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# Comparative study on antagonistic effects of low pH and cation supplementation on in-vitro activity of quinolones and aminoglycosides against *Pseudomonas aeruginosa*

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The antagonistic effects of physiological levels of $Ca^{++}$ and $Mg^{++}$ on the in-vitro activity of aminoglycosides and quinolones against *Pseudomonas aeruginosa* were studied at both pH 7·4 and 5·5. Adding $Mg^{++}$ and $Ca^{++}$ (100 mg/l) to commercial media deficient of these cations increased the MICs and MBCs of ciprofloxacin and enoxacin four-fold ($2\ P < 0{\cdot}01$), which was significantly less than the 16-fold increase found for gentamicin and netilmicin ($2\ P < 0{\cdot}01$). However, the activity of both aminoglycosides and quinolones was similarly affected by reducing the pH to 5·5 (giving eight-fold increases in MICs) or by the combination of both low pH plus cation supplementation (giving 16-fold increases in MICs). These data raise the question whether antagonizing factors should be considered not only for aminoglycosides, but also for quinolones during routine susceptibility tests on *P. aeruginosa*.

## Introduction

Supplementation of culture media with $Ca^{++}$ and $Mg^{++}$ or serum has been shown to antagonize the in-vitro efficacy of both 4-fluoroquinolone and aminoglycoside antibiotics against *Pseudomonas aeruginosa* (Medeiros *et al.*, 1971; Sabath, 1982; Blaser *et al.*, 1986). Low pH also reduces the activity of both aminoglycosides and of those quinolones containing a piperazine group at their C7 position (Sabath, 1982; Smith & Ratcliffe, 1985). The combined effect of low pH plus cation supplementation was considered in this study to establish whether the presence of both antagonizing factors further reduced the antipseudomonal activity of aminoglycosides and quinolones.

Standard procedures for in-vitro testing suggest the use of cation supplemented broth only with aminoglycosides, and not, as yet, with quinolones. Comparative data should help to clarify whether antagonizing factors should be considered not only for aminoglycosides, but also for quinolones, during routine susceptibility tests of *P. aeruginosa*.

## Materials and methods

### *Strains and media*

The in-vitro activities of gentamicin, netilmicin, ciprofloxacin and enoxacin were studied against clinical isolates and strain ATCC 27853 of *P. aeruginosa*. Mueller–Hinton Broth (5 mg/l $Mg^{++}$, 3 mg/l $Ca^{++}$; Difco Laboratories, Detroit, Mich.), Nutrient Broth No. 2 (17 mg/l $Mg^{++}$, 3 mg/l $Ca^{++}$; Oxoid, Hampshire, England),



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Tryptic Soy Broth (24 mg/l $Mg^{++}$, 16 mg/l $Ca^{++}$; Oxoid), and Todd Hewitt Broth (18 mg/l $Mg^{++}$, 21 mg/l $Ca^{++}$; Oxoid) were used for sensitivity testing. For some tests these broths were supplemented after autoclaving and refrigerating with sterile calcium chloride and magnesium chloride to obtain concentrations of 100 mg/l $Mg^{++}$ and $Ca^{++}$ and/or the pH was adjusted to 5·5 by adding HCl. In further experiments, pooled human urine (77 mg/l $Mg^{++}$, 59 mg/l $Ca^{++}$) was added after pH adjustments to 7·4 and 5·5.

When adjustment of the pH to 5·5 was required this was done by adding 0·7–2·4 ml volumes of 1 N HCl solution to 100 ml of the broth. The pH adjustment increased the $Cl^-$ concentration from 108 to 114 mmol/l for Mueller–Hinton Broth, from 79 to 86 mmol/l for nutrient broth, from 82 to 104 mmol/l for tryptic soy broth and from 40 to 64 mmol/l for Todd Hewitt broth.

*Susceptibility tests*

Overnight cultures in Mueller–Hinton Broth were used to prepare inocula of a final bacterial concentration of $5 \times 10^6$ cfu/ml. MICs and MBCs were determined by the microdilution technique with culture volumes of 100 µl. The MIC was read as the lowest drug concentration inhibiting visible growth following 18–20 h of incubation at 37°C. The MBCs were defined as the concentration giving >99·9% reduction of the inoculum in a 2 µl transfer volume. In a limited number of experiments it was found that the results of sensitivity testing in cation- or pH-modified media were similar when the inocula were prepared from overnight cultures grown either in standard Mueller–Hinton Broth or in broth which was identical to that used in the sensitivity test itself.

## Results

Table I shows the effect of cation supplementation and pH reduction on the MICs of gentamicin and ciprofloxacin for ten strains of *P. aeruginosa* in Mueller–Hinton Broth. Adding $Mg^{++}$ and $Ca^{++}$ increased the MICs and MBCs of ciprofloxacin four-fold ($2\ P < 0{\cdot}01$), which was significantly less than the 16-fold increase found with gentamicin ($2\ P < 0{\cdot}01$). Reduction of the pH to 5·5 also increased the MICs and MBCs of both drugs ($2\ P < 0{\cdot}01$) in both cation-supplemented and unsupplemented

**Table I.** Antagonistic effects of cation supplementation and pH reduction on the in-vitro activity of gentamicin and ciprofloxacin against 10 strains of *P. aeruginosa*

| | Median (range) of MICs and MBCs (mg/l) measured in MHB[a] | | Median fold increase in MIC and MBC, compared to values in MHB, in: $MHB_S$[b] | $MHB_{5·5}$[c] | $MHB_{S,5·5}$[d] |
|---|---|---|---|---|---|
| Ciprofloxacin | | | | | |
| MIC | 0·125 | (0·06–0·5) | 4 | 8 | 16 |
| MBC | 0·5 | (0·125–1) | 4 | 8 | 16 |
| Gentamicin | | | | | |
| MIC | 0·5 | (0·125–4) | 16 | 4 | 16 |
| MBC | 1 | (0·25–16) | 16 | 8 | 16 |

[a] Mueller–Hinton Broth.
[b] Broth supplemented with 100 mg/l $Ca^{++}$ and $Mg^{++}$
[c] pH of the broth adjusted to 5·5.
[d] Broth supplemented with 100 mg/l $Ca^{++}$ and $Mg^{++}$, pH 5·5.

Mueller–Hinton Broth. In contrast to the antagonistic effect of cation supplementation at pH 7·4, the activity of both compounds was compromised similarly by pH reduction to 5·5 (giving an eight-fold MIC change) or by the combination of both low pH plus cation supplementation (giving a 16-fold increase in MIC).

These results were confirmed in experiments using three other commercial media (Table II). The combination of both antagonizing factors reduced the median activity of both ciprofloxacin and gentamicin by a factor of 16 (ranges 8–32- and 4–32-fold for the quinolone and the aminoglycoside, respectively). Data obtained in pooled human urine were similar to those determined in the supplemented broths, reflecting the relatively high concentrations of $Mg^{++}$ and $Ca^{++}$ (77 and 59 mg/l, respectively) in the urine.

The data obtained with ciprofloxacin and gentamicin were confirmed in sensitivity tests using other quinolone and aminoglycoside antibiotics. With enoxacin the median increase of MICs and MBCs in modified media was either the same, or differed by a factor of two, as compared to the results shown for ciprofloxacin in Table I. The median MICs and MBCs of netilmicin increased in the presence of the antagonizing factors similarly to those of gentamicin, shown in Table I. For both aminoglycosides the median changes in MICs were either identical or differed by a factor of two.

The antagonistic effect of $Mg^{++}$ and $Ca^{++}$ on the in-vitro activity of quinolones and aminoglycosides was concentration dependent and was most pronounced when both cations were present (Table III).

The antagonistic effects of cations and low pH were similar when MBCs, instead of MICs, were considered as endpoints. In 90% of the tests the MBCs were 2 two-fold dilution steps, or less, above the MICs.

The stability of pH was studied in 112 wells after incubation periods of 4 h and 20 h. A slight increase of the pH occurred within 4 h in all cultures with media of an initial pH of 5·5, including sterile and inoculated controls. In wells with aminoglycoside or quinolone concentrations between 0 and 64 mg/l the pH ranged from 5·6 to 5·8. After 20 h of incubation the pH in all turbid cultures ranged from 8·0 to 8·6, for both media with an initial pH of 5·5 or of 7·4. In contrast, the pH remained relatively stable at quinolone or aminoglycoside concentrations of 64 mg/l, ranging from 5·6 to 6·0 for the low pH media and from 7·3 to 7·8 for the standard media. These changes were similar to those observed after 20 h in wells with sterile culture media. However, in cultures with an initial pH of 5·5 the pH increased to 6·5 or more in all cultures with quinolone or aminoglycoside concentrations of eight times the MIC or less. As shown in Figure 1, remarkable pH changes occurred at concentrations of up to 64 times the MIC, suggesting some bacterial activity even in the presence of such high quinolone and aminoglycoside concentrations.

In summary, the pH remained relatively stable for more than 4 h in all cultures and for the entire incubation period in cultures with very high drug concentrations. At lower concentrations the pH gradually increased and pH values of more than 8·0 were measured in all turbid cultures. No differences were observed between quinolones or aminoglycosides with respect to the stability of the pH during MIC determinations.

## Discussion

Cation supplementation at pH 7·4 antagonized the antipseudomonal effect of quinolones less than that of aminoglycosides. However, the activity of quinolones and

**Table II.** Antagonistic effect of cation supplementation and pH reduction on in-vitro activity of ciprofloxacin and gentamicin against three strains of *P. aeruginosa* in five culture media

| | | Median MIC (mg/l) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | ciprofloxacin | | | | gentamicin | | | |
| Broth | $Ca^{++}/Mg^{++}$ in (mg/l) | B[a] | $B_s$[b] | $B_{5\,5}$[c] | $B_{s,5\,5}$[d] | B | $B_s$ | $B_{5\cdot5}$ | $B_{s,5\cdot5}$ |
| Mueller Hinton | 3/ 5 | 0·25 | 0·5 | 1 | 2 | 0·25 | 4 | 2 | 4 |
| Nutrient | 3/17 | 0·06 | 0·5 | 0·25 | 1 | 0·06 | 4 | 1 | 2 |
| Tryptic Soy | 16/24 | 0·12 | 1 | 1 | 4 | 1 | 8 | 4 | 4 |
| Todd Hewitt | 21/18 | 0·12 | 0·25 | 2 | 2 | 0·25 | 2 | 4 | 4 |
| Urine | 59/77 | 0·5 | ND[e] | 2 | ND | 4 | ND | 8 | ND |

[a]Broth, not modified
[b]Broth supplemented with 100 mg/l $Ca^{++}$ and $Mg^{++}$.
[c]pH of the medium adjusted to 5·5.
[d]Broth supplemented with 100 mg/l $Ca^{++}$ and $Mg^{++}$, pH 5·5.
[e]Not done.

Table III. Antagonistic effect of different cation supplementations on in-vitro activity of gentamicin and ciprofloxacin against eight strains of *P. aeruginosa*

| | Median (range) of MICs (mg/l) in unsupplemented MHB[a] | Median fold increase in MICs (mg/l) after supplementation of $Ca^{++}$ and $Mg^{++}$ according to concentrations of: | | | | |
|---|---|---|---|---|---|---|
| | | 100[b]/3[c] | 5/25 | 100/25 | 5/100 | 100/100 |
| Ciprofloxacin | 0·25 (0·06–1) | 2 | 2 | 2 | 4 | 4 |
| Enoxacin | 1·5 (0·25–8) | 1 | 1·5 | 2 | 2 | 6 |
| Gentamicin | 0·5 (0·25–8) | 16 | 3 | 32 | 16 | 32 |
| Netilmicin | 0·5 (0·25–32) | 32 | 4 | 32 | 16 | 64 |

[a]Mueller Hinton Broth with 5 and 3 mg/l of $Ca^{++}$ and $Mg^{++}$.
[b]Concentration of $Ca^{++}$ in mg/l.
[c]Concentration of $Mg^{++}$ in mg/l.

aminoglycosides was similarly affected by low pH or by the combination of cation supplementation plus low pH. Commercial media are deficient of $Mg^{++}$ and $Ca^{++}$ compared to conditions prevailing *in vivo* and the pH in infected sites and in urine is generally lower than in in-vitro cultures. Thus, standard in-vitro sensitivity tests may overestimate the in-vivo activity of quinolones against *P. aeruginosa* by a factor of 4- to 16-fold.

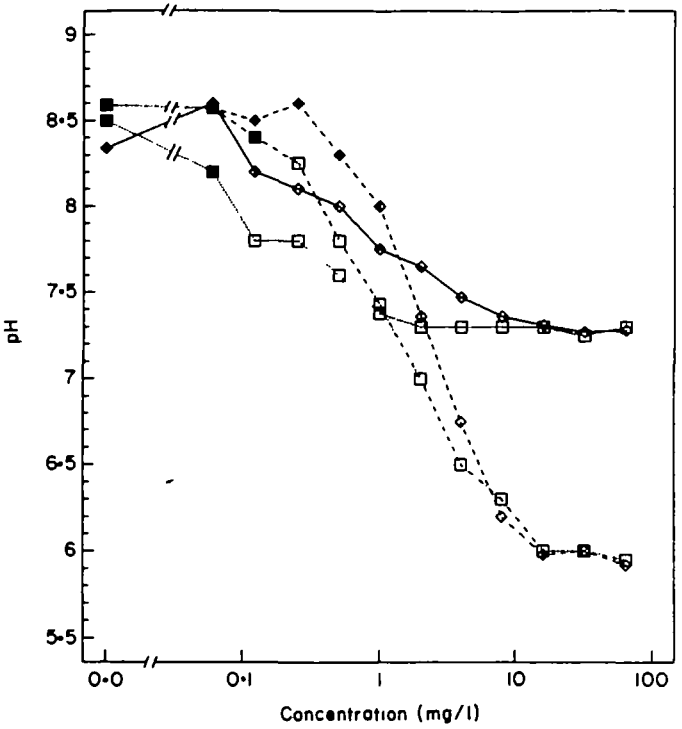


Figure 1. Effect of drug concentration on pH during MIC determinations of gentamicin and ciprofloxacin against *P aeruginosa* ATCC 27853, after an incubation period of 20 h. Regular Mueller–Hinton Broth, or Mueller–Hinton Broth with an initial pH adjusted to 5·5 was used. ◇———◇, Gentamicin 7 4 (initial pH), ◇ — — — ◇, gentamicin 5·5; □·····□, ciprofloxacin 7·4; □———□, ciprofloxacin 5 5 Solid symbols indicate cultures which were turbid after 20 h of incubation.

In areas of inflammation the local pH is always lower than the normal arterial pH of 7·42. In empyema fluid the pH is often 6–7, occasionally even lower (Sabath, 1982). In pus aspirated from 7-day-old experimental abscesses with *Staphylococcus aureus* a mean pH of 6·7 (range 6·2–7·0) was reported (Hays & Mandell, 1974). In the fluid of steel net cages implanted subcutaneously into rabbits and infected with Gram-negative rods the pH was 6·86 (Rylander *et al.*, 1981). In early morning urine specimens the pH is generally between 5 and 6 (Asscher *et al.*, 1966).

Mean concentrations of $Mg^{++}$ and $Ca^{++}$ in serum are 22 and 50 mg/l. In infected sites these concentrations may vary with time. In a rat 'granuloma pouch' model $Mg^{++}$ concentrations more than doubled over a period of 72 h after infection, whereas $Ca^{++}$ concentrations decreased to less than half (Davey & Barza, 1985).

Since high drug concentrations are reached in the urine, a 16-fold reduction of the activity determined in standard sensitivity tests may not be critical in the treatment of cystitis with quinolones, unless relatively low doses are administered. However, an over-estimation of quinolone activity by a factor of 4 or 8 may be relevant for the clinical treatment of systemic infections, particularly since most strains of *P. aeruginosa* are inhibited by quinolones *in vitro* at or near the susceptibility breakpoints. It has been shown that resistant subpopulations are rapidly selected during quinolone and aminoglycoside treatments which provide peak concentrations of less than eight times the MIC (Blaser *et al.*, 1987). This is consistent with reports on the emergence of resistance during quinolone therapy. This phenomenon mostly occurs in the clinical treatment of infections due to borderline sensitive species, such as *P. aeruginosa* and *Staph. aureus* (Wolff, M. *et al.*, 1985; Follath *et al.*, 1986; Scully *et al.*, 1986).

It has been proposed that the antagonistic effect of $Ca^{++}$ and $Mg^{++}$ on the activity of aminoglycosides is due both to an inhibition of ionic binding of the drug to an outer membrane site (possibly a phosphate site on the lipopolysaccharide) and to inhibition of the energy-dependent uptake of the drug (Damper & Epstein, 1981; Hancock, 1981; Nicas & Hancock, 1983). All aminoglycosides have a large net positive charge at a physiological pH due to the presence of multiple amino groups. Positively-charged molecules experience a strong driving force for entry into bacteria, which maintain a large electrical potential across their cytoplasmic membrane. The inside of the cytoplasmic membrane is electrically negative and alkaline, and the outside of the membrane is electrically positive and acidic. Damper & Epstein (1981) observed a decrease of the electrical potential difference following a reduction of the external pH. The results were consistent with the concept that a reduction of the extracellular pH increases the pH gradient, resulting in a compensatory decrease in the electrical membrane potential. The proton-motive force, being the sum of both of the above factors, remained virtually unchanged. A reduction in the electrical potential difference reduces the driving force for entry of the drug into the cell and thus could decrease the rate of aminoglycoside uptake.

It is tempting to postulate similar transport mechanism for the aminoglycosides and the quinolones studied in this investigation. The degree of ionization may explain the striking pH effect for both aminoglycosides and the piperazine-containing quinolones ciprofloxacin and enoxacin. However, the biochemical basis of the antagonistic effect of pH and cations on the activity of quinolones has not yet been studied in as much detail as have the effects on aminoglycosides.

Smith & Ratcliffe (1985) studied the effect of pH on the activity of ten 4-quinolone

antibacterials against *Escherichia coli* and observed two completely different responses, governed by the nature of the substituents at the C7 position of the 4-quinolone nucleus. With the more modern 4-quinolones with piperazine at C7 (e.g. ciprofloxacin, ofloxacin, or norfloxacin), pH reduction progressively decreased their antimicrobial activity. On the other hand, acrosoxacin, oxolinic acid, flumequine, cinoxacin, and nalidixic acid (drugs lacking piperazine at C7) became progressively more active as the pH fell. At a high pH all the 4-quinolones would tend to be negatively charged. It seems that negatively-charged piperazine-containing drugs, and neutral non-piperazine drugs are the most active species. In other words, the piperazine-containing drugs are least active when positively charged, but drugs lacking piperazine are least active when negatively charged. These conclusions suggest a major difference between the two groups of 4-quinolones, with respect either to their mode of action, or to their mechanism of penetration into bacteria (Smith & Ratcliff, 1985).

Standard in-vitro sensitivity testing of quinolones neglects the antagonizing effects of cations, pH or hypoxia. The role of these antagonizing factors in predictions of in-vivo efficacy for the treatment of infections caused by *P. aeruginosa* remains to be established. It is conceivable that host defence mechanisms, either alone or in combination with subinhibitory concentrations of quinolones or aminoglycosides, may be sufficient to achieve clinical and microbiological cure. Some in-vitro studies suggest such positive interactions between leucocytes and quinolones or aminoglycosides (McDonald, Wetherall & Pruul, 1981; Blaser, Gilbert & Zinner, 1988; Schlaeffer *et al.*, 1988). However, the data obtained in this study lead to questions as to whether it is correct to account for antagonistic factors with aminoglycosides but not with quinolones. In particular, such antagonistic effects may be of consideration in the development of sensitivity breakpoint values.

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## References

Asscher, A. W., Sussman, M., Waters, W. E., Davis, R. H. & Chick, S. (1966). Urine as a medium for bacterial growth *Lancet ii*, 1037–41.

Blaser, J., Dudley, M. N., Gilbert, D. & Zinner, S. H. (1986). Influence of medium and method on the in vitro susceptibility of *Pseudomonas aeruginosa* and other bacteria to ciprofloxacin and enoxacin. *Antimicrobial Agents and Chemotherapy* **29**, 927–9.

Blaser, J., Gilbert, D. & Zinner, S. H. (1988). Effect of enoxacin with and without leukocytes against *Staphylococcus aureus* in a pharmacokinetic model. *Reviews of Infectious Diseases* **10**, *Suppl. 1*, 33–4.

Blaser, J., Stone, B. B., Groner, M. C. & Zinner, S. H. (1987). Comparative study with enoxacin and netilmicin in a pharmacodynamic model to determine importance of ratio of antibiotic peak concentration to MIC for bactericidal activity and emergence of resistance. *Antimicrobial Agents and Chemotherapy* **31**, 1054–60.

Damper, P. D. & Epstein, W. (1981). Role of the membrane potential in bacterial resistance to aminoglycoside antibiotics. *Antimicrobial Agents and Chemotherapy* **20**, 803–8.

Davey, P. & Barza, M. (1985). *Programme and Abstracts of 25th Interscience Conference on Antimicrobial Agents and Chemotherapy*, Abstract 574.

Follath, F., Bindschedler, M., Wenk, M., Frei, R., Stalder, H. & Reber, H. (1986). Clinical efficacy of ciprofloxacin in *Pseudomonas* infections. In *Proceedings of the 1st International Ciprofloxacin Workshop, Leverkusen, 1985,* (Neu, H. C. & Weuta, H., Eds), pp. 411–3. Excerpta Medica, Amsterdam.

Hancock, R. E. W. (1981). Aminoglycoside uptake and mode of action—with special reference to streptomycin and gentamicin. I. Antagonists and mutants. *Journal of Antimicrobial Chemotherapy* **8,** 249–76.

Hays, R. C. & Mandell, G. L. (1974). $pO_2$, pH, and redox potential of experimental abscesses. *Proceedings of the Society for Experimental Biology and Medicine* **147,** 29–30.

McDonald, P. J., Wetherall, B. L. & Pruul, H. (1981). Post-antibiotic leukocyte enhancement: increased susceptibility of bacteria pretreated with antibiotics to activity of leukocytes. *Reviews of Infectious Diseases* **3,** 38–44.

Medeiros, A. A., O'Brien, T. F., Wacker, W. E. C. & Yulug, N. F. (1971). Effect of salt concentration on the apparent in-vitro susceptibility of *Pseudomonas* and other gram-negative bacilli to gentamicin. *Journal of Infectious Diseases* **124,** *Suppl.,* S59–64.

Nicas, T. I. & Hancock, R. E. W. (1983). Alteration of susceptibility to EDTA, polymyxin B and gentamicin in *Pseudomonas aeruginosa* by divalent cation regulation of outer membrane protein H1. *Journal of General Microbiology* **129,** 509–17.

Rylander, M., Brorson, J. E., Holm, S. E. & Norrby, R. (1981). Studies on some variables influencing aminoglycoside efficacy in vivo and in vitro. *Scandinavian Journal of Infectious Diseases* **13,** 217–25.

Sabath, L. D. (1982). Antagonism of antimicrobial agents by products of inflammation. In *Action of Antibiotics in Patients* (Sabath, L. D., Ed.) pp. 74–83. Hans Huber Publishers, Berne.

Schlaeffer. F., Laxon, J., Blaser, J. & Zinner, S. H. (1988). Ciprofloxacin enhanced leukocyte killing of drug induced resistant bacteria. *Reviews of Infectious Diseases* **10,** *Suppl. 1,* 32–3.

Scully, B. E., Neu, H. C., Parry, M. F. & Mandell, W. (1986). Oral ciprofloxacin therapy due to Pseudomonas aeruginosa. *Lancet i,* 819–22.

Smith, J. T. & Ratcliffe, N. T. (1985). Activity of the 4-quinolone antibacterials at physiological pH values. In *Recent Advances in Chemotherapy, Antimicrobial Section 2. Proceedings of the 14th International Congress of Chemotherapy, Kyoto, 1985* (Ishigami, J., Ed.), pp. 1861–2. University of Tokyo Press, Tokyo.

Wolff. M., Pathe, J. P., Paugon, B., Bure, A., Reigner, B. & Vachon, F. (1985). *Programme and Abstracts of 25th Interscience Conference on Antimicrobial Agents and Chemotherapy,* Abstract 654.

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