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Potential Unreliability of Nitrofurantoin Disks in Susceptibility Testing

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The Kirby-Bauer disk diffusion antimicrobial susceptibility test was studied with three lots of commercial nitrofurantoin disks by using 50 strains of *Proteus mirabilis*. One lot of disks consistently gave zone diameters 3 to 6 mm larger than the other two and resulted in a substantial number of *Proteus mirabilis* strains being erroneously categorized as sensitive. Antimicrobial content in disks of the three lots did not account for these differences. The pH of eluate from the unreliable disks was higher than from disks of the other two and resulted in more rapid diffusion of the antimicrobial and consequently larger zones of inhibition.

The disk diffusion method of Bauer et al. (2) is commonly used to determine the susceptibility of bacteria to antimicrobials. Many variables (1; A. W. Bauer, Proc. Third Int. Congr. Chemother., p. 466, 1964) which influence test results have been defined including inoculum size, pH, and ionic concentration of media, as well as the content of antibiotics in disks. During a circumscribed period of 1972, an inordinate number of *Proteus* species appeared to be susceptible to nitrofurantoin when tested in our laboratory. The present investigation was undertaken to discover the cause of these "falsely" large zones of inhibition.

MATERIALS AND METHODS

Susceptibility testing. Susceptibility tests were performed by the disk diffusion method of Bauer et al. (2). Commercially prepared 150-mm plates containing Mueller-Hinton (M-H) agar at a depth of 5 mm were obtained from Scott Associates, San Diego, Calif. Three-hundred-microgram nitrofurantoin disks, lots number 2211 (I), number 2359 (II) (Pfizer Diagnostic Div., Pfizer Inc. N.Y.), and lot number 506856 (III) (Difco Laboratories, Detroit, Mich.) were used throughout the tests. Disks were kept either in original containers or containers with desiccant and stored at 4 C. The bacteria studied were 50 strains each of *Proteus mirabilis* and *Escherichia coli* isolated from clinical specimens. The M-H agar plates were incubated at 35 C, and zones of inhibition were measured after 18 h of incubation.

Nitrofurantoin content of disks. Nitrofurantoin in disks of each lot was eluted in 50 ml of distilled water by overnight incubation at 37 C, and spectrophotometric absorption was measured at 400 nm with a Beckman DU spectrophotometer after nitromethane extraction and the addition of hyamine reagent (3). Complete elution of nitrofurantoin was checked by a repeat cycle of elution into fresh distilled water. The concentration of nitrofurantoin was then extrapolated from a standard plot of nitrofurantoin at various concentrations. Standards were prepared by dissolving nitrofurantoin powder in dimethyl formamide, followed by dilution in distilled water, pH 6.0.

pH effect. Antimicrobial in disks of each lot was eluted in 1 ml of deionized water during 2 h of incubation at 37 C, and the pH was measured with a glass electrode potentiometer. To study the effect of pH on nitrofurantoin activity, the antimicrobial was eluted from lot I disks in 1 ml of distilled water, adjusted to pH values between 4 and 11 with either 0.01 N NaOH or 0.01 N HCl, and then reabsorbed onto the original disks by evaporation under nitrogen. Complete recovery of antimicrobial was accomplished by repeatedly washing the test tubes with 0.05 ml of distilled water and adding the wash to the respective disks. These disks were then tested on M-H agar plates streaked with *E. coli*, ATCC 25922, by the method of Bauer et al. (2).

Effect of varying disk content of nitrofurantoin at constant pH. Solutions of nitrofurantoin were prepared in dimethyl formamide. Final concentrations of nitrofurantoin varied from 200 to 400 μg/ml. Blank paper disks, 6 mm in diameter, were soaked in these solutions and evaporated under nitrogen. These disks were then tested by the method of Bauer et al. (2) against representative *E. coli* and *P. mirabilis* strains.

Rate of elution of nitrofurantoin from disks. Nitrofurantoin disks from lot II were adjusted to pH values between 4 and 8 by adding 0.01 N HCl or 0.01 N NaOH solution onto disks and by evaporating the disks to dryness under nitrogen. The disks were then suspended in 50-ml portions of distilled water at 35 C, and at various intervals 3 ml of solution was removed

and measured spectrophotometrically at 400 nm with a Beckman DU spectrophotometer. After obtaining each spectrophotometric reading, the 3-ml portion was restored to the original solution to maintain a total volume of 50 ml. Since direct spectrophotometric measurement of nitrofurantoin solution at 400 nm yields the same result as measurement after nitromethane extraction and hyamine reagent treatment, the 3-ml portion was measured directly in order to permit restoration of solution to its original volume.

RESULTS

Distribution of zones of inhibition for 300-μg nitrofurantoin disks. The three different lots of nitrofurantoin disks were tested against 50 strains each of *P. mirabilis* and *E. coli*, and the diameters of zone inhibition were compared (Fig. 1). Results were interpreted as follows: inhibitory zones that fell between 15 and 16 mm were considered indicative of an intermediate degree of susceptibility to nitrofurantoin, inhibitory zones below 15 mm were indicative of resistance, and those above 16 mm were indicative of susceptibility. *E. coli* strains were uniformly susceptible (100%) to the three lots of nitrofurantoin disks. The zone size diameters of lots II (average 21.9 ± 2.2 mm) and III (average 21.3 ± 1.9 mm) were comparable, but lot I disks gave larger zone sizes (average 26.3 ± 1.8 mm).

The results of nitrofurantoin disk susceptibility tests with strains of *P. mirabilis* also showed larger inhibition zone sizes with disks of lot I as compared with lots II and III. The average diameters of inhibition zones for the different lots were: lot I = 19.4 ± 2.3 mm; lot II = 15.1 ± 1.4 mm; and lot III = 13.5 ± 1.7 mm. In contrast to *E. coli*, the larger zone sizes with lot I disks were sufficient to change the susceptibility category of *Proteus* strains. A large number of *Proteus* strains, which were actually resistant or intermediately susceptible when studied with disks of lots II and III, were susceptible with lot I.

Disk potency. The content of nitrofurantoin eluted from disks when measured spectrophotometrically is shown in Table 1. The actual nitrofurantoin content of disks in lot I (350 μg) and lot II (310 μg) were higher than the 300 μg specified on the disk, whereas lot III (256 μg) contained less than 300 μg per disk. Complete elution of nitrofurantoin occurred; no further nitrofurantoin could be recovered by a second cycle of elution.

Effect of the pH of nitrofurantoin disks on inhibitory zone sizes. The pH of eluted antimicrobial from disks of lot I (pH 7.4) was higher than that of lot II (pH 5.8) and III (pH 6.0). The effect upon zone diameters of deliberate changes in the pH of the antimicrobial solutions used to prepare disks is shown in Fig. 2.

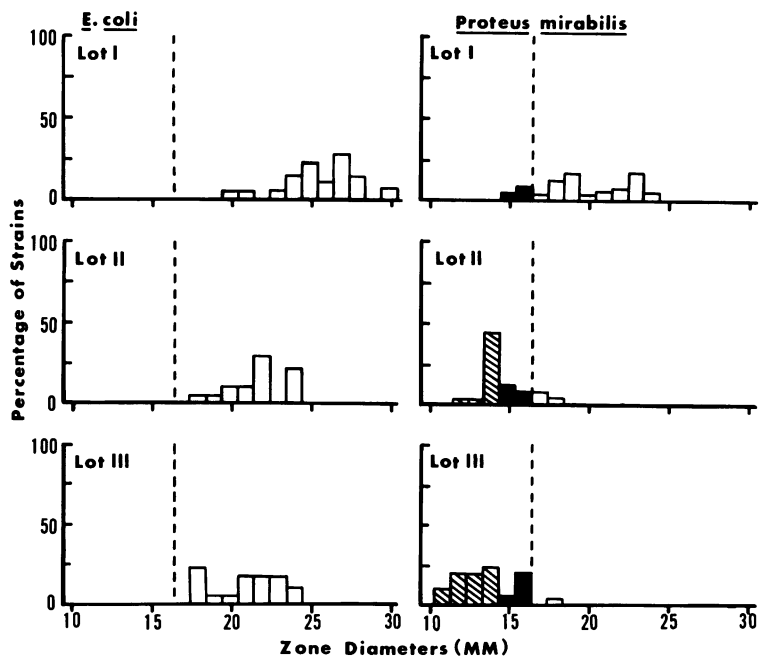

FIG. 1. *Distribution of zone diameters, using 300-μg nitrofurantoin disks. Cross-hatched bars represent strains which are interpreted as resistant; black bars, intermediate; and clear bars, susceptible. The dotted line represents the break point between susceptible and intermediate zones.*

TABLE 1. *Nitrofurantoin content and pH on disk*

Lot no.	Manufacturer	Disk content (μg/disk)	pH of eluate in deionized water
I (2211)	Pfizer	350	7.4
II (2359)	Pfizer	310	5.8
III (506856)	Difco	256	6.0

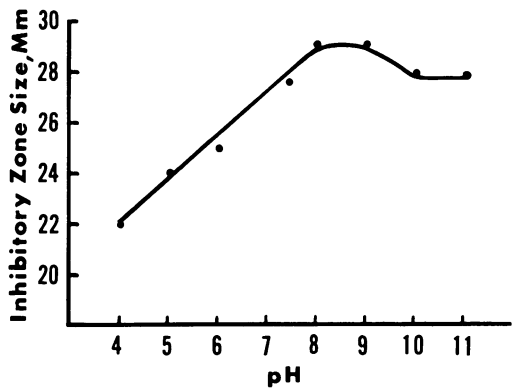

FIG. 2. *Effect of pH of nitrofurantoin disks on inhibitory zone size with E. coli ATCC 25922.*

Between pH 8.0 and 9.0, there were maximal zones of inhibition which decreased at higher and lower pH values. There was a decrease in solubility at lower pH (pH 7.0) as noted by the occurrence of a precipitate. The inhibitory zone size increased by 3.0 mm when the pH of antimicrobial solution on disks was raised from 6.0 to 7.5.

When pH of the nitrofurantoin solution was held constant at 7.0, increasing the disk content of drug from 200 to 400 μg did not appreciably affect the diameters of inhibition zones. These data are presented in Table 2.

Rate of nitrofurantoin elution from disks. The rates of nitrofurantoin diffusion from disks of lot II adjusted to various pH values are shown in Fig. 3. All the antimicrobial was eluted within 40 min from nitrofurantoin disks of pH 8.0. In the same time period, only 55% of nitrofurantoin was eluted from disks of pH 7.0, and only 20% was eluted from disks of pH 5.0 and 6.0. In a separate experiment, it was found that all the antimicrobial was eluted from disks of lot I (pH 7.4) within 20 min, whereas only approximately 50% of antimicrobial was eluted from disks of lots II and III in the same time period.

DISCUSSION

In this study, one commercial lot (lot I) of 300-μg nitrofurantoin disks was found to give unusually large zones of inhibition with *E. coli* and *P. mirabilis* strains when compared with two other lots (II and III) of commercial nitrofurantoin disks. The zones of inhibition with the same strains of these species were 3 to 6 mm larger in diameter when tested with disks of lot I. The amount of nitrofurantoin in disks of lot I (350 μg, 118%) was similar to that of lot II (310 μg, 103%) and higher than that of lot III (256 μg, 85%), but all were within the limits of 65 to 150% allowed by the Food and Drug Administration (FDA). Turck et al. (5) reported a difference of only 1 to 2 mm between 300- and 100-μg nitrofurantoin disks with susceptible *E. coli* strains. In the present study, disk content was increased from 200 to 400 μg without appreciably affecting inhibition zone diameters of either *Proteus* species or *E. coli*. Thus it is unlikely that differences in antibiotic content account for the observed discrepancy in diameters of zones of inhibition. The pH of eluates from lot I disks was found to be substantially higher (pH 7.4) than those from disks of lots II (pH 5.8) and III (pH 6.0). Although alteration of pH changes color of nitrofurantoin solutions, it does not affect the absorbance of the solutions at 400 nm; thus, pH per se does not affect the

TABLE 2. *Effect of varying disk content at constant pH upon inhibition zone diameter*

Nitrofurantoin (μg/disk)	Inhibition zone diam (mm)				
	E. coli		*P. mirabilis*		
	Strain 1	Strain 2	Strain 1	Strain 2	Strain 3
200	18.5	21.0	13.5	13.0	11.0
250	19.0	21.0	13.5	13.5	11.5
300	20.0	21.0	13.5	12.0	11.5
350	20.0	22.0	14.5	13.0	11.0
400	20.5	22.5	14.5	14.0	12.5

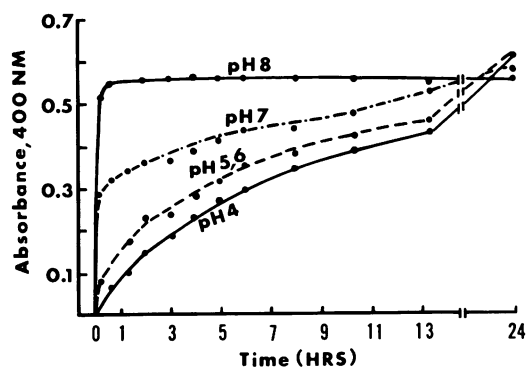

FIG. 3. *Effect of pH upon rate of nitrofurantoin elution from paper disks, 35 C.*

content of nitrofurantoin in the disk. Nitrofurantoin (4) shows a marked increase in solubility with increasing pH; there is a threefold increase in solubility as the pH is increased from 6.0 to 7.0. This increased solubility could result in more drug being adsorbed in a paper disk but, as noted, differences in disk content of nitrofurantoin were not great. The slightly alkaline pH of antibiotic eluates from disks of lot I appears to be more important in influencing the diffusion characteristics of the antimicrobial in medium. Rates of diffusion are in turn critically important determinants of the ultimate zone size of inhibition. Diameters of zones of bacterial inhibition due to antibiotics diffusing from disks may be visualized as the result of a race between the diffusion rate of antibiotic and the growth rate of bacteria. Although the ultimate quantitative diffusion of nitrofurantoin from disks of lower pH is similar to that of disks with higher pH, the rate of diffusion is the more important zone size determinant and is much more rapid with disks of higher pH. The present study indicates that diffusion of nitrofurantoin is most rapid from disks at pH 7.0, but disks of this pH are least discriminatory for the determination of the antimicrobial susceptibility of *Proteus* species. Perhaps lowering the nitrofurantoin content of disks with a pH of 7.0 should be explored to improve the overall performance of nitrofurantoin disks.

Strains of *E. coli* are generally susceptible to nitrofurantoin, and "falsely" increased zone diameters do not result in a change of susceptibility category. The zone diameters of *P. mirabilis* strains are poised very closely at or below the breakpoint for susceptible and intermediate or resistant strains. An increase of three or more millimeters in zone diameter will cause a categorical shift of a substantial number of *P. mirabilis* strains into the susceptible range.

Since nitrofurantoin is classified as a chemotherapeutic agent and not an antibiotic, the performance of these disks is not regulated by the FDA. Deplorably, there are at present no federal regulations regarding the content or performance of disks for such chemotherapeutic agents as sulfonamides, nalidixic acid, trimethoprim-sulfamethoxazole, as well as nitrofurantoin. In the case of nitrofurantoin, it seems clear that standards for the pH of reference nitrofurantoin solutions are also necessary. If performance tests of such agents were included within the jurisdiction of the FDA, the release of unreliable nitrofurantoin disks might have been avoided. Daily testing of a standard strain of *E. coli* with nitrofurantoin disks should readily detect increases in the diameter of zones of inhibition and would alert laboratories to disks which perform erroneously.

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