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The preservation of ophthalmic solutions with antibacterial combinations

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Solutions of pilocarpine, atropine and physostigmine preserved with benzalkonium, chlorhexidine, phenylmercuric nitrate (PMN), chlorocresol and chlorbutol in simple solution and in combination with either phenylethanol or disodium edetate (EDTA), were contaminated on two separate occasions with high inocula of *P. aeruginosa*. Phenylethanol-antibacterial combinations were consistently effective in killing the inoculum within 15 min except for one formulation with chlorhexidine and physostigmine salicylate and formulations with PMN for which sterilization times ranged from 30 to 90 min.

Phenylethanol enhances the activity of benzalkonium, chlorhexidine, PMN, chlorocresol, chlorbutol and a hydroxybenzoate mixture against sensitive and resistant *Pseudomonas aeruginosa* (Richards, Suwanprakorn & others, 1969; Richards & McBride, 1971a; Richards, 1971). Furthermore, disodium edetate (EDTA) has been shown to enhance the activity of benzalkonium, chlorhexidine, chlorbutol, polymyxin and chlorocresol, but not PMN, against sensitive and resistant *P. aeruginosa* (Brown & Richards, 1965; Brown, 1968; Richards & McBride, 1971a; Richards, 1971).

We have evaluated the preservation of ophthalmic alkaloidal solutions against heavy contamination with *P. aeruginosa* using antibacterial agents alone and in combination with phenylethanol (0·4%) or EDTA (0·05%) as recommended by Richards (1971).

MATERIALS AND METHODS

Atropine sulphate B.P. was supplied by Koch Light Labs. Ltd., Colnbrook, benzalkonium chloride solution B.P., physostigmine sulphate B.P.C. by Macarthy Ltd., Glasgow, physostigmine salicylate B.P. by MacFarlane Smith,* Edinburgh, and chlorhexidine acetate from ICI. The disodium edetate (EDTA), chlorbutol, chlorocresol, phenylethanol, phenylmercuric nitrate (PMN) and pilocarpine hydrochloride were all BDH laboratory reagents.

The test organism was *P. aeruginosa* NCTC 6750 and Oxoid nutrient broth No. 2, the medium for liquid cultures with Oxoid nutrient agar for the solid cultures: incubation was at 37°. Cell numbers were estimated by colony counts as already described (Richards & others, 1969) and stock cultures maintained as before (Brown & Richards, 1964). The inactivating medium used to prevent any carry over of antibacterial action consisted of thioglycollate medium U.S.P. containing lecithin and polysorbate 80 (Riegelman, Vaughan & Okumoto, 1956).

* A gift from Dr. D. Ritchie.

Preparation of the solutions

The solutions were prepared according to the B.P.C. by mixing aliquots of concentrated solutions of the alkaloids, the various antibacterials and additives before diluting to volume. The pH of the solutions was measured before and after sterilization. The solutions containing either atropine sulphate or pilocarpine hydrochloride

Table 1. *pH after autoclaving (A) and resterilization time (B; min) for pilocarpine hydrochloride and atropine sulphate solutions* (1·0% *w/v*), *and physostigmine sulphate and salicylate* (0·25% *w/v*) *solutions contaminated with* P. aeruginosa *NCTC* 6750. Each solution was initially inoculated* and sampled, and later re-inoculated† and re-sampled. Samples for each solution were sterile in the tabulated time except for EDTA and the physostigmine salts.

Antibacterial(s) concentration(s) (% w/v)‡		Pilocarpine HCl		Atropine sulphate		Physostigmine sulphate		Physostigmine salicylate	
		A	B	A	B	A	B	A	B
Benzalkonium chloride	0·01	3·4	15	4·58	60	3·3	15	—	—
Benzalkonium chloride EDTA	0·01 0·05	3·75	15	4·47	15	3·58	15	—	—
Benzalkonium chloride phenylethanol	0·01 0·4	3·4	15	4·13	15	3·23	15	—	—
Chlorhexidine acetate	0·01	3·85	60	—	—	—	—	3·9	180
Chlorhexidine acetate EDTA	0·01 0·05	4·0	30	—	—	—	—	3·96	90
Chlorhexidine acetate phenylethanol	0·01 0·4	3·83	15	—	—	—	—	3·84	30
Phenylmercuric nitrate	0·002	3·75	240	4·32	24 h	3·38	90	3·87	90
Phenylmercuric nitrate EDTA	0·002 0·05	3·85	120	4·39	300	3·91	90	3·92	60
Phenylmercuric nitrate phenylethanol	0·002 0·4	3·6	60	4·23	90	3·3	45	3·73	45
Chlorocresol	0·05	3·58	45	4·27	30	3·62	30	3·89	45
Chlorocresol EDTA	0·05 0·05	4·0	45	4·3	15	3·61	45	3·94	30
Chlorocresol phenylethanol	0·05 0·4	3·85	15	4·13	15	3·57	15	3·78	15
Chlorbutol	0·5	2·25	15	2·42	15	3·22	45	3·44	30
Chlorbutol EDTA	0·5 0·05	2·23	15	—	—	3·3	60	3·56	60
Chlorbutol phenylethanol	0·5 0·4	2·25	15	2·45	15	3·1	30	3·41	15
EDTA	0·05	3·85	24 h	—	—	3·62	>300	3·8	>300
Phenylethanol	0·4	3·75	24 h	—	—	3·6	90	3·61	60

* Inoculations: $5{\cdot}2 \times 10^6$ orgs/ml $9{\cdot}8 \times 10^6$ orgs/ml $8{\cdot}4 \times 10^6$ orgs/ml $1{\cdot}2 \times 10^7$ orgs/ml
† Re-inoculations: $1{\cdot}6 \times 10^6$ orgs/ml $6{\cdot}8 \times 10^6$ orgs/ml $1{\cdot}2 \times 10^7$ orgs/ml $8{\cdot}4 \times 10^6$ orgs/ml
‡ Benzalkonium and phenylethanol % v/v.

were sterilized by heating at 115° for 30 min, and the solutions containing physostigmine sulphate or salicylate were heated at 98–100° for 30 min.

The determination of the sterilization times has already been described (Richards & McBride, 1971b). In the present determinations, a second inoculum was added to solutions some days after the first and the sampling procedure repeated.

RESULTS AND DISCUSSION

Table 1 shows benzalkonium and chlorbutol were the only two antibacterials, as simple solutions, to effect sterility of the contaminated pilocarpine solutions within 15 min. Phenylethanol-antibacterial agent combinations were all sterile within 15 min, with the exception of the combination with PMN which was sterile within 1 h. EDTA-antibacterial agent combinations were always as effective or more effective than the antibacterial agent in simple solution, but only with chlorbutol and benzalkonium was the EDTA-antibacterial combination as effective as the phenylethanol-antibacterial combination. In both of these formulations, however, the simple solution was effecting sterilization as rapidly as the combinations. Therefore the phenylethanol-antibacterial combination showed greater effectiveness in the pilocarpine hydrochloride formulations than the EDTA-antibacterial combination.

A similar pattern of results was obtained with atropine sulphate, except that benzalkonium with atropine had a longer sterilization time. The 60 min sterilization time for benzalkonium indicates either that the benzalkonium is less active in the presence of atropine sulphate than in the presence of the other two alkaloids, or that the inoculum into the atropine solutions contained cells of higher resistance to benzalkonium. However, both of the combinations with benzalkonium were effective within 15 min under the same conditions.

The results with physostigmine also show the phenylethanol-antibacterial combinations to be consistently effective, while benzalkonium was the only antibacterial in simple solution to have as fast a sterilization time.

Chlorbutol in simple solution has much slower sterilization times with physostigmine than with pilocarpine and atropine. This can be explained in terms of pH because pH both influences the activity of chlorbutol and is also affected by the heat treatment to which the chlorbutol is subjected. Heat treatment causes chlorbutol to break-down with the production of hydrochloric acid and consequent lowering of pH (Murphy, Allen & Mangiaracine, 1955; Riegelman & Vaughan, 1958). Therefore, autoclaved solutions would be expected to have a lower pH and consequently a greater antibacterial activity than steamed solutions. This is seen in the results in the Table. The susceptibility of chlorbutol to thermal decomposition, with the possibility of increased antibacterial activity is not, however, a desirable property.

EDTA-benzalkonium combinations are known to be effective against *P. aeruginosa* (Brown & Richards, 1965; Monkhouse & Groves, 1967), but the results in this present work show that EDTA-antibacterial combinations are not always effective. The EDTA-PMN combinations with physostigmine sulphate is no more effective than PMN alone and this agrees with Brown (1968) and Richards & McBride (1971a). The EDTA-chlorbutol combination has a longer sterilization time with both physostigmine salts than chlorbutol alone, although the pHs are similar. Richards (1971) also found that EDTA-chlorbutol combinations against resistant *P. aeruginosa* to be no more effective than chlorbutol alone. EDTA-chlorocresol combinations are also of doubtful benefit (Table 1). The mode of action of the antibacterial agent

and the state of resistance of the *P. aeruginosa* cells may be important factors in determining whether EDTA can enhance antibacterial activity against *P. aeruginosa.*

The slow sterilization time of 180 min for chlorhexidine in physostigmine solution again indicates that chlorhexidine can have reduced activity in final ophthalmic solutions when compared with its activity in simple solutions (Richards, 1964). Kohn, Gershenfeld & Barr (1963) found chlorhexidine in simple solution to have a sterilization time of 15 min against heavy contamination with *P. aeruginosa.* The sodium metabisulphite in the physostigmine solution may be reducing the effectiveness of the chlorhexidine which is known to be incompatible with sulphates (Stock, 1965).

Physostigmine salicylate with phenylethanol-chlorocresol or phenylethanol-chlorbutol combinations is preserved as effectively as physostigmine sulphate with benzalkonium alone or in combination. Therefore it is not necessary to limit the choice of salt to the sulphate for compatibility reasons. This makes physostigmine salicylate the salt of choice because, unlike the sulphate, it is not hygroscopic.

The double inoculum was used to investigate if any of the antibacterial agents were removed from the solution by the first heavy inoculum thus leaving the formulations inadequately protected against further contamination. The results, however, showed no significant difference in the sterilization times for the first and second inoculum.

The results, in conjunction with previous work (Richards & others, 1969; Richards & McBride, 1971a, b; Richards, 1971) support the use of antibacterial combinations in the preservation of ophthalmic solutions against contamination with *P. aeruginosa.* Phenylethanol 0·4%, in combination with the other antibacterial agents, has a potential usefulness in all the formulations tested.

Kohn & others (1963) considered that "An antibacterial substance which has a sterilizing time of greater than 1 hour may be arbitrarily considered to be too slow acting for use as a preservative in multidose ophthalmic preparations". We agree that 1 h is a reasonable time in which to expect the antibacterial agent to effect a kill of contaminating organisms. Our results, in combination with results already published (Richards & others, 1969; Richards & McBride, 1971a), show that it is possible by the use of phenylethanol-preservative combinations to achieve sterilization times within the hour for each of the five final ophthalmic preparations tested.

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