

Interaction Between Acetohydroxamic Acid and 12 Antibiotics Against 14 Gram-Negative Pathogenic Bacteria

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Received for publication 9 July 1973

Acetohydroxamic acid (AHA) is a potent inhibitor of urease which prevents alkalization of urine and stone formation in rats in the presence of infection caused by urease-producing bacteria. Because an antibacterial effect of AHA, and synergy between kanamycin and AHA have also been described, we studied the interaction between AHA and 12 antibiotics against 14 gram-negative bacteria. Synergy, sometimes to a striking degree, was found in 17% of interactions; however, antagonism was detected in 5%. Infecting organisms would need to be studied individually before the antibacterial effect of AHA and an antibiotic could be predicted.

Acetohydroxamic acid (AHA) has been shown to be an effective inhibitor of bacterial urease in vitro (1, 2, 5, 5b). Administration of AHA to rats greatly reduced the degree of stone formation in urinary infections caused by *Proteus mirabilis* (5a). These data suggest that AHA may be useful in treating chronic urinary tract infections that are accompanied by urolithiasis.

Studies of antibacterial synergy have been prompted by two sets of observations: (i) AHA has bacteriostatic effects against many pathogenic gram-negative bacteria (5b); (ii) synergy between hydroxamic acids and kanamycin for several strains of *Proteus* has previously been described (5). In the present paper we present data on the interaction between AHA and 12 commonly used antibiotics against 14 pathogenic gram-negative bacteria.

MATERIALS AND METHODS

Organisms. Fourteen gram-negative pathogenic bacteria that had been isolated from patients with urinary tract infections were selected from the Diagnostic Microbiology Laboratory, V.A. Hospital, Houston. These included the following: five strains of *Proteus* (two of *morganii* and one each of *mirabilis*, *rettgeri*, and *vulgaris*); three of *Pseudomonas aeruginosa*; two each of *Escherichia coli* and *Klebsiella*; and one each of *Enterobacter* and *Providencia*. Disk sensitivity testing showed each organism to be resistant to several antibiotics.

Culture medium. Bacteria were grown in Trypticase soy broth (TSB). After overnight growth approximately 10^9 colony-forming units were present. All antibiotic sensitivity testing was carried out using TSB.

AHA synthesis and chemical determinations.

AHA was synthesized in our laboratory as described by Fishbein et al. (3).

Antibiotics. Standard antibiotics for in vitro sensitivity testing were graciously provided by pharmaceutical companies. Antibiotic concentrations were chosen to include levels that might be achieved in the urine of patients on standard dosages. The range of concentrations studied for each drug was as follows: nalidixic acid, 6 to 12,500 $\mu\text{g/ml}$; carbenicillin, 5 to 10,000 $\mu\text{g/ml}$; ampicillin, cephalothin, and streptomycin, 2.5 to 5,000 $\mu\text{g/ml}$; kanamycin, 1.25 to 2,500 $\mu\text{g/ml}$; gentamicin, tetracycline, and tobramycin, 1 to 2,000 $\mu\text{g/ml}$; colymycin, 0.6 to 1,250 $\mu\text{g/ml}$; chloramphenicol and clindamycin, 0.5 to 1,000 $\mu\text{g/ml}$.

Antibiotic testing and synergy. Antibiotic susceptibility was assayed by serial twofold dilutions in TSB with an inoculum of 10^8 viable organisms/ml. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of antibiotic which prevented turbidity of the broth after 18 h. Subcultures were made at 18 h by streaking 0.02 ml of nonturbid suspensions onto brain heart infusion (BHI) agar; the minimum bacterial concentration (MBC) was reported as the lowest concentration at which no bacteria grew.

To study synergy or antagonism by AHA, this compound was added to TSB to give a final concentration of 1.95 mg/ml, a level which would be attainable in human urine in the presence of relatively normal renal function (6, 8). Synergy or antagonism was said to be present if at least a two-tube (fourfold) difference in MIC or MBC was observed in the presence of AHA. Control tubes containing broth, 10^8 organisms, and 1.95 mg of AHA per ml were included for every organism studied; using these methods this concentration of AHA was not bacteriostatic.

In the initial study standard twofold dilutions were used. Because of the large number of assays involved (12 antibiotics, 12 dilutions, and 14 bacterial strains)

a microtiter system was developed. Disposable microtiter plates and dispensing pipettes (Cooke Engineering Co.) were rinsed in 95% ethanol and shaken dry. Antibiotic solution (50 μ liters) was distributed into the first well and 25 μ liters of TSB were dropped into the 2nd through 12th wells. Serial dilutions were carried out with 25- μ liter prong dilutors, after which 25 μ liters of TSB containing about 2×10^8 bacteria with and without AHA (3.9 mg/ml) was dropped into every well in alternate rows. The final concentration of bacteria was about 10^6 /ml and the final concentration of AHA was 1.95 mg/ml. Plates were covered with transparent tape and incubated overnight at 37 C. The MBC was determined by culturing 0.01 ml in the same manner as for tube dilutions.

At first this microtiter method was used simultaneously with the tube dilution studies. Simultaneous studies were repeated several times with the same organisms and antibiotics; the microtiter system was found to give reliable, reproducible results that varied by no more than one tube from the standard tube dilutions. In later studies the microtiter method was used exclusively.

RESULTS

In comparing the effect of AHA and an antibiotic on various bacteria to that of the antibiotic alone, four kinds of results may be observed: (i) synergy; (ii) antagonism; (iii) no difference; or (iv) indeterminate (seen when a bacterium is so susceptible that it is killed by the lowest concentration of the antibiotic present, or so resistant that it grows at the highest concentration).

Results of studies with 12 antibiotics and 14 gram-negative organisms are summarized in Table 1. Of 168 observations on MIC values, synergy was noted 32 times and antagonism 7. In 96 cases the addition of AHA did not affect the MIC. The remaining 33 studies gave indeterminate results. Similar data were obtained for MBC values. There were 24 instances of synergy and 9 of antagonism. In 91 cases there was no difference and in 44 results were indeterminate.

Synergy was seen more frequently with carbenicillin, chloramphenicol, clindamycin, and gentamicin than with other drugs (Table 2). The synergistic effect was usually of modest degree. However, with some antibiotics, synergism was relatively high grade and may have made the difference between effective and non-effective antimicrobial concentrations (Fig. 1). A striking degree of synergy was detected with some bacteria; one strain of *P. aeruginosa* appeared to be particularly sensitive to AHA in combination with most antibiotics studied (Fig. 2).

Isolated instances of antagonism were observed with many of the antibiotics studied

TABLE 1. Interaction between AHA and 12 antibiotics against 14 gram-negative bacteria

Effect	Occurrence of effect	
	MIC ^a	MBC ^b
Synergy	32 (19) ^c	24 (14)
Antagonism	7 (4)	9 (5)
No difference	96 (57)	91 (55)
Indeterminate	33 (20)	44 (26)

^a MIC, minimum inhibitory concentration.

^b MBC, minimum bactericidal concentration.

^c Numbers in parentheses indicate percentages.

(Table 2). When addition of AHA produced antagonism, the difference in MIC or MBC never exceeded three dilutions (an eightfold difference).

The interaction between AHA and four antibiotics (ampicillin, chloramphenicol, kanamycin, and tetracycline) was studied in urine using four *Proteus* (one each of *mirabilis*, *morganii*, *rettgeri*, and *vulgaris*). Urine was provided by a healthy volunteer and used for serial tube dilutions at the initial pH (range 5.4 to 5.7) or after alkalization by adding 2 N NaOH (final pH 8.5). Tube dilutions using the same antibiotics were also carried out simultaneously in TSB. The pH of all tubes was measured after the MIC was read, and 0.01 ml was removed for subculture. As would have been expected, ampicillin was less effective and kanamycin more effective in alkaline urine. Adding together the data for MIC and MBC, synergy between an antibiotic and AHA was observed in acid urine in nine, alkaline urine in two, and TSB in five instances. The difference between results in acid and alkaline urine was chiefly related to the effect of kanamycin; in five instances a high degree of synergy was detected in acid urine whereas in alkaline urine the kanamycin was 10 to 100 times as effective and synergy was not observed. There were two instances of antagonism in these studies, one each with gentamicin and kanamycin.

DISCUSSION

The hydroxamic acids are specific inhibitors of bacterial urease (1, 2, 5, 5b). Of the congeners studied to date, AHA appears to have the greatest pharmacological potential (2). It is rapidly and completely absorbed from the gastrointestinal tract of the experimental animals (4, 6) and man (7, 8). One-half to two-thirds of an administered dose is excreted unchanged into the urine (4), in the presence of normal renal function (7). Rats have been given 500 mg per kg per day for periods of time up to 3

TABLE 2. Interaction between each antibiotic and AHA against fourteen gram-negative bacteria

Antibiotic	Synergy		Antagonism		No difference		Indeterminate	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Ampicillin	2	2	0	0	9	8	3	4
Carbenicillin	5	2	0	0	4	7	5	5
Cephalothin	1	1	0	0	10	10	3	3
Chloramphenicol	4	3	0	0	9	9	1	2
Clindamycin	3	4	0	0	9	5	2	5
Colistin	2	2	1	1	3	1	8	10
Gentamicin	5	3	0	1	3	4	6	6
Kanamycin	1	0	2	2	9	10	2	2
Nalidixic acid	1	1	1	2	12	11	0	0
Streptomycin	3	1	1	1	10	10	0	2
Tetracycline	2	2	1	1	10	9	1	2
Tobramycin	3	3	1	1	8	7	2	3

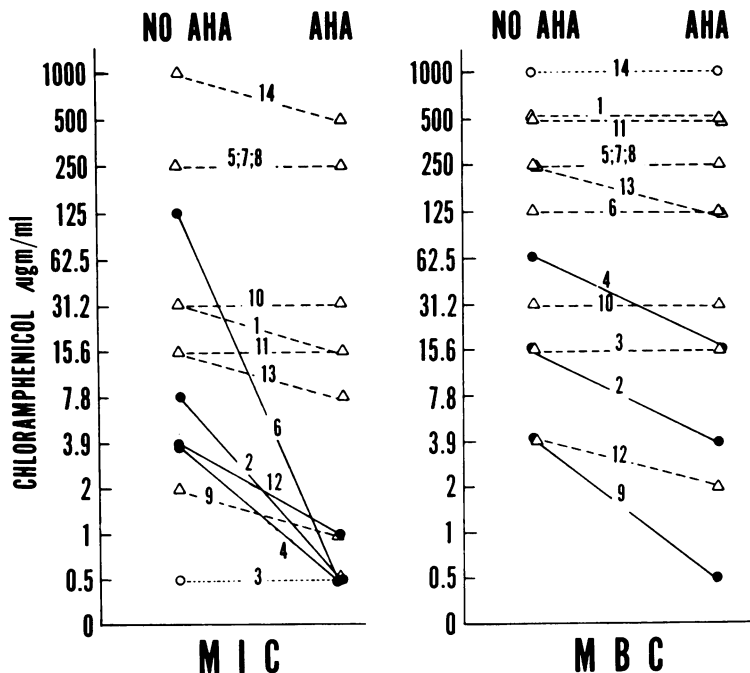


FIG. 1. Interaction between AHA and chloramphenicol against 14 gram-negative pathogens. Symbols: ●, synergy; Δ, no difference; ○, indeterminate. Antagonism between chloramphenicol and AHA was not observed with these 14 bacteria. Organisms are numbered as follows: 1, *Proteus mirabilis*; 2, *Proteus morganii* #1; 3, *Proteus morganii* #2; 4, *Proteus rettgeri*; 5, *Proteus vulgaris*; 6, *Pseudomonas aeruginosa* #1; 7, *Pseudomonas aeruginosa* #2; 8, *Pseudomonas aeruginosa* #3; 9, *Escherichia coli* #1; 10, *Escherichia coli* #2; 11, *Klebsiella* #1; 12, *Klebsiella* #2; 13, *Enterobacter*; 14, *Providencia*.

months without adverse effects (2). Up to 150 mg per kg per day have been administered to human subjects with hepatic coma in an attempt to reduce absorption of ammonia from the gut with no obvious toxicity (7, 8).

We have shown previously (5b) that AHA has two effects in vitro which are of potential pharmacological importance: (i) by blocking

the effect of bacterial urease AHA prevents alkalization of urine by *Proteus* species; (ii) it is bacteriostatic for many common pathogenic gram-negative bacteria. In vivo, AHA greatly inhibits the formation of bladder and renal stones in rats that have urinary infection caused by *P. mirabilis* (5a).

Using 12 antibiotics and 14 gram-negative

pathogens in TSB we observed a synergistic effect between AHA and an antibiotic in 56 of 336 observations (17%). A modest degree of antagonism was seen in 16 instances (5%). Similar results were obtained when the interaction between AHA and four antibiotics against four species of *Proteus* was studied in urine. Instances of synergy and antagonism were found with many of the antibiotics and nearly all of the organisms. In contrast with the finding of Gale (5), synergy was not detected more frequently with kanamycin nor was antagonism found more frequently with ampicillin. The reason for this apparent discrepancy is not understood. It may be attributed to the use of: (i) different strains of *Proteus*; (ii) medium with a relatively low pH; (iii) different assay techniques (Gale incorporated the hydroxamates into medium and used disk sensitivities or, alternatively, measured turbidity in broth over 7 h to demonstrate synergy); or (iv) different hydroxamic acids (Gale

did not study AHA and although five of the six hydroxamic acids that he used produced similar effects, three were isomers of alanyl hydroxamic acid).

We have previously suggested (5a) that AHA or a related hydroxamic acid might prevent further deposition of calculus in patients whose urinary tract is infected with urease-producing bacteria. Such pharmacological control of urinary alkalinity and ammonia production may make it possible to treat infected urinary stones by medical means. The data presented here suggest that in certain cases a synergistic effect with commonly used antibiotics may have a part in the pharmacological potential of AHA. Because antagonism was also observed in 5% of interactions, each infecting organism would need to be examined individually before the antibacterial effect of AHA and the antibiotic could be predicted.

Gale (5) proposed that the synergy resulted

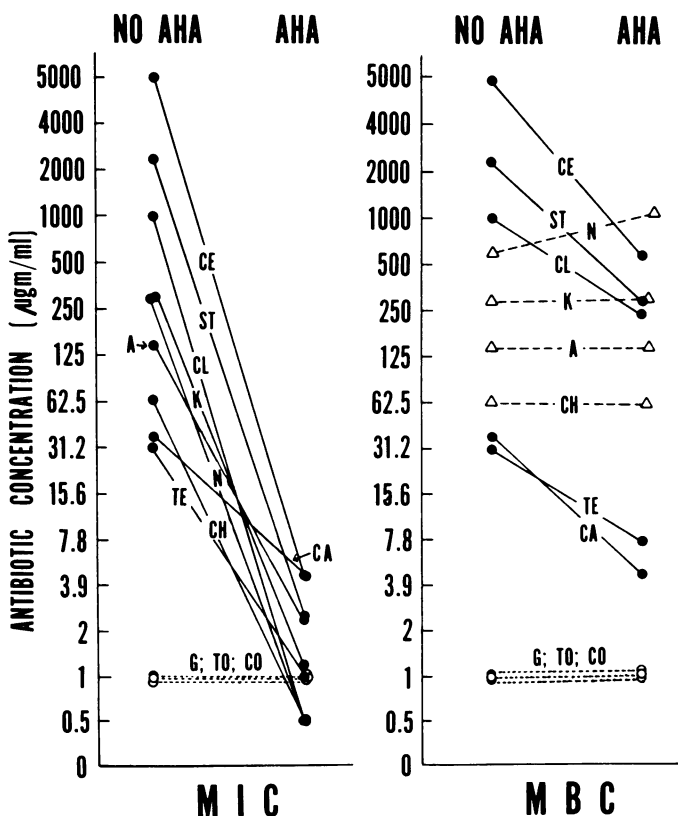


FIG. 2. Synergistic effect between AHA and 12 antibiotics against a sensitive *Pseudomonas aeruginosa*. Symbols: ●, synergy; Δ, no difference; ○, indeterminate. Antagonism against this organism was not observed. Antibiotics are as follows: A, ampicillin; Ca, carbenicillin; Ce, cephalothin; Ch, chloramphenicol; Cl, clindamycin; Co, colymycin; G, gentamicin; K, kanamycin; N, nalidixic acid; St, streptomycin; Te, tetracycline; To, tobramycin.

from increased penetration of hydroxamic acids into bacterial cells in the presence of aminoglycosides. Although this explanation would be consistent with our observation that AHA exerts a dose-related antibacterial effect against a number of gram-negative pathogenic bacteria, it fails to explain antagonism between AHA and kanamycin that we observed in two instances, or the dependency of the synergistic interaction upon pH.

ACKNOWLEDGMENT

We are indebted to R. R. Martin for his critical review of the manuscript.

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