Radioenzymatic Assays for Aminoglycosides with Kanamycin 6'-Acetyltransferase

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To facilitate the rapid and accurate quantitation of parenterally administered aminoglycosides, we defined the optimum conditions (pH, duration of incubation, and cofactor concentrations) to permit radioenzymatic assays with kanamycin acetyltransferase. The accuracy in quantitating tobramycin, netilmicin, kanamycin, and amikacin at concentrations in the therapeutic range was greater than 90%, with a mean recovery of 102.8%. The mean of the interassay coefficient of variation was 7.8%. Typical standard curves at six different concentrations resulted in a correlation coefficient (*r* value) of >0.99 for each aminoglycoside. The radioenzymatic assay correlates well with the bioassay (tobramycin and netilmicin) and radioimmunoassay (amikacin and kanamycin); the correlation coefficient is greater than 0.90 for all. We conclude that the radioenzymatic assay utilizing kanamycin 6'-acetyltransferase is feasible for all commercially available parenterally administered aminoglycosides.

Aminoglycosides are broad-spectrum antibiotics that are active against a variety of gram-negative and certain gram-positive bacteria. Monitoring the aminoglycoside concentration in serum is necessary because of unpredictable pharmacokinetics in seriously ill patients (15), the relatively narrow therapeutic index, and reduced elimination in patients with renal failure (3, 7). Aminoglycosides such as gentamicin, tobramycin, and netilmicin are associated with ototoxicity and nephrotoxicity at peak serum concentrations of greater than 12 µg/ml and with trough concentrations of 2 µg/ml; amikacin and kanamycin trough values of 10 µg/ml and peak serum concentrations of >50 µg/ml are also associated with toxicity.

Numerous assays have been developed for aminoglycosides (4, 8, 9, 11, 16, 18). Radioenzymatic assays (REA) with gentamicin adenyl transferase is one technique used for the quantitation of gentamicin (17). Other aminoglycoside-inactivating enzymes acetylate, adenylylate, or phosphorylate the antibiotic with the appropriate coenzyme. Adenylylation and acetylation have been used to quantitate gentamicin and tobramycin with [¹⁴C]adenosine triphosphate, and netilmicin and amikacin have been assayed with ¹⁴C- or ³H-acetyl-coenzyme A (1, 9). The adenylylation reactions have also been linked with other enzyme systems to produce bioluminescence which is quantitated (5, 13); this approach bypasses the use of radioactive isotopes. Gentamicin 3'-acetyltransferase and kanamycin 6'-acetyltransferase (KAT) are now commercially available (P-L Biochemicals, Inc., Milwaukee, Wis.), thereby eliminating the need to purify the enzymes.

We describe here an REA for tobramycin, kanamycin, netilmicin, and amikacin with KAT and ¹⁴C-acetyl-coenzyme A (¹⁴C-acetyl-CoA). The assay is rapid, precise, and accurate, and it correlates well with other established assays. This assay is ideal for large numbers of samples, and little technical experience is needed for the procedure. Data reduction is greatly simplified, since the concentration versus the assay response is linear. This assay is suitable for clinical monitoring of serum levels and for research pur-

poses. The use of a single enzyme capitalizes on its intrinsic, broad-substrate specificity.

MATERIALS AND METHODS

Antibiotics were obtained from the following sources: tobramycin (potency, 958 µg/mg), cephalothin, and cefazolin, Eli Lilly & Co., Indianapolis, Ind.; kanamycin (potency, 775 µg/mg), cefamandole, and carbenicillin, USP, Rockville, Md.; ticarcillin, Beecham Laboratories, Bristol, Tenn.; netilmicin (potency, 603 µg/mg), Schering Corp., Bloomfield, N.J.; and amikacin (potency, 905 µg/mg), Bristol Laboratories, Syracuse, N.Y. Stock solutions for each aminoglycoside at 100 µg/ml in double-distilled water were stored at -70°C.

Quality control samples, at two concentrations, were prepared with known amounts of aminoglycosides in fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.).

The buffers used were morpholineethanesulfonic acid (MES) from Sigma Chemical Co., St. Louis, Mo., and succinic acid from Eastman Kodak Co., Rochester, N.Y. ¹⁴C-acetyl-CoA (40 to 60 mCi/mmol, [NEC-313], purchased from New England Nuclear Corp., Boston, Mass., and KAT EC 2.3.1.55 (prepared from *Escherichia coli* W677/R5; specific activity, 20 U/mg) from P-L Biochemicals were used in the reaction mixture. Phosphocellulose paper squares (type P-81; Whatman, Inc., Clifton, N.J.) were cut into squares (0.75 by 0.75 in. [ca. 1.9 by 1.9 cm]). Scintillation fluid was made from 15.2 g of PPO (2,5-diphenyloxazole) and 0.19 g of POPOP (1,4-bis[2]-(5-phenyloxazolyl)benzene), both from New England Nuclear, in scintillation-grade toluene. Bovine serum albumin (0.1% concentration) was dissolved into 0.1 M succinic acid or MES buffer. Nonradioactive acetyl-coenzyme A from P-L Biochemicals was only used in the amikacin reaction mixture. Radioimmunoassay kits for amikacin and kanamycin were obtained from Monitor Science, Newport Beach, Calif. A bioassay kit for amikacin was also purchased from the same supplier.

REA are based on the enzymic acetylation of the aminoglycoside, with radioactive acetyl coenzyme A. Acetylated aminoglycosides retain a net positive charge at a pH of >5.0.

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Acetyl coenzyme A and acetic acid are acids at this pH. Cation exchange paper, phosphocellulose, is used to selectively bind acetylated aminoglycoside while not binding acetyl coenzyme A. Acetylation of the aminoglycoside is assessed by determining the amount of radioactivity bound to the phosphocellulose in the presence of the enzyme. A 10- μ l serum sample or standard aminoglycoside solution was incubated with 50 μ l of buffer containing ^{14} C-acetyl-CoA at various concentrations for each aminoglycoside and 1.67 U of KAT per ml. Each sample was assayed in duplicate, including blanks and standards. After incubation at 37°C, a 50- μ l reaction mixture sample was pipetted onto 3/4-in.² (ca. 1.9-cm²) phosphocellulose paper. The papers were batch washed ($n \leq 30$) with 1 liter of distilled water with appropriate buffer at 1.0 mmol/liter, then washed twice for 6 and 2 min to remove unbound ^{14} C-acetyl-CoA, and allowed to air dry. The papers were then placed into 10 ml of scintillation fluid, and the contained radioactivity was determined with a scintillation spectrometer.

Optimum conditions for quantitation of each antibiotic was determined for the following: time of incubation, pH and ^{14} C-acetyl-CoA concentration. A sample containing 30 μ l of tobramycin and netilmicin (20 μ g/ml) and 30 μ l of kanamycin and amikacin (40 μ g/ml) was incubated with 10 μ l of KAT and 150 μ l of buffer. A total of 16 buffer combinations at eight different pHs were tested.

Solutions of MES and of the sodium salt of succinic acid (0.1 M) were prepared at the following pHs: 5.1, 5.3, 5.5, 5.7, 5.9, 6.1, 6.3, and 6.5. The acetylation of each aminoglycoside was tested at each pH. Optimum pH was defined as that yielding the greatest amount of acetylated product with 30 min of incubation at 37°C.

The optimum time of incubation was determined by removing a 50- μ l sample from a mixture containing 500 μ l of the appropriate buffer, 50 μ l of ^{14} C-acetyl-CoA, 50 μ l of KAT, and 100 μ l of the appropriate antibiotic standard solution at 0, 2, 4, 6, 10, 20, 30, 40, and 60 min at 37°C.

After the determination of optimum pH and duration of incubation, the optimum ^{14} C-acetyl-CoA concentration was determined for each aminoglycoside. A sample containing 100 μ l of buffer, 10 μ l of KAT (20 U/ml per mg), and 20 μ l of aminoglycoside (20 μ g of tobramycin and netilmicin per ml and 40 μ g of kanamycin and amikacin per ml) was incubated with 10 μ l of ^{14} C-acetyl-CoA at concentrations of 2.76, 5.42, 13.8, 27.6, and 82.9 μ mol/liter. Each was incubated at the optimum pH for the optimum length of time (determined above).

Normal human serum containing each aminoglycoside at 5 and 15 μ g/ml was spiked with the following selected β -lactam antibiotics: cephalothin, cefazolin, and cefamandole at 100 μ g/ml and ticarcillin and carbenicillin at 200 μ g/ml. Each spiked sample was assayed in duplicate. Known amounts of each aminoglycoside were dissolved in pooled human serum at concentrations of 0, 2.5, 5.0, 10, and 15 μ g/ml for netilmicin and tobramycin and 0, 5, 10, 20, 30, and 40 μ g/ml for kanamycin and amikacin. Each sample was assayed for the added aminoglycoside in duplicate under the optimum conditions. Quality control samples were assayed each day in duplicate.

Samples derived from patients receiving aminoglycosides were assayed by methods other than the REA for comparative purposes. Tobramycin was quantitated by bioassay (12). Kanamycin was assayed by radioimmunoassay (11). Netilmicin was assayed by microbiological assay by E. Oden of Schering-Plough, Kenilworth, N.J. Amikacin was assayed by bioassay (2) and radioimmunoassay (11). Linear regres-

sion analysis was used to determine the correlation between the methods.

RESULTS

The optimum pH for each aminoglycoside is shown in Fig. 1. P-L Biochemicals, a supplier of KAT, suggests that the optimum pH for the acetylation of kanamycin is pH 5.7. We confirmed this observation. The pH optimum for the other aminoglycosides was in the same range, but differences were observed. The maximum acetylation of tobramycin occurred with succinic acid buffer (0.1 M) at pH 5.5. The maximum acetylation of netilmicin was with MES buffer (0.1 M) at pH 5.9. Amikacin was maximally acetylated at a pH of 6.3 with both succinic acid and MES buffer. MES buffer adjusted to pH 5.9 and 6.3 was chosen for the assay of netilmicin and amikacin, respectively. Succinic acid at pH 5.5 and 5.7 was chosen for tobramycin and kanamycin, respectively.

The effect of incubation time on acetylation is shown in Fig. 2. The acetylation of tobramycin and netilmicin at a concentration of 20 μ g/ml increased linearly for 20 min;

FIG. 1. The effect of pH between 5.1 and 6.5 on acetylation (counts per minute) at one concentration of aminoglycoside. Tobramycin and netilmicin were tested at 20 μ g/ml; kanamycin and amikacin were tested at 40 μ g/ml. Tests were carried out with 0.1 M MES (Δ) and 0.1 M succinic acid (●).

acetylation did not measurably increase with longer periods of incubation. The acetylation of kanamycin and amikacin at a concentration of 40 $\mu\text{g/ml}$ increased linearly to 40 min of incubation; further acetylation did not increase substantially (<5%) with longer incubation times. For a routine assay, a 20-min incubation was used for tobramycin and netilmicin, and a 40-min incubation was used for kanamycin and amikacin.

The optimum ^{14}C -acetyl-CoA concentration was determined for each aminoglycoside (Fig. 3). Tobramycin and netilmicin were similar; both had maximum acetylation with a ^{14}C -acetyl-CoA concentration of 13.8 $\mu\text{mol/liter}$. At higher concentrations (values of up to three times the optimum concentration), the amount of acetylation decreased or increased by less than 2%. Kanamycin had a maximum acetylation with ^{14}C -acetyl-CoA of 27.6 $\mu\text{mol/liter}$ and did not increase more than 5% at higher concentrations. Maximum acetylation for amikacin occurred at a ^{14}C -acetyl-CoA concentration of 82.9 $\mu\text{mol/liter}$, and there was apparent substrate inhibition at higher concentrations. In routine assays of the aminoglycosides, the optimum and efficient ^{14}C -acetyl-CoA concentration found in these experiments was utilized. The optimum assay conditions are summarized in Table 1.

Interassay precision for each aminoglycoside in which a quality control sample was assayed on separate days is shown in Table 2. Quality control samples were stored at -70°C for 6 months; tobramycin was stored for up to 2 years at this temperature. A stored sample was assayed in parallel

FIG. 2. Effect of length of incubation at 37°C on acetylation. Top graph represents kanamycin (▲) and amikacin (○) at 40 $\mu\text{g/ml}$. Bottom graph represents tobramycin (■) and netilmicin (●) at 20 $\mu\text{g/ml}$.

FIG. 3. Effect of cofactor concentration on acetylation (counts per minute). Counts per minute were plotted with various concentrations of ^{14}C -acetyl-CoA (micromoles per liter). Symbols: (■) amikacin, (▲) tobramycin, (○) netilmicin, and (●) kanamycin.

with freshly prepared samples for each aminoglycoside. The coefficient of variation was less than 10%; except for the low-concentration quality control sample containing tobramycin or kanamycin. In the case of kanamycin, the high coefficient of variation could be due to the low number of samples assayed, i.e., $n = 8$. It was observed that the apparent concentration in the low-tobramycin quality control decreased during 2 years of storage.

TABLE 1. Optimum assay conditions for aminoglycoside quantitation

Antibiotic	Type of buffer	pH	Incubation time (min)	Acetyl coenzyme A concn ($\mu\text{mol/liter}$)
Tobramycin	Succinic acid	5.5	20	13.8
Netilmicin	MES	5.9	20	13.8
Kanamycin ^a	Succinic acid	5.7	40	27.6
Amikacin	MES	6.3	40	82.9 ^b

^a Standard assay modified (see text).

^b Cold acetyl coenzyme A, 27.6 $\mu\text{mol/liter}$, was used to dilute ^{14}C -acetyl-CoA for a total acetyl coenzyme A concentration of 82.9 $\mu\text{mol/liter}$.

Accuracy data are shown in Table 3. Mean recoveries of tobramycin, netilmicin, kanamycin, and amikacin were 107, 105.6, 99.2, and 93.3%, respectively. The range of recoveries of all aminoglycosides was 82 to 125%.

The linearity of typical standard curves is shown in Table 4. On regression analysis, r was greater than 0.996 for all of the aminoglycosides. Kanamycin posed problems in quantitation because the aqueous standard curve was nonlinear in concentrations between 30 and 100 $\mu\text{g/ml}$. We therefore sought the accurate quantitation of kanamycin at concentrations of up to 50 $\mu\text{g/ml}$. Serum samples containing known concentrations of kanamycin did not correlate well with measured concentrations. Modifications of the standard assay, heating all serum samples at 60°C for 15 min, and diluting the standards, quality control samples, and the unknowns with equal volumes of water resulted in a linear standard curve to 50 $\mu\text{g/ml}$ in water or serum. With this modification, known concentrations correlated with measured concentrations (Table 3). This revised method for assaying kanamycin was adopted and used routinely.

Five selected β -lactam antibiotics were dissolved in serum samples containing indicated concentrations of aminoglycosides to determine their effect on the assay. Previous authors (2, 6, 14) have reported that carbenicillin and ticarcillin form complexes with gentamicin and tobramycin, thereby inactivating the latter compounds. The data presented in Table 5 confirm this observation. The apparent concentration of tobramycin and kanamycin was significantly decreased ($P < 0.01$) when carbenicillin and ticarcillin were added at a final concentration of 200 $\mu\text{g/ml}$. Assayable kanamycin concentration decreased 50% or more. The amount of amikacin decreased at the higher concentrations of ticarcillin and carbenicillin, but the decrease was not statistically significant. There was no detectable change in netilmicin concentration when mixed with carbenicillin or ticarcillin. The other three β -lactam antibiotics tested, cephalothin, cefazolin, and cefamandole, did not have a detectable effect on assayable aminoglycoside concentration.

The optimum conditions for the quantitation of each aminoglycoside were used for the analysis of patient specimens (Table 1). Figure 4 depicts the results obtained when patient specimens were assayed by the method described and alternative methods. The values obtained by the method described here (REA) were plotted against values obtained by a reference method. The correlation coefficients of the comparisons are shown in Fig. 4. The r values were greater than 0.9 for all reference methods when compared with REA, and the slopes ranged from 0.8 to 1.2. Radioimmunoas-

TABLE 3. Recovery of aminoglycoside added to human serum

Aminoglycoside	Concn ($\mu\text{g/ml}$)	
	Actual	Assayed
Tobramycin	0.0	0.2
	0.0	0.1
	1.0	1.1
	5.0	5.1
	5.0	5.6
	7.5	7.7
	10.0	10.7
	10.0	11.0
	15.0	15.4
	15.0	15.6
Netilmicin	1.6	2.0
	3.0	3.5
	5.2	5.6
	6.5	6.7
	8.8	9.0
	10.0	10.5
	12.6	12.6
	18.0	18.1
Kanamycin	5.0	4.4
	5.0	4.7
	5.0	4.7
	5.0	5.0
	10.0	9.8
	10.0	10.6
	12.0	12.7
	12.0	13.8
Amikacin	40.0	37.3
	40.0	39.2
	10.0	8.2
	10.0	9.6
	20.0	19.2
	20.0	19.7
	25.0	22.2
	25.0	22.8
	30.0	28.7
	30.0	28.8
	40.0	36.8
	40.0	38.7

say of amikacin ($n = 17$) was also compared with REA. The results (data not shown) were $r = 0.9843$, with a slope of 0.9631.

DISCUSSION

Tobramycin, kanamycin, amikacin, and gentamicin are the aminoglycosides most frequently administered parenterally in the United States; netilmicin and sisomicin are widely used in Europe. Monitoring the serum concentration to

TABLE 2. Interassay precision

Drug	n	Concn ($\mu\text{g/ml}$) (mean $\pm$ SD)	% CV ^a
Tobramycin	63	2.1 $\pm$ 0.30	14.3
	63	9.2 $\pm$ 0.72	7.9
Netilmicin	11	3.3 $\pm$ 0.30	9.1
	11	10.8 $\pm$ 0.26	2.4
Kanamycin	8	7.3 $\pm$ 1.23	16.8
	8	18.5 $\pm$ 0.57	3.1
Amikacin	12	5.2 $\pm$ 0.25	5.0
	12	15.0 $\pm$ 0.65	4.4

^a CV, Coefficient of variation.

TABLE 4. Typical standard curves (cpm^a versus concentration)

Aminoglycoside	r	y Intercept	Slope
Tobramycin	0.9966	721.1	1,035.6
Netilmicin	0.9983	-26.8	959.4
Kanamycin ^b	0.9991	657.0	485.4
Amikacin	0.9992	2,708.1	727.5

^a cpm, Counts per minute.

^b Revised methodology (see text).

ensure efficacy and avoid toxic concentrations is commonly performed when this class of antibiotics is administered. The REA utilizing gentamicin 3'-adenylyltransferase is not suitable for netilmicin, kanamycin, and amikacin, as these compounds are poor substrates.

KAT, which is now commercially available, acetylates tobramycin, netilmicin, kanamycin, and amikacin. Optimum conditions, i.e., pH, incubation time, and ^{14}C -acetyl-CoA concentration, were defined which increased efficiency and sensitivity of the assay. With purified standards, common buffers, the enzyme KAT, ^{14}C -acetyl-CoA, a scintillation counter, and common clinical laboratory equipment, a well-characterized and simple assay was established. The REA correlated well with reference methods. With the agar-disk diffusion assay and radioimmunoassay, r was greater than 0.9. The REA technique requires only 10 μl of serum and 2 to 3 h for performance; of this time, only half is used for technical procedures. The speed of the assay allows clinicians to perform dose adjustments within the dosing interval.

TABLE 5. Apparent effect of selected β -lactam antibiotics on aminoglycoside concentrations

Aminoglyco- side	β -lactam	Concn ($\mu\text{g/ml}$)		
		β -lactam	Amino- glycoside	Measured
Tobramycin	Cephalothin	100	5.0	5.1
	Cephalothin	100	15.0	14.7
	Cefazolin	100	5.0	5.0
	Cefazolin	100	15.0	14.4
	Carbenicillin	200	5.0	3.9 ^a
	Carbenicillin	200	15.0	11.2 ^a
	Ticarcillin	200	5.0	3.7 ^a
Netilmicin	Ticarcillin	200	15.0	11.2 ^a
	Cephalothin	100	5.0	5.8
	Cephalothin	100	15.0	15.8
	Cefazolin	100	5.0	5.8
	Cefazolin	100	15.0	15.6
	Carbenicillin	200	5.0	5.7
	Carbenicillin	200	15.0	15.5
Kanamycin	Ticarcillin	200	5.0	5.6
	Ticarcillin	200	15.0	15.1
	Cephalothin	100	5.0	5.6
	Cephalothin	100	15.0	15.7
	Cefazolin	100	5.0	5.3
	Cefazolin	100	15.0	15.9
	Cefamandole	100	5.0	4.5
Amikacin	Cefamandole	100	15.0	14.8
	Carbenicillin	200	5.0	1.4 ^a
	Carbenicillin	200	15.0	6.3 ^a
	Ticarcillin	200	5.0	2.3 ^a
	Ticarcillin	200	15.0	7.5 ^a
	Cephalothin	100	5.0	5.8
	Cephalothin	100	15.0	14.3
Amikacin	Cefazolin	100	5.0	5.0
	Cefazolin	100	15.0	14.4
	Cefamandole	100	5.0	5.7
	Cefamandole	100	15.0	14.0
	Carbenicillin	200	5.3	5.3
	Carbenicillin	200	15.0	12.4
	Ticarcillin	200	5.0	5.2
Amikacin	Ticarcillin	200	15.0	13.3

^a Significant change in aminoglycoside concentration (Student t test; $P \leq 0.05$).

FIG. 4. Comparison studies of REA developed here (y-axis) versus established commercial assays (x-axis).

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