Zag. J. Pharm. Sci., Vol. 3, No. 2, August 1994

INADEQUANCY OF MODIFIED CHECKERBOARD TECHNIQUE TO DETECT ANTAGONISM BETWEEN NITROFURANTOIN AND NALIDIXIC ACID AGAINST ESCHERICHIA COLI

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ABSTRACT

The *in vitro* combined antibacterial effect of nitrofurantoin and nalidixic acid was assessed using seven strains of *Escherichia coli*. Checkerboard broth dilution methods for determining bacteriostatic or bactericidal effects as well as the agar diffusion method were used. The simplified checkerboard broth dilution method did not only fail to detect antagonism, but conversely demonstrated partial synergism. Agar diffusion method (paper strip test) and the viable count tests clearly demonstrated an antagonism between the two drugs. Such antagonism could apparently be attributed to partial protection of cells, by nitrofurantion-induced bacteriostasis, from the lethal effect of nalidixic acid. Failure of checkerboard method to detect this antagonism is probably due to its inability to assess bactericidal effect.

INTRODUCTION

Different methods have been used for measurement of combined antibacterial action[1,2]. The checkerboard (chessboard) method is the technique most frequently used, because it is easy to understand and for the simplicity of mathematics necessary to calculate and interpret the results. The better clinical outcome of synergistic combinations based on checkerboard technique criteria is considered as a further advantage[1]. Agar diffusion methods, on the other hand, are simple and easy to perform but are not quantitative.

The combined antibacterial activity of nitrofurantoin and nalidixic acid as determined by agar diffusion methods was reported to be antagonistic against Klebsiella aerogenes, Proteus morganii and Proteus mirabilis.[2]. However, there was an apparent increase in the inhibition area[3]. Although this apparent potentiation of the drug combination had been explained by the authors to be due to inhibition of swarming of the organism to the nitrofurantoin inhibition zone. Yet it was felt that other methods and other test organisms are required to assess this combination of drugs. In the present study, the drug combination was assessed by different methods, including the most favoured checkerboard method, against E. coli, in order to conclude the combined antimicrobial activity of both drugs. Moreover, the work aims also to check the reliability of the modificed checkerboard technique in assessing the combind antimicrobial activity.

EXPERIMTENAL

Chemicals :

Nalidixic acid was the product of Sigma (Poole, Dorest, England) while nitrofurantoin (Macrodantin) was a market product of Kahira Pharmaceutical & Chemical Industrial Company, Egypt.

Media :

Nutrient broth used in all experiments contained (in one litre) : peptone (Oxoid), 5.0 g; sodium chloride, 5.0 g; and beef extract (Difco laboratories, Detroit), 3.0 g; pH 6.8-7.2. Nutrient agar was prepared from nutrient broth by hardening with 1.2% w/v agar (Oxoid).

Microorganisms :

Seven strains of Escherichia coli were used in this study. These strains comprised four local isolates (No. 2,3,4,5) and three standard strains namely ATCC 14169 (No. 1), ATCC 10536 (No. 6) and ATCC 8739 (No. 7).

Solutions of antimicrobial agents :

Nitrofurantoin and nalidixic acid were dissolved in the minimum volume of 0.01 N NaOH, pH was adjusted to 7.2 with 0.01 N HCl and distilled water was added to make the required volume. Antimicrobial solutions were sterilsed by filtration and used freshly prepared.

Determination of MIC :

Two-fold serial dilution technique was used. Five ml portions of nutrient broth were inoculated with each 0.1 ml of ten fold dilution of an overnight culture of test organism and incubated at 37°C for 48 h.

Determination of combined bacteriostatic effects of broth dilution :

The simplified checkerboard technique as described before[1] was used with slight modification. Portions (5 ml) of nutrient broth medium containing either a single drug or both in varying concentrations,were inoculated each with 50µl of overnight culture of the test organism and incubated at 37°C. Results were recorded after 24 and 48 hrs.

Determination of the fractional inhibitory concentration (FIC):

The FIC interaction index was calculated according to the formula of Hallander et al.(1982)[4].

Determination of combined antibacterial effect by agar diffusion method :

The paper strip test technique[2] was used. Filter paper strips, impregnated with either nitrofurantoin (2000 µg/ml) or nalidixic acid (1000 µg/ml) solutions were placed at right angle to one another on the surface of nutrient agar medium seeded with the test organism (0.1 ml culture/10 ml nutrient agar). The plates were inverted and incubated for 3 days and the inhibition zone pattern was observed daily.

Determination of bactericidal activity:

The viable count tests of previously recorded methods[1,2] were used. Portions (10 ml) of nutrient broth containing varying concentrations of either drug or both (in combination) were inoculated with an overnight culture of the test organism to a final level of approximately 10^6-10^8 CFU/ml and incubated at 37°C. At zero time and as convenient, samples were taken for viable counting by surface viable counting as previously described[5].

RESULTS

MICs of nalidixic acid (NAL) and nitrofurantoin (NF) against the seven strains of E. coli were determined. Results after 24 hrs are presented in Table 1.

Table (1): The MIC (µg/ml) of nalidixic acid (NAL) and nitrofuranoin (NF) against E. coli after 24 hrs.

Strain #	NF	NAL
1	300	> 100
2	200	30
3	200	30
4	300	20
5	200	10
6	200	10
7	200	30

Preliminary agar diffusion tests demonstrated a slight antagonistic effect, between nalidixic acid and nitrofurantoin against strains # 2,3,4, and 7. This antagonism was noticed in the form of reduction in the width of nalidixic acid inhibition zone at the junction with nitrofurantoin strip.

Results of the combined inhibitory effect of the two drugs against strains # 2,3,4 and 7 in broth medium are presented in Table 2.

Strains # 4 and 7 were used in viable count tests to assess the combined bactericidal effect. The combined bactericidal effect of nalidixic acid-nitrofurantoin combinations against strains # 4 and 7 as compared with both drugs are presented in Table 3 and 4, respectively.

DISCUSSION

Nitrofurantoin and nalidixic acid are synthetic antibacterial agents used as urinary antiseptics[6]. As assessed by agar diffusion method, the interaction between both drugs was antagonistic against Klebsiella aerogenes and Proteus morganii[1]. Against Proteus mirabilis, however, it was not possible to detect such an antagonism and on the contrary, there was an apparent potentiation between the two drugs[3]. Although the authors found an explanation for such apparent potentiation, the detection of antagonism could not have been possible relying solely on

Table (2): Growth inhibitory effect of nalidixic acid (NAL) and nitrofurantoin (NF), singly or in combination, against E. coli.

Drug conc. in µg/ml		E. coli 2		E. coli 3		E. coli 4		E. coli 7	
NAL	NF	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
10	-	+	+	+	+	+	+	+	+
20	-	+	+	+	+	-	-	+	+
40	-	-	-	-	-	-	-	-	+
-	100	(-)	+	+	+	+	+	-	+
-	200	-	-	-	+	+	+	-	+
-	400	-	-	-	+	-	-	-	-
2.5	50	NT	NT	NT	NT	NT	NT	-	-
5	50	NT	NT	NT	NT	+	+	-	+
5	100	-	-	NT	NT	NT	NT	-	-
10	100	-	-	-	+	-	+	+	+
10	200	-	-	-	+	NT	NT	-	-
20	200	-	-	NT	NT	-	-	-	(-)
20	400	NT	NT	-	+	NT	NT	NT	NT

+ = Growth. - = No growth. (-) = Very scanty growth. NT = Not tested.

Table (3): Bactericidal * activity of nalidixic acid (NAL) and nitrofuantoin (NF), singly or in combination, against E. coli No. 4 in nutrient broth medium.

Drug treatment / Time in (h)	NAl, 30 µg/ml	NF, 50 µg/ml	NAL, 30 µg/ml NF, 50 µg/ml	NAL, 10 µg/ml	NF, 300 µg/ml	NAL, 10 µg/ml + NF, 300 µg/ml
0	1.5×10^7	1.5×10^7	1.5×10^7	1.5×10^7	1.5×10^7	1.5×10^7
2	1.0×10^6	1.56×10^8	2.9×10^6	1.12×10^6	3.0×10^7	0.7×10^7
4	1.7×10^2	ND	1.06×10^3	ND	ND	3.2×10^5
24	2.4×10^2	1.88×10^9	$< 1 \times 10^3$	0.7×10^5	1.6×10^9	2.9×10^6
48	3.1×10^2	G	<10	1.5×10^3	G	G

* Expressed as viable count, CFU/ml. ND = Not determined. G = Growth obseved.

Table (4): Bactericidal * activity of nalidixic acid (NAL) and nitrofurantion (NF) singly or in combination against E. coli No. 7 in nutrient broth medium.

Drug treatment / Time in (h)	NAl, 40 µg/ml	NF, 400 µg/ml	NAL, 40 µg/ml NF, 400 µg/ml
0	2.5×10^6	2.5×10^6	2.5×10^6
2	$< 1 \times 10^5$	3.7×10^6	4.6×10^6
4	1.7×104	3.1×106	2.4×106
24	1.6×10^2	$< 1 \times 10^5$	1.2×10^3

* Expressed as viable count, CFU/ml.

agar diffusion method. In the present study, a similar situations was found. Using the simplified checkerboard technique recommended by many authors[1] the antagonism between nalidixic acid and nitrofurantion was not possible to be detected except in only one combination of drugs, against strain # 7, and only after 48 hr of incubation where the criteria of antagonism as interpreted according to Kerry et al. (1975)[7] were fulfilled and the fractional inhibitory concentration (FIC) index was 1.5. With the same combination after 24 hr, other combinations against the same organism or the remaining combinations against other organisms, however, the combined effect reaction could be interpreted as additive and indifferent (as with strains # 3 and 4 where FIC index was between 0.7-1.3) or even synergistic (as with strains # 2 and 7 after 24 hr, where FIC index was less than 0.7).

On the other hand, agar diffusion method demonstrated a slight antagonistic effect between the two drugs against 3 strains namely # 2,4,7. Furthermore, the results of bactericidal activity experiments revealed such antagonism between the two drug. In case of strain # 7 the bactericidal activity of nalidixic acid (40 µg/ml) was slightly reduced by the high concentration of nitrofurantoin (400 µg/ml). As for strain # 4 at the first 24 hr, low concentration of nitrofurantoin (50 µg/ml) has slightly reduced the bactericidal activity of high concentration of nalidixic acid (30 µg/ml).

However, the overall viable number after 48 hr was less in the presence of nitrofurantion than in its absence. Meanwhile, the slow bactericidal activity of low concentration of nalidixic acid (10 µg/ml) was slightly reduced by high concentration of nitrofurantoin (30 µg/ml) in the first 24 hr and growth took place in the nalidixic acid nitrofurantoin combination tube after 48 hr. The observation that antagonism was only evident in bactericidal study and agar diffusion, but not in checkerboard method can be attributed to the fact that the latter method measures only the bacteriostatic effect, whereas the other methods assess the bactericidal effect alone (viable count tests) or the net effect of bacteriostatic and bactericidal effects (paper strip test). This leads to the belief that the antagonism is related to the bactericidal rather than the bacteriostatic effect. Viable count results suggest that nitrofurantoin by vertue of bacteriostasis exerted some protective effect on E. coli from the bactericidal effect of nalidxic acid.

Similar phenomenon was reported for combinations of nalidixic acid with either chloramphenicol, rifampicin[8] or chloracetamide[9]. Moreover, the observation that the inhibition zone of nalidixic acid was more reduced than that of nitrofurantoin at the junction of the two strips may support the above hypothesis.

From the outcome of the present study as well as other studies[3] it can be concluded that relying on single method for judging the combined antimicrobial action of two drugs may be erraneous and the results of more than one method involving bacteriostatic and bactericidal studies may be required to confirm antagonism or synergism. Furthermore, the study does confirm a slight antagonistic effect of nitrofurantoin on the bactericidal activity of nalidixic acid against E. coli. The clinical significance of this antagonism awaits further in vivo studies.

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اخفاق طريقة الشطرنج المعدله فى كشف التأثير التضادى لمادتى النيتروفيورانتوين وحامض الناليديكسيك على ميكروب ايشيرشيا القولون

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بدراسة الفعل البكتيرى المضاد لمادتى حامض الناليديكسيك والنيتروفيورانتوين معا أو فرادى على كل من ٧ سلالات من ميكروب ايشيرشيا القولون ثلاث منها سلالات نمطيه وأربعة عزلات محلية وباستخدام الطرق المختلفة وجد أن مادة النيترفيورانتوين لها تأثير سلبى على فعل حامض الناليديكسيك المضاد للبكتريا وهذا التأثير أمكن اظهاره باستخدام طرق الانتشار فى المستنبت الصلب وكذلك بتتبع عدد الخلايا الحيه بينما يصعب اكتشافه بطريقة الشطرنج المعدله باستخدام الشوربه والتى هى أكثر استخداما حيث يبدو التأثير الاجمالى كحاصل جمع تأثير المادتين فرادى أو يكون مساويا لأقواهم وقد يعزى ذلك إلى أن هذه الطريقة تقيس فقط التأثير التثبيطى بينما يتم التضاد على التأثير القاتل لمادة حامض الناليدكسيك.

وبهذه النتيجة ومن نتائج سابقة يتبين أن الاعتماد على طريقة واحدة فقط لمعرفة الفعل البكتيرى المضاد لمادتين معا قد يؤدى الى استنتاج خاطىء تماما ويلزم تجربة أكثر من طريقه لمعرفة التفاعل الصحيح.