Improved Acetylating Radioenzymatic Assay of Amikacin, Tobramycin, and Sisomicin in Serum

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The acetylating radioenzymatic assay of aminoglycosides was modified by using [3H]acetyl coenzyme A as cosubstrate. This modification reduces the cost of the method by at least one-half without sacrificing rapidity or accuracy.

Aminoglycoside antibiotics are used extensively in the treatment of serious gram-negative bacillary infections. Since these antibiotics are potentially nephrotoxic and ototoxic, serial measurement of serum levels may be useful in averting toxicity and selecting adequate therapeutic doses. The most widely used methods for aminoglycoside assays have been based on inhibition of bacterial growth. These methods are relatively slow and can be adversely affected by the presence of other antimicrobial agents, and inhibitory end points in agar diffusion microbioassays are occasionally difficult to interpret. Because of these disadvantages, more rapid and specific methods have been developed, including the adenylating (5, 6) and acetylating radioenzymatic assays (2). The latter method is the more versatile of the two because in addition to gentamicin, sisomicin, and tobramycin, it can be used to measure amikacin. We will describe here a detailed modification to the original method of Haas and Davies (2) that reduces the cost of the assay by approximately one-half.

R factor-containing Escherichia coli R-5/W677 possessing kanamycin acetyl transferase, which was originally described by Okamoto and Suzuki (4), were obtained from Edward Yevak of Bristol Laboratories and grown in broth of the following composition (per liter): glucose, 5 g; peptone, 8 g; yeast extract, 5 g; and kanamycin sulfate, 5 mg, which was added to the broth after sterilization. To initiate growth, 40 ml of an overnight growth of the E. coli was added to 360 ml of broth. The suspension was shaken at 37 C for approximately 14 h to an optical density of 0.7 at 550 nm. To obtain the osmotic "shockate" containing the enzyme, techniques similar to those of Benveniste and Davies (1) and Nossal and Heppel (3) were used as follows. Cells from 400 ml of broth were obtained by centrifugation at 3,000 x g for 15 min and washed twice at room temperature with 20 ml of 0.01 M tris-[2-amino-2-(hydroxymethyl)-1,3 propanediol] and 0.3 M NaCl, pH 7.0. The washed pellet was then resuspended with 30 ml of 18% sucrose, 3 x 10^{-3} M ethylenediaminetetraacetate, and 0.033 M tris-(hydroxymethyl)aminomethane (Tris) at pH 7.8, stirred vigorously for 30 min at room temperature, and centrifuged at 16,000 x g for 15 min. The supernatant fluid was discarded, and the pellet was resuspended in 12 ml of ice-cold 5 x 10^{-4} M MgCl_{2} and then stirred vigorously at 4 C. To every 3 ml of the MgCl_{2} cell suspension we added 5 \mu l of 0.5 M dithiothreitol (DTT, Cleland reagent; Nutritional Biochemicals, Cleveland, Ohio). The suspension was centrifuged at 26,000 x g for 30 min and the supernatant fluid (osmotic shockate) was dialyzed at 4 C overnight against 3 liters of distilled water. Dialysis was necessary to remove any residual kanamycin that might increase the background of the assay procedure. The osmotic shockate was first lyophilized, then dissolved with 6 ml of 5 x 10^{-4} MgCl_{2} containing 10 \mu l of 0.5 M DTT, and filtered through a 0.45-\mu m membrane filter (Millipore Corp.), and aliquots were stored and frozen at -80 C.

The assay procedure was as follows. The reagent mixture was prepared by combining the following three mixtures: (i) 0.6 ml of 0.3 M Tris-maleate buffer containing 0.03 M MgCl_{2} and 0.01 M DTT at a pH of 5.7; (ii) 0.2 ml of osmotic shockate; and (iii) 0.2 ml of [3H]acetyl coenzyme A (CoA) (50 \mu Ci) per 0.55 \mu mol per ml of Tris-maleate, pH 5.7 ([3H]acetyl CoA was obtained from New England Nuclear Corp. and unlabeled acetyl CoA lithium salt was obtained from Nutritional Biochemicals). Substitution of [3H]acetyl CoA for the 4C-labeled compound is the major improvement to the original method,
effecting a minimum fourfold reduction in isotope cost, though the need to use a more expensive scintillation medium makes the overall cost reduction about one-half. Twenty microliters of each standard or sample was placed in microtiter wells, which were kept on ice. To this we added 25 μl of the reagent mixture. This reaction mixture was mixed thoroughly and incubated in a water bath at 30°C for 30 min. After the incubation, the microtiter wells were placed on ice and 20 μl of each reaction mixture was spotted onto squares (1.25 by 1.25 cm) of Whatman P-81 phosphocellulose paper, which binds the acetylated aminoglycoside. Papers were dried for 15 s and immersed in distilled water at 80°C to stop the reaction. The papers were then washed three times in 200 ml of distilled water to remove the excess [3H]acetyl CoA. After the washing, the papers were placed in scintillation vials containing 1 ml of 0.3 N NaOH and shaken for 15 min. The use of NaOH probably reduces the positive charge of the 3H-labeled acetylated aminoglycoside and removes it from the paper so that it can be counted efficiently. To determine whether the 3H-labeled acetylated aminoglycoside was efficiently removed, we applied [3H]gentamicin (1.5 μg) to the paper, eluted as described above, and obtained 100% recovery. This elution step replaces the drying step as described originally (2) without altering the rapidity of the method. The 3H-labeled acetylated aminoglycoside was counted by adding 10 ml of counting medium composed of 15 g of 2,5-diphenyloxazole, 0.9 g of 1,4-bis-2-(5-phenyloxazole)-benzene, and 500 ml of Bio-Solv BBS-3 (Beckman Instruments, Fullerton, Calif.) dissolved in 3 liters of toluene. The efficiency of counting was 42%. The standard curve was constructed to relate the disintegrations per minute of 3H-labeled acetylated aminoglycoside bound to phosphocellulose paper and the concentration of the aminoglycoside in micrograms per milliliter. Levels of aminoglycoside in unknown serum samples were interpolated from the standard curve.

Figures 1 through 3 represent typical stan-
standard curves for amikacin, sisomicin, and tobramycin at serum concentrations that would be expected clinically. The method is specific for those aminoglycosides that can be acetylated, and no interference of the assay was observed when either carbenicillin, penicillin, tetracycline, or clindamycin was present in a serum sample at levels 5 to 10 times that which would be expected clinically. Figure 4 compares the improved acetylating radioenzymatic assay (ARA) to a standard microbiological agar well diffusion method (7) using Klebsiella as the test organism. Twenty-six sera from patients receiving amikacin were analyzed in duplicate on two assay days. The mean of the duplicate determinations is plotted for each assay day. Correlation coefficient (r) as determined by linear regression analysis was 0.93 for the two methods and was significant at \( P = 0.0005 \). The y intercept of 3.16 was not significantly different from 0 at a \( P = 0.05 \).

Therefore, there was no significant difference in the measurement of amikacin by these methods. Good agreement between these methods was also obtained in the measurement of serum tobramycin and sisomicin.

Consequently, the improved ARA can be used confidently in the assay of serum amikacin, tobramycin, and sisomicin. The ARA has been demonstrated to be accurate, rapid (3 h for 12 patient determinations), specific, and versatile in the measurement of several aminoglycosides. The modifications presented here have reduced the reagent cost of the ARA to about $0.25 per sample, making it an even more attractive and practical method for the measurement of serum aminoglycosides.

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**LITERATURE CITED**