accordingly, we took up bronopol and chlorhexidine in consideration of the possibility of their practical use and investigated their bacteriostatic activity against 56 strains of *P. aeruginosa*. The MIC of bronopol was 12.5 μg/ml and did not differ from strain to strain. On the other hand, the MIC of chlorhexidine fell between 12.5 and 100 μg/ml, differing considerably among strains. However, it was 50 μg/ml in the majority of strains. Those values were smaller than those of CROSHAW *et al.*[1] (bronopol, 25~50 μg/ml; chlorhexidine, 100>100 μg/ml) and larger than those of OGURA *et al.* which were mentioned above. On the other hand, when we determined the MIC of both drugs using heart infusion broth and nutrient agar, no significant difference was found in MIC of bronopol between both culture media. In contrast, the MIC of chlorhexidine was far smaller in heart infusion broth than in nutrient agar and smaller than that of bronopol in some strains. In addition, we followed a change in survival cell count of *P. aeruginosa* with the lapse of time in heart infusion broth containing bronopol or chlorhexidine at MIC against the organism. In the broth containing chlorhexidine, bactericidal effect continued up to 9 hours after the addition and then survival cells increased in number up to 24 hours of culture. Under those conditions, however, bronopol scarcely showed bactericidal effect. As for the effect of bronopol on organisms other than *P. aeruginosa*, CROSHAW *et al.*[1] reported that this agent manifested a strong bacteriostatic activity against both Gram-negative and positive bacteria and a weak one against yeasts and fungi. We could confirm their observation. In contrast, OGURA *et al.*[18] indicated that bronopol had a stronger antibacterial effect on Gram-negative bacteria than on Gram-positive ones. However, this agent was inferior to chlorhexidine in antibacterial activity against either Gram-positive or negative bacteria. In this respect our results coincide with theirs. In the present observation bronopol showed a stronger antibacterial effect on *V. parahaemolyticus* than chlorhexidine. However, this result was not conclusive, because it was obtained in the test using only one strain.

KOMEDA *et al.*[9] reported that the bacteriostatic activity of bronopol and chlorhexidine was not so decreased in existence of a serum preparation, plasmonate (Midori-juji Co., Ltd., Osaka) and that bronopol did not produce color change and precipitate even when incubated at 37°C with such proteins. CROSHAW *et al.*[1] indicated that the addition of 50 per cent calf serum had little influence upon the bactericidal activity of bronopol. In the present observation, moreover, an addition of 10 per cent human serum also had no significant effect on it.

ARAI *et al.*[20] determined the phenol coefficients of various bacteriostatic and bactericidal agents using *P. aeruginosa* and reported that those of widely used inverted soaps were relatively small, those of mercurials varied from strain to strain and cresol was relatively excellent. According to OGURA *et al.*[21], chlorhexidine digluconate, benzethonium chloride and benzalkonium chloride had large phenol coefficients and bronopol had a value far lower than these. They suggested that bronopol might not belong to quick disinfectants but rather belong to bacteriostatics. Our results support their suggestion.

O'FLYNN *et al.*[22] reported that 39 per cent of *Proteus mirabilis* strains isolated from materials from hospitals could survive against chlorhexide at a concentration of 1 : 5000. Recently, KIN *et al.*[23] reported that chlorhexidine resistant *Alcaligenes faecalis* had been isolated from hands and fingers of operators owing to the contaminated ultrasonic hand washer. The antibacterial effect of bronopol

on such chlorhexidine resistant organisms and acquisition of resistance to this agent by various organisms will be interesting prospective problems.

Summary

The bacteriostatic and bactericidal activities of bronopol and chlorhexidine were compared.

Though generally inferior to chlorhexidine in bacteriostatic activity, bronopol had a relatively strong antibacterial effect on Gram-negative as well as Gram-positive bacteria. Especially, bronopol manifested an activity being not at all inferior to that of chlorhexidine against *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus*. However, bronopol was far inferior to chlorhexidine in both rapidity and strength of bactericidal effect. Addition of serum had little influence upon the bacteriostatic of bronopol.

Acknowledgement

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