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Enzymatic Method for Inactivation of Aminoglycosides during Measurement of Postantibiotic Effect

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To determine the postantibiotic effect of aminoglycosides, two methods are currently being used to remove the test drug: repeated washing and dilution. An enzymatic inactivation method of removing gentamicin and tobramycin was developed and compared with the dilution method. This enzymatic method provides a rapid and simple alternative method of removing aminoglycosides which results in reliable postantibiotic-effect values.

Three factors are relevant when determining aminoglycoside dosing schedules: MIC, the kinetics of bactericidal activity, and the postantibiotic effect (PAE) (10). The PAE can be determined in various ways, for example, by counting viable organisms, by measuring impedance or bioluminescence, by spectrophotometry, and by examining morphology (7). An important step in measuring the PAE is the rapid removal of antibiotic after a short exposure of the bacteria to the drug. In general, three methods of removing the antibiotic have been described: dilution, repeated washing, and enzymatic inactivation (2). A specific problem in measuring the PAE of aminoglycosides is the marked killing capacity of these antibiotics, particularly at high concentrations, necessitating a substantial dilution (up to 1,000-fold) to eliminate antimicrobial activity. As a result, the number of viable bacteria in the sample falls below the limit of detection (4). The second method used for removal of aminoglycosides is repeated washing. Although this method does not influence the limit of detection, washing itself may cause a temporary reduction in the rate of bacterial regrowth (2, 9), possibly resulting in less reliable PAE values. In addition, washing is time-consuming. The third method used for antibiotic removal is enzymatic drug inactivation. So far, the use of this method has been restricted to the β-lactam antibiotics. In the present study, an enzymatic method was developed for inactivation of the aminoglycosides gentamicin and tobramycin. To validate this new method, we compared the results of the enzymatic inactivation method with those of the generally used dilution method.

The strains used for measurement of the PAE by the enzymatic inactivation method were *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 43816, and *Pseudomonas aeruginosa* ATCC 27853. MICs were determined by a standard macrodilution method with Mueller-Hinton broth (Difco, Amsterdam, The Netherlands) supplemented with Ca^{2+} (25 mg/l) and Mg^{2+} (12.5 mg/l) (MHBS) (8). The MICs of gentamicin and tobramycin for the strains tested were 1.0 and 0.5 mg/l for *S. aureus*, 1.0 and 1.0 mg/l for *E. coli*, 0.5 and 0.5 mg/l for *K. pneumoniae*, and 1.0 and 0.5 mg/l for *P. aeruginosa*, respectively. *E. coli* R 176 (a clinical isolate of unknown source, resistant to gentamicin and tobramycin and susceptible to netilmicin and amikacin) was used for the extraction of an aminoglycoside-acetylating enzyme [AAC(3)-II] (6). *E. coli* R 176 was inoculated from a fresh overnight culture into Todd-Hewitt broth (Oxoid, Basingstoke, Hampshire, England) containing either gentamicin (5 mg/l; Schering-Plough, Amstelveen, The Netherlands) or tobramycin (5 mg/l; Eli Lilly and Company, Nieuwegein, The Netherlands) and incubated with shaking (200 rpm) at 37°C until the optical density at 660 nm was 0.63. After centrifugation (15 min at 6,000 × *g* and 4°C), the pellets were resuspended in phosphate-buffered saline and the bacteria were disrupted by ultrasonic treatment at an amplitude of 14 (Soniprep; MSE, Bughborough/Leics, Great Britain) 10 times for 10 s each with cooling on ice for 10 s between treatments. The cell debris was then centrifuged for 2 h at 100,000 × *g* (L-70 centrifuge; Beckman, Palo Alto, Calif.). After the addition of dithiothreitol (Sigma, St. Louis, Mo.) to a final concentration of 5 mM, the supernatant was filtered (0.45-μm-pore-size filter; Schleicher and Schuell, 's-Hertogenbosch, The Netherlands) and stored as a stock solution at −80°C. The number of units of activity in the enzyme preparation was determined in tobramycin solutions of 10 and 20 mg/l in MHBS at 37°C. There was no difference in the initial rates of enzyme activity measured at both tobramycin concentrations (data not shown). The enzymatic activity was further determined under several sets of environmental conditions: four tobramycin solutions of 5 mg/l were made in MHBS at pH 7.2 (method 1), MHBS from a 24-h culture of *S. aureus* at an adjusted pH of 7.2 (method 2), MHBS from a 24-h culture of *S. aureus* at pH 5.3 (method 3), and MHBS from a logarithmic culture of *S. aureus* at pH 7.2, exposed for 1 h to 5 mg of tobramycin per liter (method 4). To each solution were added 0.02 U of enzyme and 0.2 mmol of acetyl coenzyme A, and the solutions were incubated in a water bath at 37°C with shaking (200 rpm). Samples were taken at time zero, 10 s, 1 min, and 5 min and immediately heated to 90°C to inactivate the enzymes. Tobramycin concentrations were determined by a standard microbiological agar diffusion test, with *S. aureus* ATCC 29213 as a test strain. The enzymatic activity of the stock solution stored at −80°C was tested every month.

In order to detect a possible influence of the enzyme and acetyl coenzyme A on the bacterial growth rate, growth curves were determined by a standard method (1). Briefly, a logarith-

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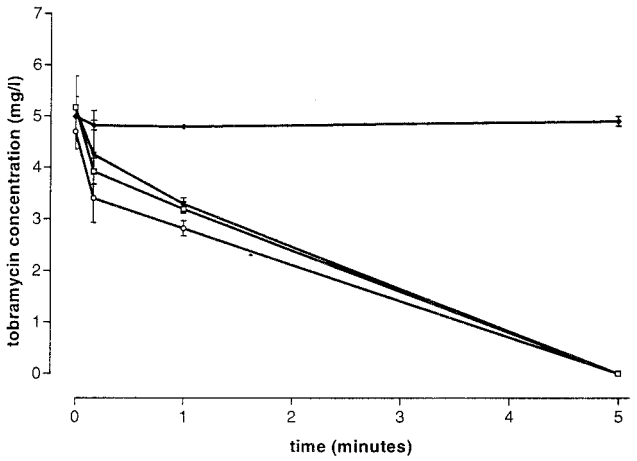

FIG. 1. Enzymatic activity, shown as the removal of tobramycin over time under different environmental conditions, i.e., in MHBS at pH 7.2 (□), in MHBS from a 24-h culture of *S. aureus* at pH 5.3 (◆), in MHBS from a 24-h overnight culture of *S. aureus* at an adjusted pH of 7.2 (▼), and in MHBS from a logarithmic culture of *S. aureus* at pH 7.2, exposed for 1 h to a 5-mg/l tobramycin solution (○) (simulating PAE test conditions). Data are means ± standard deviations from four experiments.

mic culture of each strain, with an inoculum of approximately 5×10^5 CFU/ml, was incubated with 0.01 U of enzyme per ml and 0.2 mmol of acetyl coenzyme A (95%, prepared enzymatically; Sigma) per ml. Samples were taken at 0, 1, 2, 3, 4, 6, and 24 h. Control growth curves were obtained similarly, but the enzyme and acetyl coenzyme A were not added to the cultures.

To determine the PAE, a logarithmic culture of approximately 5×10^6 CFU/ml in MHBS was exposed to gentamicin or tobramycin at a concentration of four times the MIC for 1 h at 37°C. After the incubation period, the antibiotic was removed by two methods: 500-fold dilution in prewarmed MHBS (2) and enzymatic inactivation. For enzymatic inactivation, 0.01 U of enzyme per ml and 0.2 mmol of acetyl coenzyme A per ml were added to the samples. The control cultures, which were not exposed to the antibiotics, were treated similarly. The cultures were further incubated for 6 h at 37°C. Samples were taken each hour and diluted serially 10-fold in cold sterile saline on ice. Each dilution was plated on tryptone soy agar (Oxoid) and incubated overnight at 37°C. To decrease the variability at low colony counts, near the lower limit of detection, 20-ml samples were taken from the cultures at 0, 1, 2, and 3 h, treated by the dilution method, and filtered with the Sensor II Milliflex-100 system (0.45-μm-pore-size filter; Millipore Corporation, Bedford, Mass.). At 4 to 6 h, 1-ml samples were plated on tryptone soy agar and incubated overnight at 37°C. Each experiment was performed six times. The PAE was defined as described by Craig and Gudmundsson (2) as $T - C$, where T is the time required for the CFU count of the antibiotic-exposed culture to increase 1 $\log_{10}$ unit above the count immediately after drug removal and C is the corresponding time for the control cultures. The SAS computer package was used for statistical analysis (11).

Extraction of enzyme from 1 liter of bacterial culture yielded 90 ml of a solution containing 0.1 U of enzyme per ml (standard deviation = 0.01, $n = 3$). Figure 1, a plot of the enzymatic activities under several sets of environmental conditions, shows no differences among the enzymatic activities in MHBS at pH 7.2 (method 1), in MHBS from a 24-h culture of *S. aureus* at an adjusted pH of 7.2 (method 2), and in MHBS from a logarithmic culture of *S. aureus* at pH 7.2 (method 4). The enzymes were almost inactive in MHBS containing a 24-h culture of *S. aureus* at pH 5.3 (method 3). The results show that the enzymatic solution was able to inactivate the tobramycin under several test conditions, including the simulation of the PAE test conditions (method 4). The enzymatic stock solution stored at −80°C was stable for at least 2 years.

No significant differences were found between the growth curves of the strains in the presence or in the absence of the enzyme and acetyl coenzyme A (data not shown). The mean PAE values determined by the dilution and enzymatic inactivation methods are shown in Table 1. The difference between the results of the two methods is expressed as the mean change in PAE ($\Delta PAE = PAE_{dilution} - PAE_{enzyme}$ for each individual pair of experiments) with 95% confidence intervals. There was no significant difference between the values determined by the two methods. The observed aminoglycoside PAEs for both methods were highly comparable, and differences were not statistically significant. The between-sample, between-day variabilities of the PAE values, expressed as the mean coefficient of variation of each of the four strains, were 7.5% (gentamicin) and 9.3% (tobramycin) for the dilution method and 8.3% (gentamicin) and 9.2% (tobramycin) for the enzymatic inactivation method.

The PAE has been used to support the use of alternative dosing schedules, i.e., larger doses with longer intervals (5, 12). Because of the supposed significance of the PAE, the method for measuring the PAE has to be as accurate as possible. There was no significant difference in PAE values determined by the above-described enzymatic inactivation method and the dilution method at a concentration of four times the MIC. In addition, the PAE values found were comparable to those mentioned in the literature (2, 3). An important drawback of

TABLE 1. In vitro PAEs obtained for gentamicin and tobramycin by dilution and enzymatic inactivation

Strain	Gentamicin			Tobramycin		
	PAE[a] (h)		ΔPAE[b]	PAE[a] (h)		ΔPAE[b]
	Dilution	Enzymatic inactivation		Dilution	Enzymatic inactivation	
S. aureus ATCC 29213	2.4 (0.1)	2.5 (0.1)	−0.1 (−0.30–0.04)	2.0 (0.3)	2.0 (0.2)	0.0 (−0.33–0.26)
E. coli ATCC 25922	1.6 (0.2)	1.5 (0.2)	0.1 (−0.04–0.18)	1.6 (0.1)	1.6 (0.2)	0.0 (−0.29–0.22)
K. pneumoniae ATCC 43816	2.1 (0.1)	2.1 (0.2)	0.0 (−0.23–0.13)	2.2 (0.1)	2.2 (0.2)	0.0 (−0.29–0.29)
P. aeruginosa ATCC 27853	1.9 (0.1)	1.9 (0.1)	0.0 (−0.30–0.17)	2.1 (0.1)	2.0 (0.1)	0.1 (−0.10–0.23)
Overall			0.0 (−0.12–0.03)			0.0 (−0.10–0.10)

[a] Data are mean PAEs after a 1-h exposure to four times the MICs of the antibiotics, based on six experiments. Values in parentheses are standard deviations.
[b] Data are mean differences in PAEs obtained by the dilution and the enzymatic inactivation methods in paired parallel experiments. Values in parentheses are 95% confidence intervals.

the dilution method is the relatively high limit of detection, and in order to get reliable colony counts, a large sample must be filtered because of the 500-fold dilution. In contrast, with the enzymatic inactivation method, no problems were encountered with respect to the limit of detection.

We conclude that the enzymatic inactivation method is a simple, rapid, and more accurate method of measuring the PAEs of gentamicin and tobramycin, especially at high concentrations. With the appropriate enzymes, the same method should be applicable for the study of PAEs of other aminoglycosides.

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REFERENCES

1. **Carlberg, D. M.** 1985. Determining the effects of antibiotics on bacterial growth by optical and electrical methods, p. 64–92. *In* V. Lorian (ed.), Antibiotics in laboratory medicine—1985, 2nd ed. Williams and Wilkins, Baltimore.
2. **Craig, W. A., and S. Gudmundsson.** 1986. The postantibiotic effect, p. 515–536. *In* V. Lorian (ed.), Antibiotics in laboratory medicine—1986, 2nd ed. The Williams and Wilkins Co., Baltimore.
3. **Gudmundsson, A., H. Erlendsdottir, M. Gottfredsson, and S. Gudmundsson.** 1991. Impact of pH and cationic supplementation on in vitro postantibiotic effect. Antimicrob. Agents Chemother. **35:**2617–2624.
4. **Isaksson, B., R. Maller, L. E. Nilsson, and M. Nilsson.** 1993. Postantibiotic effect of aminoglycosides on staphylococci. J. Antimicrob. Chemother. **32:** 215–222.
5. **Isaksson, B., L. Nilsson, R. Maller, and L. Sören.** 1988. Postantibiotic effect of aminoglycosides on gram-negative bacteria evaluated by a new method. J. Antimicrob. Chemother. **22:**23–33.
6. **Leitner, F., and K. E. Price.** 1982. Aminoglycosides under development, p. 29–36. *In* A. Whelton and H. C. Neu (ed.), The aminoglycosides. Marcel Dekker, Inc., New York.
7. **Mackenzie, F. M., and I. M. Gould.** 1993. The post-antibiotic effect. J. Antimicrob. Chemother. **32:**519–537.
8. **National Committee for Clinical Laboratory Standards.** 1990. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 2nd ed. Approved standard M7-A2. National Committee for Clinical Laboratory Standards, Villanova, Pa.
9. **Parker, R. F., and H. C. Marsh.** 1946. The action of penicillin on *Staphylococcus*. J. Bacteriol. **51:**181–186.
10. **Roosendaal, R., and I. A. J. M. Bakker-Woudenberg.** 1992. The impact of antibiotic dosage schedules on the efficacy of treatment in serious infection, p. 5–22. Roche Scientific Service. Editiones Roche, Basel.
11. **SAS Institute Inc.** 1990. SAS users guide. SAS Institute Inc., Cary, N.C.
12. **Ter Braak, E. W., P. J. de Vries, K. P. Bouter, S. G. van der Vegt, G. C. Dorrestein, J. W. Nortier, A. van Dijh, R. P. Verkooyen, and H. A. Verbrugh.** 1990. Once-daily dosing regimen for aminoglycoside plus beta-lactam combination therapy of serious bacterial infections: a comparative trial with netilmicin plus ceftriaxone. Am. J. Med. **89:**58–66.