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Antimicrobial Activity of Borate-Buffered Solutions

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A minimal salts medium adjusted to physiological pH and osmolality was buffered with either 0.3% phosphate or 1.2% borate and evaluated for antimicrobial activity. The borate-buffered medium, either with or without a carbon source, exhibited significant antimicrobial activity against 15 *Pseudomonas* strains, 12 strains of enteric bacteria, and 7 strains of staphylococci. The borate-buffered system appears suitable for use as a generic vehicle for ophthalmic pharmaceutical agents.

In 1958, Kingma summarized the pharmacology and toxicology of boron compounds (10). Known as *sal sedativum*, borates were used in the eighteenth century to soothe the skin. Even before this time, borates were used as cleansing agents and by Arab physicians, who used them as early as 875 A.D. for internal medication.

More recently physicians have used boric acid to produce both microbiostatic and microbicidal effects. Boric acid solutions have been used to soften and dry irritated and infected tissues. In addition, borates at concentrations of 0.75 to 3% have been found to exert marked bacteriostatic and weak bactericidal effects when added to nutrient broth cultures of *Staphylococcus aureus*, *Staphylococcus haemolyticus*, and *Escherichia coli* (10). These findings agree with a previous study by Fisher (4), which describes the pronounced bacteriostatic action of borates at concentrations of 0.2 to 4% against common microbial contaminants and pathogens found in many media used in wet dressings. Moreover, Swate and Weed found that 40 patients with vulvovaginal candidiasis responded well to treatments with 600 mg of boric acid in pessaries and ointments containing 5% boric acid in lanolin (15).

During the last 15 years new uses for borates have been discovered. Fayinka (3), Porter and Brodie (13), and Watson and Duerden (16) have used borates to preserve urine samples for 24 h before culturing. These authors have found that the optimum boric acid concentration was approximately 2% for urine preservation at room temperature. Longer exposure times or higher concentrations, especially above 3%, can cause reductions in gram-negative bacteria.

Some toxic manifestations of borate use have been reported. Most of these toxic reactions can be traced to inappropriate use or accidental ingestion. The review by Kingma in 1958 described cases in which boric acid compounds were accidentally fed to children, given as antiepileptic remedies, given by injections that resulted in overdose, and used for wound and bladder irrigation. External poisoning resulted from application to infants with external areas of atopic eczema, to patients with large areas of burned skin, and to adults with generalized exudative or exfoliative dermatitis. Additional cases of accidental poisoning in infants were reported by Ginsburg (6), Adelhardt (1), O'Sullivan and Taylor (11), and Bosio and Bertoncini (2). However, the safety of boric acid in ordinary use is supported by a survey of dermatologists (4), by recent studies of ophthalmic ointments (8), by dermatologic use of ointments on neonates (5), and by two pharmacokinetic studies of oral (14) and intravenous (9) administration.

The present study evaluated the antimicrobial activity of 1.22% borate buffer in a minimal salts medium, with and without a carbon source (glucose). For comparison, phosphate-buffered and unbuffered minimal salts media were also evaluated. The antimicrobial activity of the solutions was evaluated as a function of the log change in viable cell count over 28 days. The significance of the results as they relate to the preservative effectiveness test described by the United States Pharmacopeia (USP XXI) and their application to ophthalmic pharmaceuticals is discussed.

MATERIALS AND METHODS

Micoorganisms. The microorganisms that were tested are listed in Tables 1 to 4. They were obtained from several different sources, including clinical, environmental, and product isolates.

Defined media. Minimal media used with the *Pseudomonas* strains contained 0.04% $(NH_4)_2SO_4$ and 0.05% $MgSO_4$, either unbuffered, buffered with 0.10% $KH_2PO_4 \cdot 7H_2O$ and 0.02% Na_2HPO_4, or buffered with 1.00% boric acid and 0.22% $Na_2B_4O_7 \cdot 10H_2O$. The minimal medium for the enteric bacteria and staphylococci contained 0.04% $(NH_4)_2SO_4$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% $CaCl_2 \cdot 2H_2O$, 0.0025% $Na_2MoO_4 \cdot 2H_2O$, and 1.00% vitamin solution. The vitamin solution was prepared separately at 100× concentration and filter sterilized. The final concentrations were 0.0005% calcium pantothenate, 0.0001% thiamine hydrochloride, 0.0001% pyridoxine hydrochloride, 0.0001% biotin, and 0.0001% nicotinic acid.

Each solution was made isotonic with 0.9% NaCl by calculating the salt equivalent of each ingredient and adding the appropriate amount of NaCl. The pH of each solution was adjusted to 7.00 ± 0.2 with hydrochloride or KOH. The glucose, $MgSO_4$, $CaCl_2$, and $NaMoO_4$ were autoclaved separately at 100× concentration and then aseptically transferred to the minimal media.

The microorganisms were transferred from nutrient broth to the defined minimal medium containing phosphate and glucose. After the microorganisms adapted and began growing in the minimal medium, a second 24-h culture was used for each experiment. The respective cultures were harvested by centrifugation, washed three times in distilled water, and standardized at A_{500} to give approximately 10^6 CFU/ml.

The staphylococci were unable to grow in the minimal medium without the addition of amino acids. They were therefore harvested directly from the tryptic soy broth, washed three times in distilled water, and standardized by

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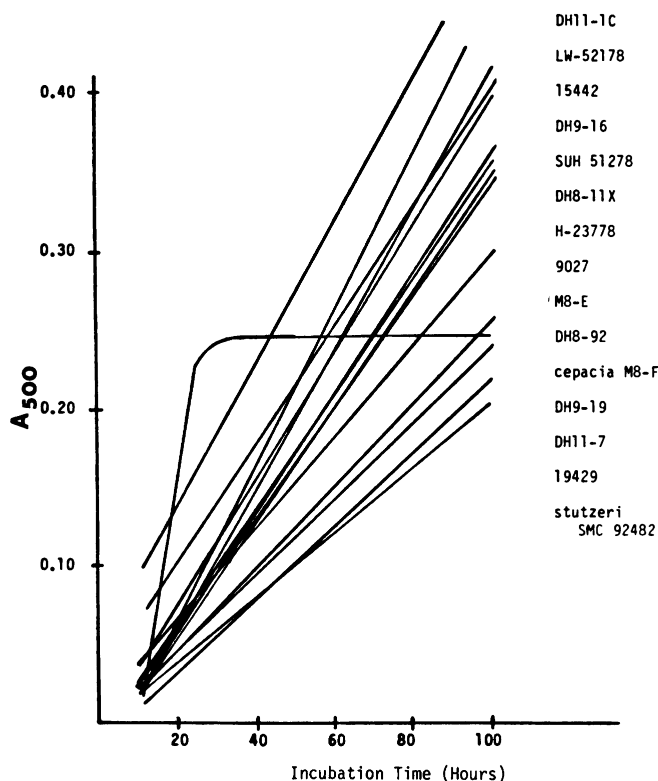

FIG. 1. Growth response of *Pseudomonas* strains in minimal salts medium containing phosphate and glucose at 32 to 34°C. *P. aeruginosa* strains designated by number only.

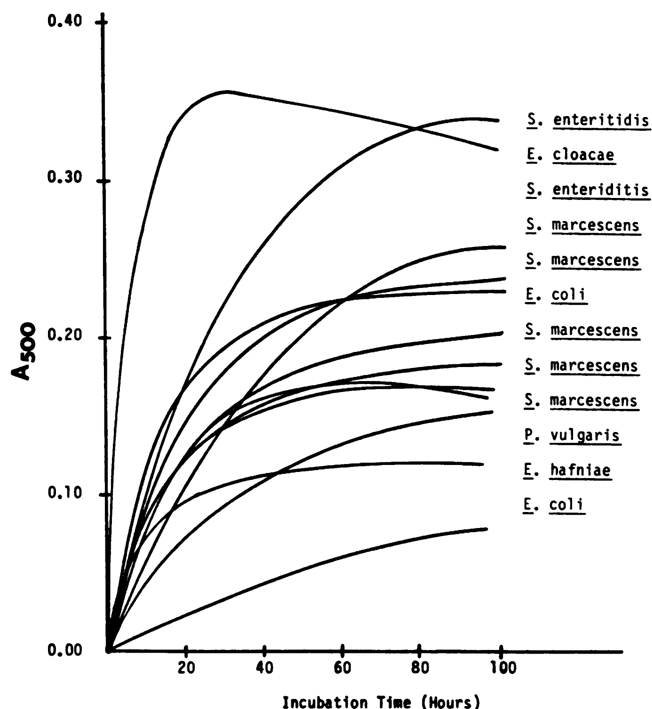

FIG. 2. Growth response of enteric bacteria in minimal salts medium containing phosphate and glucose at 32 to 34°C.

optical density measurements to give approximately 10^6 CFU/ml of sample. From 0.1 to 0.5 ml of the standardized suspension was then added to each 100-ml portion of minimal medium. The medium was incubated at 32 to 34°C for 28 days. Appropriate dilutions of each medium were subcultured in pour plates of nutrient agar at various times for 28 days. Growth or survival in each minimal medium was evaluated by the change in viable cell count.

RESULTS

The *Pseudomonas* strains and enteric bacteria grew well in the minimal salts media containing phosphate and glucose. Their growth response during the first 100 h of incubation is given in Fig. 1 and 2. The growth response of the staphylococci could not be measured because of their requirement of amino acid supplements. These supplements were purposely omitted so as not to interfere with the experiment. Thus, the minimal salts medium served merely to maintain the staphylococci.

The growth response of 13 strains of *Pseudomonas aeruginosa* was evaluated by log difference in CFU per milliliter over 28 days (Table 1). The 28-day interval was chosen in accordance with the industrial standard for preservative evaluation described by USP XXI. *P. aeruginosa* increased its growth by an average of 3.1 logs in the medium containing phosphate and glucose. The presence of phosphate alone resulted in a slight increase of 0.6 log, similar to the slight increase observed in the minimal medium alone or when combined with glucose. *P. aeruginosa* appears to require both a source of carbon and phosphate to proliferate in the minimal salts medium. When the phosphate buffer is replaced with a borate buffer, the *Pseudomonas* strains die slowly. Moreover, with the addition of a carbon source, glucose, to the borate-buffered minimal medium, the bacteria are reduced an average of 5.0 logs in 28 days. This reduction was in marked contrast to an average reduction of

TABLE 1. Growth response of *P. aeruginosa* strains in phosphate- and borate-buffered media[a]

P. aeruginosa strain	Growth differential[b]					
	Phosphate buffer	Phosphate buffer plus glucose	Borate buffer	Borate buffer plus glucose	No additives	Glucose only
9027	0.4	3.0	−0.6	−4.0	0.2	0.3
15442	0.4	2.7	−0.4	−3.5	0.0	1.5
SUH-51278	1.4	4.3	−5.0	−5.0	1.1	1.7
H-23778	0.9	3.4	−5.4	−5.4	1.2	2.2
M8-E	0.2	3.2	−1.0	−5.9	−0.2	0.6
LW-52178	0.4	3.4	−0.5	−5.8	−0.2	0.8
DH9-19	1.1	−5.6[c]	−2.0	−5.6	0.1	−1.3
DH8-11X	0.1	2.5	−4.2	−4.2	0.6	1.5
DH11-1C	−0.1	2.3	−1.0	−4.9	0.1	0.1
DH9-16	0.1	2.1	−0.5	−6.3	0.1	−0.1
DH8-92	2.0	3.8	−2.4	−4.4	1.6	1.6
DH11-7	0.7	2.9	−0.4	−4.5	0.3	0.2
19429	0.7	3.1	−0.4	−5.2	0.1	0.8
$\bar{X}$	0.6	3.1	−1.8	−5.0	0.4	0.8
SEM	0.2	0.9	−0.5	−1.4	0.1	0.2
Relative SEM (%)	30	29	28	28	29	28

[a] Buffers and glucose were added to minimal salts media; strains were grown 28 days at 32 to 34°C.

[b] Log differences are reported in CFU per milliliter on day 28 compared with day 0.

[c] Data excluded from calculations.

TABLE 2. Growth response of *P. cepacia* and *P. stutzeri* in phosphate- and borate-buffered media[a]

Pseudomonas species	Growth differential[b]					
	Phosphate buffer	Phosphate buffer plus glucose	Borate buffer	Borate buffer plus glucose	No additives	Glucose only
P. cepacia	−1.0	2.1	−2.3	−2.5	−2.5	−2.7
P. stutzeri	1.0	2.6	−5.3	−5.3	−5.3	−5.3

[a] Buffers and glucose were added to minimal salts media; bacteria were grown 28 days at 32 to 34°C

[b] Log differences are reported in CFU per milliliter on day 28 compared with day 0.

1.8 logs over that time in the absence of the glucose. The substrate-accelerated death (12) was observed for each strain of *P. aeruginosa* exposed to the borate buffer. However, the addition of glucose did not increase the death rate of *Pseudomonas cepacia* or *Pseudomonas stutzeri* (Table 2).

The 12 strains of enteric bacteria were more susceptible to the presence of borates than were the other bacteria (Table 3). They showed an average decrease of 5.1 to 5.4 logs after 28 days in the borate-buffered minimal medium. Although the effect of glucose could not be observed after 28 days of exposure, the intermittent recoveries of the enteric bacteria revealed that substrate-accelerated death did occur. Enteric bacteria in the unbuffered medium also appeared to die more rapidly when glucose was present, as shown by a mean decrease of 4.1 logs in the presence of glucose, in contrast to a mean increase of 0.31 log in the absence of glucose. The phosphate-buffered minimal medium provided the same degree of survival, except for *Enterobacter hafniae* (*Hafniae alvei*), that the unbuffered minimal medium did: after 28 days, there was a mean increase of 0.3 log in growth of

TABLE 3. Growth response of enteric bacteria in phosphate- and borate-buffered media[a]

Enteric bacteria species	Growth differential[b]					
	Phosphate buffer	Phosphate buffer plus glucose	Borate buffer	Borate buffer plus glucose	No additives	Glucose only
E. coli	1.9	−4.5	−4.5	−4.5	0.2	−3.9
E. coli	0.0	−0.3	−4.6	−5.8	0.2	−5.8
S. marcescens	−0.3	2.1	−5.9	−4.3	0.0	−6.3
S. marcescens	−0.1	2.7	−4.8	−6.2	−0.1	−6.2
S. marcescens	0.1	2.1	−5.9	−5.9	0.1	−5.9
S. marcescens	1.5	4.6	−4.6	−4.9	1.3	−2.1
S. marcescens	0.1	2.2	−5.9	−5.9	0.2	−5.9
P. vulgaris	1.5	−4.5	−4.5	−4.5	1.4	−4.5
S. enteritidis	−0.7	−6.0	−6.0	−6.0	0.0	−1.9
S. enteritidis	0.2	−6.2	−6.2	−6.2	−0.2	−6.2
E. hafniae	−0.1	−3.6	−5.8	−5.8	−5.8[c]	−0.2
E. cloacae	0.0	2.9	−2.0	−5.2	0.1	−1.2
$\bar{X}$	0.3	−0.7	−5.1	−5.4	0.3	−4.1
SEM	0.2	1.1	0.3	0.2	0.2	0.7
Relative SEM (%)	67	157	6	4	67	17

[a] Buffers and media were added to minimal salts media; bacteria were grown 28 days at 32 to 34°C.

[b] Log differences are reported in CFU per milliliter on day 28 compared with day 0.

[c] Data excluded from calculations

TABLE 4. Growth response of *Staphylococcus* spp. in phosphate- and borate-buffered media[a]

Staphylococcus species	Growth differential[b]					
	Phosphate buffer	Phosphate buffer plus glucose	Borate buffer	Borate buffer plus glucose	No additives	Glucose only
S. aureus	−6.3	−6.3	−6.3	−6.3	−6.3	−6.3
S. aureus	−6.2	−6.2	−6.2	−6.2	−6.2	−6.2
S. aureus	−6.2	−6.2	−6.2	−6.2	−6.2	−6.2
S. aureus	−6.2	−6.2	−6.2	−6.2	−6.2	−6.2
S. epidermidis	−5.9	−5.9	−6.2	−5.9	−5.9	−5.9
S. epidermidis	−6.2	−6.2	−6.2	−6.2	−6.2	−6.2
S. epidermidis	−6.0	−5.0	−6.0	−6.0	−6.0	−6.0
$\bar{X}$	−6.1	−6.0	−6.1	−6.1	−6.1	−6.1
SEM	0.1	0.2	0.1	0.1	0.1	0.1
Relative SEM (%)	1	3	1	1	1	1

[a] Buffers and glucose were added to minimal salts media; bacteria were grown 28 days at 32 to 34°C.

[b] Log differences are reported in CFU per milliliter on day 28 compared with day 0.

bacteria in both media. The addition of glucose to the phosphate-buffered medium gave mixed results. All strains proliferated during the first 100 h (Fig. 2), although survival for 28 days at levels above the initial concentration occurred for only 6 of the 12 strains tested. Of the six strains, five were different strains of *Serratia marcescens*. Significant decreases were observed for *E. coli*, *Proteus vulgaris*, *E. hafniae*, and two strains of *Salmonella enteritidis*.

The seven strains of staphylococci neither proliferated initially nor survived well during 28 days of exposure to any of the combinations of minimal media (Table 4). The recoveries made between 0 and 28 days indicated that staphylococci may die slightly sooner in the borate-buffered medium than in the phosphate-buffered or unbuffered media.

DISCUSSION

The purpose of this study was to investigate the suitability of a generic, borate-buffered vehicle as a preservative system for ophthalmic pharmaceutical agents. The duration of the study and cell recovery corresponded to terms specified by the USP preservative effectiveness test. Minimal media containing trace salts necessary for the growth of *Pseudomonas* strains, enteric bacteria, and maintenance of staphylococci were chosen to pose a worst-case challenge for a preservative system. The buffer strength, pH, and osmolality of the minimal media were adjusted to conditions found in many ophthalmic formulations.

Gram-negative rods, especially *P. aeruginosa* and *S. marcescens*, represent some of the most common contaminants of commercially produced eye care products. Many of these products are formulated in a phosphate buffer preserved with benzalkonium chloride. Others, designed for use with hydrogel contact lenses, are preserved with thimerosal, chlorhexidine, or sorbic acid. Inexpensive and widely available, these eye care products are often subjected to widespread consumer abuse. Their preservative systems are weak in comparison to those of topical products because of the extreme sensitivity of ocular tissues to chemicals in general. The borate-buffered vehicle investigated in this study appears better suited for use in ophthalmic products than phosphate-buffered formulations. All of the microor-

ganisms evaluated—15 *Pseudomonas* strains, 12 enteric bacteria strains, and 7 *Staphylococcus* strains—decreased in number when exposed to a borate-buffered minimal medium. Even in the presence of a carbon source, the viable cells decreased, often to a significant extent.

The lack of proliferation and survival of these microorganisms in a borate-buffered vehicle provides an increased level of safety for ophthalmic products. However, this preservative system does not, by itself, meet the criteria for effectiveness given by USP XXI. The kill rate of the vehicle is too slow, even at the elevated temperatures of 32 to 34°C. Thus, the borate buffer requires augmentation by other agents, probably at very low concentrations, to meet USP specifications. Furthermore, other ingredients in the formulation may interfere with the preservative system. Thus, the ingredients of each formulation must be balanced to achieve the desired kill rate.

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