A reappraisal of the antibacterial action of cotrimoxazole *in vitro*

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SYNOPSIS The *in-vitro* action of the sulphamethoxazole/trimethoprim combination (cotrimoxazole) against coliforms, *Proteus* spp, and *Staphylococcus aureus* was re-examined. In nutrient broth the components of cotrimoxazole did not produce bacteriostatic synergy against most sulphonamide-resistant and a significant proportion of sulphonamide-sensitive strains. Any bactericidal action appeared to be due to the trimethoprim component. After addition to urine the combination was only bacteriostatic against most coliforms and *Proteus* spp over six hours. Further clinical trials are needed to determine the circumstances under which cotrimoxazole is superior to either trimethoprim or sulphonamides against some infections, particularly of the urinary tract.

When a combination of sulphamethoxazole with trimethoprim (cotrimoxazole) was introduced in 1968, its chief advantage was thought to be the antibacterial synergism between the two components (Darrell, Garrod, and Waterworth, 1968). This synergism was found to produce not only a more effective bacteriostatic effect (bacteriostatic synergy) when the drugs were used together rather than singly, but often a bactericidal activity (Darrell *et al*, 1968; Bushby, 1969). However, trimethoprim alone has been found to be as effective as the combination in treating urinary infections in outpatients (Brumfitt and Pursell, 1972), and has some bactericidal activity *in vitro* (Darrell *et al*, 1968). These findings prompted us to re-examine the activity of sulphamethoxazole and trimethoprim *in vitro*. We have already reported some results of the bactericidal action over 24 hr (Lewis and Lacey, 1973). Here we have extended our observations that in nutrient broth the bacteriostatic synergy is unreliable and we have found that any bactericidal action resulting from the combination seems to be due to the trimethoprim component. Furthermore, cotrimoxazole is rarely bactericidal in urine. We therefore suggest that the advisability of invariably combining the drugs in treatment should be subjected to further clinical evaluation. Combined therapy increases the cost and the danger of toxic effects (Brumfitt and Pursell, 1972) and presents problems for the laboratory in sensitivity testing.

Materials and Methods

ORGANISMS

Cultures of coliforms, *Proteus* spp, and *Staphylococcus aureus* were obtained from infected material of hospital and non-hospital patients. Care was taken to avoid replicate isolates of the same strain. Approximately 70% of the coliforms were *E. coli*, 20% *Klebsiella* spp, and 10% *Enterobacter* spp. Since the relevant properties of these three organisms were similar, the group will be referred to as 'coliforms'. *E. coli* strain CN 314 was supplied by Burroughs Wellcome, Beckenham, Kent. All the bacteria were sensitive to trimethoprim (MIC < 1.6 µg/ml) but of variable sulphonamide sensitivity. Resistance to sulphonamides was defined as MIC > 200 µg sulphamethoxazole/ml.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

Sulphamethoxazole and trimethoprim were incorporated in doubling dilutions singly or in combination (usually in a ratio of 1 part trimethoprim: 20 parts sulphamethoxazole) into plates containing Oxoid diagnostic sensitivity test agar with the addition of 5% (v/v) lysed horse blood. The plates were spot-inoculated with 10-20 colony-forming units of each culture and then incubated for 24 hr at 37°C. The endpoint was defined as the concentration that completely inhibited growth. Bacteriostatic synergy was defined as a four-fold or more reduction in the level of trimethoprim required to inhibit growth on

the addition of sulphamethoxazole. For example, if the growth of a culture was inhibited by 1 μg trimethoprim/ml or 20 μg sulphamethoxazole/ml, synergy would be indicated by inhibition of growth by 0.25 μg trimethoprim combined with 5 μg of sulphamethoxazole/ml. (A two-fold reduction denotes additive antibiotic action rather than synergy.)

Sulphamethoxazole and trimethoprim lactate were obtained from Burroughs Wellcome, Beckenham, Kent.

DETERMINATION OF MINIMUM BACTERICIDAL CONCENTRATIONS

Sulphamethoxazole and/or trimethoprim were added generally to final levels of 32 times that of the MIC to broths (Oxoid no. 2) containing 5% (v/v) lysed horse red cells and stored overnight at 4°C. After prewarming to 37°C, each was then inoculated with an overnight broth culture of the test organism to a final cell density of 10^4 /ml. The broths were sampled immediately and then at intervals by spreading a loopful (0.01 ml) on nutrient agar plates by the method of Darrell *et al* (1968). Several cultures were also sampled by spreading 0.025 ml over the surface of nutrient agar plates. These methods of sampling were adopted (a) to dilute out residual drugs, (b) to enable an easy check on the presence of contaminants to be made, and (c) to make our results comparable with those of Darrell *et al* (1968). The broths were considered to be free of sulphonamide and trimethoprim inhibitors because some cultures of *Staph. aureus* were destroyed by as little as 0.4 μg trimethoprim/ml or 0.4 μg trimethoprim/ml and 8 μg sulphamethoxazole/ml (for example see table IV). Sulphamethoxazole alone was bacteriostatic at levels as low as 8 μg /ml. Effective bactericidal action was defined as the reduction in the number of viable bacteria to 10% or less of the inoculum. In cultures that were not completely killed by the drugs, a proportion of the surviving organisms were checked for their identity.

BEHAVIOUR OF ORGANISMS IN HUMAN URINE

Twenty-four-hr collections of midstream urine from a healthy individual were sterilized by filtration and then pooled. Sulphamethoxazole (1 mg/ml) and trimethoprim (50 μg /ml) were added to 25 ml aliquots (producing little change in pH) and then bacteria were added to a final density of between about 10^3 and 10^4 organisms/ml. The inoculated urine was immediately sampled for viable bacteria (Miles and Misra, 1938) on MacConkey agar and then after two, four, six, and 24 hours' incubation at 37°C. All experiments were done in duplicate, and the arithmetic means of the results were calculated.

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Counts on several of the inoculated urine samples were confirmed by an independent worker. In other experiments, urine was collected from each of two healthy subjects on the third day of a course of treatment with cotrimoxazole (two tablets Septrin, Burroughs Wellcome, twice a day), pooled, and then treated as above. Controls included urine inoculated with the same bacteria but containing no antibacterial agents; counts of viable organisms in these were usually between 10^7 and 10^9 /ml after 24 hr at 37°C.

Results

BACTERIOSTATIC SYNERGY IN NUTRIENT AGAR

The components of cotrimoxazole were not synergistic against nearly all sulphonamide-resistant coliforms and staphylococci, and a considerable minority of sulphonamide-sensitive coliforms, staphylococci, and *Proteus* spp (table I).

Organism	Reaction to Sulphonamide ¹	No. Tested	Percentage Showing Bacteriostatic Synergy ²
<i>Staphylococcus aureus</i>	R	14	7
	S	45	85
Coliforms	R	56	7
	S	48	75
<i>Proteus</i> spp	S	10	60

Table I *Bacteriostatic synergy between sulphamethoxazole and trimethoprim against strains of Staphylococcus aureus, coliforms, and Proteus spp*

¹R = resistant (MIC > 200 μg /ml), S = sensitive

²Synergy defined in Methods

BACTERICIDAL ACTION OF COTRIMOXAZOLE IN NUTRIENT BROTH

The reliability of the method was demonstrated by examining the bactericidal action of cotrimoxazole

Organism	Reaction to Sulphonamide ¹	No. Tested	No. Cultures Destroyed by Cotrimoxazole at ²		
			2Hr	4Hr	6Hr
<i>Staphylococcus aureus</i>	R	6	2	4	5
	S	9	2	8	9
<i>Proteus</i> spp	S	10	0	0	0
<i>Escherichia coli</i>	R	7	0	0	0
	S	10	0	1	1
<i>Klebsiella</i> spp	S	1	0	0	0
<i>Enterobacter</i> spp	S	2	0	0	0

Table II *Bactericidal action of cotrimoxazole against strains of Staphylococcus aureus, coliforms, and Proteus spp in nutrient broth*

See footnote to table I

Bactericidal action defined in Methods

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on three strains each of *Staph. aureus*, *E. coli*, and *Proteus* spp on between three and six occasions. Results were similar for all the strains when tested at two, four, and six hours. But at 24 hr results were sometimes variable. To see if this variation at 24 hr could be due to resistant mutants occurring in some cultures and not in others, the MICs of colonies from the 24-hr cultures were compared with those of the wild strains. There was no significant increase of resistance to the combination.

The bactericidal action of cotrimoxazole was variable towards different species; 14 out of 15 isolates of *Staph. aureus* were efficiently destroyed by six hours. None of the 10 *Proteus* isolates and only one of 20 coliforms showed destruction to < 10% of the inoculum (table II). The coliform that was efficiently destroyed was the strain CN 314. Although results of viable counts at 24 hr for individual strains were rather variable, they were generally, similar to those at six hours. Out of 50 coliforms

Organism and Strain No.	Drug	Reaction to Sulphonamide	Viable Bacteria/ml at				
			0 Hr	2 Hr	4 Hr	6 Hr	24 Hr
<i>S. aureus</i> 1073	Trimethoprim	S	5×10^8	3×10^8	1×10^1	0	1×10^8
<i>S. aureus</i> 1073	Cotrimoxazole		4×10^8	3×10^8	1×10^1	0	0
<i>S. aureus</i> 1931	Trimethoprim	S	$> 10^4$	4×10^8	0	0	$> 10^8$
<i>S. aureus</i> 1931	Cotrimoxazole		$> 10^4$	6×10^8	4×10^1	0	6×10^8
<i>S. aureus</i> 609	Trimethoprim	S	2×10^8	2×10^8	0	0	1×10^8
<i>S. aureus</i> 609	Cotrimoxazole		1×10^8	2×10^8	3×10^8	8×10^8	0
<i>S. aureus</i> 2273	Trimethoprim	R	2.5×10^8	8×10^1	0	0	0
<i>S. aureus</i> 2273	Cotrimoxazole		3×10^8	7×10^1	0	0	$> 10^8$
<i>S. aureus</i> 53	Trimethoprim	R	3×10^8	1×10^8	8×10^1	0	0
<i>S. aureus</i> 53	Cotrimoxazole		2.5×10^8	9×10^8	0	0	1×10^1
<i>S. aureus</i> 11164	Trimethoprim	R	3×10^8	4×10^8	3×10^8	1×10^1	2×10^8
<i>S. aureus</i> 11164	Cotrimoxazole		3×10^8	5×10^8	3×10^8	0	$> 10^8$
<i>S. aureus</i> 56	Trimethoprim	R	4×10^8	8×10^8	1.2×10^8	1.4×10^8	6×10^8
<i>S. aureus</i> 56	Cotrimoxazole		6×10^8	3×10^8	2×10^1	0	0
<i>E. coli</i> CN 314	Trimethoprim	S	4×10^8	0	4×10^1	4×10^1	0
<i>E. coli</i> CN 314	Cotrimoxazole		$> 10^4$	3×10^8	3×10^8	2×10^8	8×10^8

Table III Comparison of the rate of destruction of seven strains of *Staphylococcus aureus* and of one *E. coli* by trimethoprim (1.6 µg/ml) and by cotrimoxazole (1.6 µg trimethoprim and 32 µg sulphamethoxazole/ml)

Strain No. (Reaction to Sulphonamide)	Concentration (µg/ml) of		No. of Colonies per Plate ¹ at				
	Sulphamethoxazole	Trimethoprim	0 Hr	2 Hr	4 Hr	6 Hr	24 Hr
R3 (resistant)	8	0.4	64	80	50	> 200	C ^a
	32	0.4	19	23	130	52	C
	8	1.6	52	62	26	37	C
	32	1.6	18	32	49	8	C
	128	1.6	63	110	19	130	C
	32	6.4	> 39	40	> 200	185	SC ^a
	128	6.4	69	143	183	> 200	135
	512	6.4	33	75	> 200	60	> 200
	128	25.6	54	78	85	70	SC ^a
	256	12.8	16	38	> 200	86	SC ^a
	1024	12.8	70	86	50	133	61
	256	51.2	50	46	120	> 200	SC
1073 (sensitive)	8	0.4	> 200	121	10	1	90
	32	0.4	> 200	100	12	0	65
	8	1.6	> 200	92	26	1	150
	32	1.6	> 200	73	9	4	90
	128	1.6	> 200	70	6	1	0
	32	6.4	> 200	73	7	1	0
	128	6.4	> 200	66	9	1	0
	512	25.6	> 200	90	24	1	0
	256	12.8	> 200	118	14	1	1
	1024	12.8	> 200	103	10	2	0
	256	51.2	> 200	98	10	3	0

Table IV Effect of various concentrations and proportions of sulphamethoxazole and trimethoprim on a strain of *E. coli* (R3) and a strain of *Staphylococcus aureus* (1073) in nutrient broth

¹A standard loopful (0.01 ml) was spread over the surface of agar plates.
C^a = confluent; SC^a = semi-confluent

Isolate No.	Organism	Sulphonamide Sensitivity ¹	Viable Counts (organisms/ml) at				
			0 Hr	2 Hr	4 Hr	6 Hr	24 Hr
1	<i>Enterobacter</i> spp	S	3×10^3	4×10^3	3×10^3	1×10^3	3×10^3
2	<i>Enterobacter</i> spp	S	9×10^3	9×10^3	1×10^4	7×10^3	1×10^3
3	<i>Klebsiella aerogenes</i>	S	8×10^3	1×10^4	1×10^4	8×10^3	1×10^4
4	<i>E. coli</i>	S	1×10^4	1×10^4	1×10^4	6×10^3	3×10^3
5	<i>E. coli</i>	R	2×10^4	2×10^4	2×10^4	2×10^4	8×10^3
6	<i>E. coli</i>	R	9×10^3	8×10^3	9×10^3	7×10^3	7×10^3
7	<i>E. coli</i>	R	3×10^3	6×10^3	6×10^3	2×10^3	2×10^3
8	<i>E. coli</i>	R	9×10^3	9×10^3	7×10^3	8×10^3	5×10^3
9	<i>E. coli</i> (strain CN 314)	S	3×10^4	2×10^4	3×10^4	3×10^4	6×10^2
10	<i>Proteus</i> spp	S	5×10^4	5×10^4	5×10^4	6×10^4	0
11	<i>Proteus</i> spp	S	6×10^4	6×10^4	4×10^4	4×10^4	0

Table V Effect of cotrimoxazole (1 mg sulphamethoxazole, 50 µg trimethoprim/ml) upon clinical isolates of Enterobacteriaceae in urine

¹See footnote to table I

Isolate No	Organism	Sulphonamide Sensitivity ¹	Subject	Viable Counts (organisms/ml)				
				0 Hr	2 Hr	4 Hr	6 Hr	24 Hr
1	<i>Enterobacter</i> spp	S	R.W.L.	1.2×10^4	1.0×10^4	5.2×10^4	4.6×10^3	0
2	<i>Enterobacter</i> spp	S	R.W.L.	1.2×10^5	1.1×10^5	5.0×10^4	2.6×10^4	8.0×10^2
3	<i>Klebsiella aerogenes</i>	S	R.W.L.	9.0×10^4	7.0×10^4	3.4×10^4	1.7×10^4	4.0×10^2
4	<i>Escherichia coli</i>	S	R.W.L.	7.0×10^4	7.6×10^4	3.2×10^4	2.4×10^4	8.8×10^3
5	<i>E. coli</i>	R	R.W.L.	5.6×10^4	8.4×10^4	7.8×10^4	6.8×10^4	9.6×10^3
6	<i>E. coli</i>	R	J.D.A.	2.4×10^4	2.3×10^4	2.2×10^3	4.0×10^2	0
7	<i>E. coli</i>	R	J.D.A.	8.4×10^4	7.6×10^4	3.2×10^4	1.3×10^4	0
8	<i>E. coli</i>	R	J.D.A.	4.4×10^4	5.0×10^4	4.8×10^4	2.6×10^4	0
9	<i>E. coli</i> strain CN 314	S	J.D.A.	1.5×10^5	1.1×10^5	7.6×10^3	3.8×10^3	2.4×10^3
10	<i>Proteus</i> spp	S	J.D.A.	5.4×10^4	6.8×10^4	4.8×10^4	1.3×10^4	0
11	<i>Proteus</i> spp	S	J.D.A.	6.4×10^4	6.2×10^4	7.0×10^4	2.9×10^4	0

Table VI Effect of urine obtained from healthy individuals receiving a course of treatment with cotrimoxazole upon various clinical isolates of Enterobacteriaceae

¹See footnote to table I

only seven were efficiently destroyed (to $< 10\%$) at 24 hr, and out of 38 staphylococci 34 were destroyed. The 14 staphylococci and the one coliform killed by cotrimoxazole within six hr were also destroyed by trimethoprim alone at the same concentration ($1.6 \mu\text{g/ml}$) as that used in cotrimoxazole. Results for seven of the staphylococci and the one *E. coli* strain to be destroyed are shown in table III. Sulphamethoxazole alone was always bacteriostatic. It therefore seems that the bactericidal activity of cotrimoxazole is due to the trimethoprim component. Alteration in the ratio of trimethoprim to sulphamethoxazole or increasing the concentrations of the drugs more than 10-fold had little influence on the bactericidal activity of the mixture, at least over six hr (table IV).

ACTIVITY OF COTRIMOXAZOLE IN URINE

The activity of cotrimoxazole in urine was found to be mainly bacteriostatic. Of nine coliforms and two *Proteus* spp tested, all survived in undiminished numbers for six hr in urine containing cotrimoxazole, regardless of their sulphonamide sensitivity (table V).

The two *Proteus* isolates, but none of the coliforms, were destroyed in 24 hours.

Urine obtained from healthy individuals receiving therapeutic doses of cotrimoxazole also produced very little bactericidal action within six hr, although some bactericidal activity was found at 24 hr (table VI).

Discussion

Reasons for the differences in our results from those obtained by other workers are uncertain, since the methods used for some of the experiments were similar. The discrepancy in bactericidal activity of the combination for our strains and those of Darrell *et al* (1968) could be due to differences in strains (the latter tested only four strains for bactericidal effect). Strain differences may also account for the discrepancy between our findings and those of Bushby (1969), because the only coliform (strain CN 314) that we have found to be reliably destroyed within six hr by cotrimoxazole was obtained from his laboratory.

One advantage claimed for using a mixture of trimethoprim and sulphamethoxazole is in the prevention of resistance of bacteria to both drugs simultaneously. The value of this effect, however, is less than it might appear, because (1) many coliforms, *Proteus* spp, and staphylococci are already resistant to sulphonamides; (2) trimethoprim-resistant mutants with normal growth properties are difficult to isolate *in vitro*, even in strains already resistant to sulphonamides except for *Strep. faecalis* (Lewis and Lacey, 1973); (3) trimethoprim resistance has remained relatively uncommon in clinical strains, including those resistant to sulphonamides (Lacey, Gillespie, Bruten, and Lewis, 1972; Lewis and Lacey, 1973); (4) if resistance to trimethoprim does become common in coliforms, we expect that it will be mediated by R factors—one such R factor that determines resistance to trimethoprim also confers resistance to sulphonamides and has recently been detected in strains isolated in London (Fleming, Datta, and Grüneberg, 1972).

We do not dispute that combinations of trimethoprim and sulphamethoxazole are of value in treating some infections. The two drugs show bacteriostatic synergy against many organisms. But the use of the combination when a bactericidal agent is called for,

and the efficiency of the combination in preventing the acquisition of drug resistance, calls for further clinical evaluation.

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