ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1988, p. 1002–1004
0066-4804/88/071002-03$02.00/0

Vol. 32, No. 7

Determination of Pyrazinamide MICs for *Mycobacterium tuberculosis* at Different pHs by the Radiometric Method

MAX SALFINGER† AND LEONID B. HEIFETS*

National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado 80206

Received 2 November 1987/Accepted 19 April 1988

The MICs of pyrazinamide (PZA) were determined for *Mycobacterium tuberculosis* cultivated under different pH conditions in 7H12 liquid medium. Mycobacterial growth was monitored by the radiometric method (BACTEC system; Johnston Laboratories, Inc., Towson, Md.). We observed a predictable eightfold difference between the MICs determined at pH 5.5 and those determined at pH 5.95. The highest MICs for 21 susceptible strains were 50.0 μg/ml at pH 5.5 and 400 μg/ml at pH 5.95. This eightfold difference enabled us to predict MICs at pH 5.5 from the values observed at pH 5.95. The use of 7H12 broth at pH 5.95 simplified the radiometric PZA susceptibility test by avoiding the addition of acid solutions in the course of cultivation, which was required when the test was performed at pH 5.5. An additional benefit of using pH 5.95 instead of pH 5.5 was that all tested strains grew at pH 5.95, while some of them, especially PZA-resistant strains, did not grow at pH 5.5.

Isoniazid, rifampin, and pyrazinamide (PZA) are the three most important drugs in the modern chemotherapy of tuberculosis. The inclusion of PZA in treatment regimens has made possible the shortening of the duration of therapy to 6 months. PZA has been alleged to effect tubercle bacilli within macrophages (4).

Testing of the susceptibility of *Mycobacterium tuberculosis* to PZA is technically difficult because PZA is active only at acid pHs (7, 9). Unfortunately, the low pH (5.5) required itself prevents up to 50% of strains from growing in 7H10 Middlebrook agar medium (13). Even with recent improvements in the use of this medium, about 10% of clinical isolates do not grow at pH 5.5 (2, 3).

PZA itself has no discernible activity against tubercle bacilli. PZA-susceptible strains of *M. tuberculosis* deaminate PZA to pyrazinoic acid, the metabolite with antituberculous activity. Therefore, determination of the activity of a given strain of *M. tuberculosis* to elaborate pyrazinamidase (1) has been suggested as a rapid method for PZA susceptibility testing (3, 6, 8) and for screening PZA-susceptible strains (10). Unfortunately, this method fails to give information regarding the proportion of a test population that is PZA resistant, and it is also independent of the PZA concentration.

In the early 1980s (11, 12), a radiometric method (BACTEC system; Johnston Laboratories, Inc., Towson, Md.) for rapid testing of susceptibility of tubercle bacilli to isoniazid, rifampin, ethambutol, and streptomycin was introduced. Generally, it employs a 100-fold inoculum difference between the growth control and drug test vials that allows the quantitative determination of 1% of the bacterial population which is resistant (15). Heifets and Iseman (5) have described a method of delayed acidification of Middlebrook 7H12 broth that obviates the difficulties associated with the initial cultivation at low pHs. Recently, Tarrand et al. (14) were able to use this modified BACTEC system to detect a 1 or 2% population of PZA-resistant bacteria artificially mixed with a susceptible strain.

The aims of this study were (i) to ascertain whether the radiometric method could be used to determine the MIC of PZA against tubercle bacilli in 7H12 broth; (ii) to determine changes in MIC in relationship to variations of the pH of the media; and (iii) to evaluate the feasibility of determining the MIC of PZA at a pH higher than 5.5.

MATERIALS AND METHODS

Cultures. A total of 36 clinical isolates of *M. tuberculosis* (21 susceptible and 15 resistant to 50 μg of PZA per ml in a routine radiometric test) from the National Jewish Center for Immunology and Respiratory Medicine, as well as referral cultures submitted to us for drug susceptibility testing, were used. The strains were preserved in portions in 7H9 broth at −70°C, when necessary, until testing.

PZA solutions. Appropriate stock solutions of PZA (Sigma Chemical Co., St. Louis, Mo.) were prepared in distilled water, sterilized by membrane filtration, and then divided into portions for storage at −70°C for not more than 3 months.

Acidifying solution. Phosphoric acid (85.7%, 100 ml) was added to 1,900 ml of distilled water, sterilized by membrane filtration, and stored in a cold room. A 0.9-ml portion of this diluted phosphoric acid was added to 30 ml of 7H12 broth (pH 6.8; Johnston Laboratories). The acidifying solution (pH 3.64) was stored at 4°C.

MIC determination at different pHs. The strains were subcultured in 7H12 broth (4 ml, pH 6.8) and incubated at 36 ± 1°C, and the growth index (GI) was recorded daily in the BACTEC system until it reached 150 to 300 (5). Then, 0.1 ml of this culture was transferred into a special broth, 7H12 (2 ml, pH 5.95; Johnston Laboratories). An allergist syringe with a permanently affixed needle (Becton Dickinson and Co., Paramus, N.J.) was used to draw 0.7 to 0.8 ml of the culture; the plunger was then given a rapid push so that the medium was forced back into the vial. Repetition of this procedure 2 more times was sufficient to disperse the microorganisms evenly.

Susceptible strains took 3 to 8 days (mean, 4.6 days) to

* Corresponding author.
† Present address: Department of Medical Microbiology, University of Zurich, CH-8028 Zurich, Switzerland.

TABLE 1. Contents of the vials used for the PZA-susceptible *M. tuberculosis* strains at different pHs

pH	Presence of a final PZA concn (μg/ml) of:								Vol (ml) of the vials with:					
	400	200	100	50	25	12.5	6.2	0	Inoculum	7H12	Drug[a]	Acidifying solution	H_2O	Total
5.95	+	+	+	+	+	+		+	0.1	2	0.1	0	0.5	2.7
5.8		+	+	+	+	+		+	0.1	2	0.1	0.2	0.3	2.7
5.7			+	+	+	+	+	+	0.1	2	0.1	0.4	0.1	2.7
5.5			+	+	+	+	+	+	0.1	2	0.1	0.7	0	2.9

[a] Distilled water was used in control vials.

reach a GI of 100 to 150, and resistant strains took 4 to 12 days (mean, 6.3 days). At this time PZA and the acidifying solution were added to achieve the final drug concentration and the lower pH value. The standard deviation of the GI readings rarely exceeded 10% of the mean. The pH was measured with a digital pH meter (Accumet 815 MP; Allied Fischer Scientific Co., Pittsburgh, Pa.). The specific contents of the vials used for the PZA-susceptible strains are shown in Table 1. The 15 PZA-resistant strains were tested at pH 5.95 (3,200, 800, and 200 μg/ml) and pH 5.5 (3,200, 800, 200, and 50 μg/ml). One vial was used for each drug concentration at the different pHs.

The growth in all vials was recorded daily until the GI in the two control vials (acidified, distilled water instead of PZA) reached its maximum and started declining. At ph 5.95 susceptible strains began to decline within 3 to 8 days (mean, 4.9 days), and resistant strains began to decline within 3 to 9 days (mean, 6.0 days). We observed the same or a longer interval for susceptible strains at pH 5.5, whereas resistant strains had a shorter or, at least, an equal interval. The pH values were checked again with susceptible strains at the end of two experiments by using membrane-filtered broth. All four pH values remained unchanged.

The MIC was defined as the lowest concentration which produced a decline of the GI readings after the addition of PZA and acid solutions.

RESULTS

PZA-susceptible strains. Of the 21 PZA-susceptible strains, 13 (62%) had MICs of ≤12.5 μg/ml at pH 5.5; at pHs 5.7, 5.8, and 5.95 the percentages were 38, 10, and 0%, respectively (Table 2). Three strains (14%) did not grow at pH 5.5. The MICs for 90% of strains tested were 50 μg/ml at pH 5.5 and 5.7, 100 μg/ml at pH 5.8, and 200 μg/ml at pH 5.95 (Fig. 1). The MIC varied over this narrow pH range by a factor of 4, and for all 21 strains it varied by a factor of 8. The cumulative percentage curve of inhibited strains showed a similar course, except at pH 5.5.

PZA-resistant strains. At pH 5.5 one strain had a MIC of 200 μg/ml, three strains had MICs of 800 μg/ml, six strains had MICs of 3,200 μg/ml, and five strains failed to grow. The MIC shifted to higher concentrations in nonacidified vials. At pH 5.95 the MIC was 800 μg/ml for two strains and 3,200 μg/ml for four more strains, and for the remaining nine strains the MIC was ≥3,200 μg/ml.

TABLE 2. MICs for 21 PZA-susceptible strains

pH	No. of strains susceptible at the following MICs (μg/ml):							
	≤6.2	12.5	25.0	50.0	100.0	200.00	400.00	No Growth
5.5	6	7	1	4	0	0	0	3
5.7	2	6	9	2	2	0	0	0
5.8	0	2	7	8	3	1	0	0
5.95	0	0	2	6	9	2	2	0

DISCUSSION

Based on previous research performed at our institution (5), Johnston Laboratories produced a special low-pH broth, 7H12 (pH 5.9 ± 0.1; actual pH, 5.95) to avoid the acidification from pH 6.8 to 6.0 previously necessary for PZA tests. An additional change was diluting the phosphoric acid stock solution (1:20) in 7H12 broth instead of diluting fluid, resulting in more accurate pH values. In contrast to the results of Tarrand et al. (14), we did not observe a rise in pH during the experiments, probably because our medium was buffered more strongly.

It is well known that the inoculum size for PZA susceptibility testing at pH 5.5 is critical (9, 13). Too heavy an inoculum may result in false resistance because the bacterial mass locally neutralizes the acid pH in the agar. On the other hand, with too low an inoculum, the mycobacteria may not grow (J. J. Tarrand, A. Spicer, and D. H. M. Groschel, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C226, p. 349). In a preliminary experiment (data not shown), we used *M. tuberculosis* H37Rv at different GIs (100 to 129, 130 to 159, 160 to 189, and 216 to 243) for inoculation of the special 7H12 broth (pH 5.95). Subsequently, the MICs for the four different GI ranges at the four tested pHs were the same. Tarrand et al. (14) tested whether bacterial cell absorption of PZA or metabolic products interfered with PZA activity. They used UV-killed and washed bacteria or a sterile filtrate from rapidly growing cultures and found that both additives abolished PZA activity. Binding of PZA by mycobacteria could not be demonstrated spectrophotometrically. Because of the concerns mentioned above, we used a GI range of only 100 to 150.

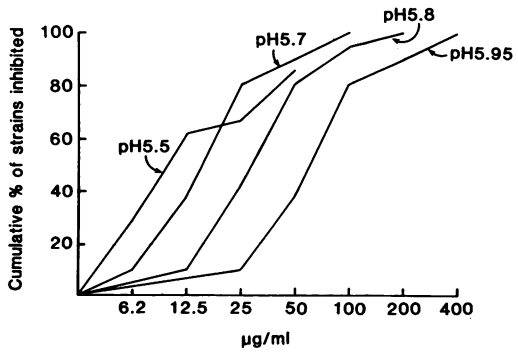

FIG. 1. Effect of pH on the inhibitory activity of PZA against 21 PZA-susceptible *M. tuberculosis* strains cultured in 7H12 broth.

The radiometric method (BACTEC system) made it possible to monitor the growth kinetics of *M. tuberculosis* in liquid medium and to detect the beginning of the logarithmic phase of growth, and thus to determine the optimal moment for adding the PZA and lowering the pH to the desirable level. The standard deviation of GI readings (susceptible strains, 29 vials; resistant strains, 11 vials) was within 10% of the mean. It exceeded 10% only for resistant strains, which needed more than 9 days to reach a GI of 100. We conclude that our procedure for the standardization of the inoculum is optimal for routine use.

Heifets and Iseman (5) have noted that it is not known whether the subpopulation that is tolerant to low pH inevitably selected by any available PZA susceptibility method reflects the population as a whole. The data presented here (Fig. 1) indicate that there is no change in the subpopulations at different pH levels. The curves had very similar slopes.

In 1954 McDermott and Tompsett (9) studied the PZA activity in Tween-albumin and in oleic acid-albumin broth and determined the MIC macroscopically (visible bacterial growth at 2 weeks). They reported a complete inhibition of growth of *M. tuberculosis* H37Rv with 125 μg of PZA per ml at pH 6.0, and with 16 μg of PZA per ml at pH 5.5. With the radiolabeled 7H12 broth, we were able to confirm this eightfold decrease of PZA activity between pH 5.5 and 5.95.

In summary, our data confirm that the MIC of PZA is pH dependent and indicate that there is a consistent relationship between the MICs determined at pH 5.5 and 5.95. Because of this relationship, it appears appropriate and practical to determine an MIC for PZA at pH 5.95 and to extrapolate from this the projected MIC at pH 5.5. Testing for the MIC of PZA by using pH 5.95 circumvents the technically troublesome and time-consuming step of adding phosphoric acid, thereby making quantitative PZA susceptibility testing more practical for routine testing in microbiology laboratories.

ACKNOWLEDGMENTS

This study was supported by biomedical research grant SO7RR05842-08 from the National Jewish Center for Immunology and Respiratory Medicine. M. Salfinger was supported by the Swiss Union Against Tuberculosis and Lung Diseases as a visiting scientist from the Department of Medical Microbiology at the University of Zurich, Zurich, Switzerland.

LITERATURE CITED

1. **Brander, E.** 1972. A simple way of detecting pyrazinamide resistance. Tubercle **53**:128–131.
2. **Butler, W. R., and J. O. Kilburn.** 1982. Improved method for testing susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. J. Clin. Microbiol. **16**:1106–1109.
3. **Butler, W. R., and J. O. Kilburn.** 1983. Susceptibility of *Mycobacterium tuberculosis* to pyrazinamide and its relationship to pyrazinamidase activity. Antimicrob. Agents Chemother. **24**:600–601.
4. **Dickinson, J. M., V. R. Aber, and D. A. Mitchison.** 1977. Bactericidal activity of streptomycine, isoniazid, rifampin, ethambutol and pyrazinamide alone and in combination against *Mycobacterium tuberculosis*. Am. Rev. Respir. Dis. **116**:627–635.
5. **Heifets, L. B., and M. D. Iseman.** 1985. Radiometric method for testing susceptibility of mycobacteria to pyrazinamide in 7H12 broth. J. Clin. Microbiol. **21**:200–204.
6. **Konno, K., F. M. Feldmann, and W. McDermott.** 1967. Pyrazinamide susceptibility and amidase activity of tubercle bacilli. Am. Rev. Respir. Dis. **95**:461–469.
7. **Mackaness, G. B.** 1956. The intracellular activation of pyrazinamide and nicotinamide. Am. Rev. Tuberc. **74**:718–728.
8. **McClatchy, J. K., A. Y. Tsang, and M. S. Cernich.** 1981. Use of pyrazinamidase activity in *Mycobacterium tuberculosis* as a rapid method for determination of pyrazinamide susceptibility. Antimicrob. Agents Chemother. **20**:556–557.
9. **McDermott, W., and R. Tompsett.** 1954. Activation of pyrazinamide and nicotinamide in acid environments in vitro. Am. Rev. Tuberc. **70**:748–754.
10. **Salfinger, M., and F. Kafader.** 1987. Susceptibility testing of *Mycobacterium tuberculosis* to pyrazinamide. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **265**: 404–407.
11. **Siddiqi, S. H., J. P. Libonati, and G. Middlebrook.** 1981. Evaluation of a rapid radiometric method for drug susceptibility testing of *Mycobacterium tuberculosis*. J. Clin. Microbiol. **13**: 908–912.
12. **Snider, D. E., R. C. Good, J. O. Kilburn, L. F. Laskowski, R. H. Lusk, J. J. Marr, Z. Reggiardo, and G. Middlebrook.** 1981. Rapid susceptibility testing of *Mycobacterium tuberculosis*. Am. Rev. Respir. Dis. **123**:402–406.
13. **Stottmeier, K. D., R. E. Beam, and G. P. Kubica.** 1967. Determination of drug susceptibility of mycobacteria to pyrazinamide in 7H10 agar. Am. Rev. Respir. Dis. **96**:1072–1075.
14. **Tarrand, J. J., A. D. Spicer, and D. H. M. Groschel.** 1986. Evaluation of a radiometric method for pyrazinamide susceptibility testing of *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **30**:852–855.
15. **Vestal, A. L.** 1981. Procedures for the isolation and identification of mycobacteria. U.S. Department of Health and Human Services, HHS Publication No. (CDC) 81-8230. Centers for Disease Control, Atlanta.