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Enzymatic Adenylylation by Aminoglycoside 4'-Adenylyltransferase and 2"-Adenylyltransferase as a Means of Determining Concentrations of Aminoglycoside Antibiotics in Serum

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A fast and accurate enzymatic estimation of the concentration of aminoglycoside antiobiotics in serum is given by aminoglycoside 4'-adenylyltransferase (synonym: tobramycin adenylyltransferase), a new enzyme recently found in tobramycin-resistant staphylococci (P. Santanam and F. H. Kayser, J. Infect. Dis. [Suppl.], in press). This enzyme is useful in assaying the kanamycins, the butirosins, amikacin, ribostamycin and, if required, the neomycins, tobramycin, and paromomycin. In combination with the assay using aminoglycoside 2"-adenylyltransferase (synonym: gentamicin adenylyltransferase) to determine the level of gentamicin, sisomicin, and tobramycin, a convenient method to estimate a wide range of aminoglycoside antibiotics in patients' sera is described.

Enzymatic adenylylation and acetylation have been used to estimate the concentration of aminoglycoside antibiotics in sera of patients undergoing treatment with these drugs (3, 5, 7-9, 14, 15, 17). The enzymatic assays are known to be fast and accurate and are valuable in the assessment for maintaining an effective and nontoxic serum level of the drugs. Aminoglycoside 3-*N*-acetyltransferase [AAC(3)] is used in assaying the gentamicin C antibiotics and sisomicin (18). Aminoglycoside 2"-adenylyltransferase [AAD(2")] is used in assaying the C gentamicins, the kanamycins, sisomicin, and tobramycin (7, 9, 15). The aminoglycoside 6'-*N*-acetyltransferase [AAC(6')], because of its broad substrate spectrum, is useful in assaying a wide range of aminoglycoside antibiotics (3, 8, 17). As an alternative to AAC(6'), we propose to use two adenylylating enzymes. With aminoglycoside 4'-adenylyltransferase [AAD(4')], serum levels of the kanamycins, the butirosins, amikacin, ribostamycin, and, if required, the neomycins and paromomycin can be determined. With AAD(2"), the gentamicin C antibiotics, sisomicin, and tobramycin can be assayed.

MATERIALS AND METHODS

Antibiotics and chemicals. Tobramycin was supplied by Eli Lilly and Co. (Indianapolis, Ind.); the kanamycins, butirosins, and amikacin were supplied by Bristol laboratories (Syracuse, N.Y.); the neomycins were provided by Upjohn Co. (Kalamazoo, Mich.); paromomycin was supplied by Parke, Davis and Co. (Detroit, Mich.); the gentamicins and sisomicin were supplied by Schering Corp. (Bloomfield, N.J.).

Commercial samples of gentamicin C complex, kanamycin, and neomycin were provided by the hospital pharmacy of the University of Zurich.

Radioactive chemicals were supplied by the Radiochemical Centre (Amersham, England). Dithiothreitol and adenosine triphosphate (ATP) were supplied by Calbiochem (Los Angeles, Calif.). All other inorganic salts of analytical grade were supplied by E. Merck AG (Darmstadt, Germany).

Production of enzymes. AAD(4') was extracted by the osmotic shock method from *Staphylococcus epidermidis* 109 (P. Santanam and F. H. Kayser, J. Infect. Dis. [Suppl.], in press). The procedure was slightly modified by using 20% sucrose in tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.3) that contained 0.006 instead of 0.003 M ethylenediaminetetraacetic acid for suspending the cells before the shock step. The enzymatic extract obtained by osmotic shock is stable for several hours at room temperature, for 1 week at 4°C, and for several months at −40°C (Santanam and Kayser, in press). AAD(2") was obtained by osmotic shock treatment of *Escherichea coli* W677/HJR66 as described elsewhere (7, 12).

Enzymatic assays. The methods used were described by Benveniste, Davies, and their associates (2, 6). Serum samples were kept for 5 min in a water bath at 60°C before use to inactivate any trace of adenosine triphosphatase.

To measure adenylylation of drugs by AAD(4'), the reaction mixture consisted of 1.5 μmol of Tris-hydrochloride buffer, 1 μmol of $MgCl_2$, 55 nmol of dithiothreitol, 20 nmol of [2-^{3}H]ATP (specific activ-

ity, 60 μCi/μmol), 10 μl of serum, and 25 μl of osmotic shockate, in a total volume of 60 μl. The pH of the buffer was adjusted to 8.3, found to be optimum for assaying the neomycins and paromomycin, and to 7.5, found to be optimum for assaying the rest, in the presence of serum. After incubation for 35 min at 35°C, 50-μl samples were pipetted onto 2-cm² phosphocellulose paper (Whatman P-81; Balsbone, Maidstone, U.K.), which was washed twice with distilled water, dried at 55°C, and counted for radioactivity in 10 ml of a toluene-based scintillation fluid. Reaction mixtures devoid of either the enzyme or the drug were used as controls for nonspecific binding of [2-³H]ATP. The adenylylation of drugs by AAD(2") was examined by a slight modification of the procedure described by Furger et al. (7). The reaction mixture contained 1.5 μmol of Tris-hydrochloride buffer at pH 8.3, 1.0 μmol of $MgCl_2$, 55 nmol of dithiothreitol, 20 nmol of [2-³H]ATP (specific activity, 60 μCi/μmol), 20 μl of serum containing the drug, and 25 μl of osmotic shockate. After 15 min of incubation at 35°C, 50-μl samples were pipetted onto 2-cm² phosphocellulose papers, which were washed, dried, and then counted for radioactivity as described above.

RESULTS

Standard solutions containing 20, 10, 5, and 2.5 μg of laboratory standard samples of amikacin, kanamycin, neomycin, paromomycin, and butirosin per ml were prepared by a twofold dilution in pooled human sera from stocks and assayed by AAD(4'). The degree of adenylylation of these antibiotics was found to be directly proportional to the drug between 2.5 and 20 μg/ml (Fig. 1). The high molar ratio of ATP to the drug (20 nmol of ATP to 0.34 to 0.68 nmol of drug) allowed the estimation of kanamycin, neomycin, and paromomycin also at serum concentrations between 20 and 40 μg/ml. Because of the slow adenylylation of amikacin and butirosin (Santanam and Kayser, in press), however, either the incubation time for assaying these drugs must be doubled or the serum sample must be appropriately diluted. Dilution to concentrations between 2.5 and 20 μg/ml was practical and gave reproducible and accurate results. The incubation time of 35 min at 35°C was necessary for completion of adenylylation in the presence of serum. The drugs, however, are not all adenylylated to the same extent, possibly because the end products differ in their ability to inhibit the enzymatic reaction. On the basis of known drug concentrations in pooled human sera and the concentrations assayed, it is possible to calculate the efficiency of assay (Table 1). The average adenylylation of amikacin, for example, was 96.3%, and the relative standard deviation was 5.5%. Similar data were obtained with the other drugs. Assays of 36 different human serum samples, containing either 5 or 10 μg of amikacin per ml, gave a relative standard deviation of 6% and 4%, respectively, which shows no significant variation between different serum samples.

Standards of commercially available gentamicin complex, tobramycin, and sisomicin were

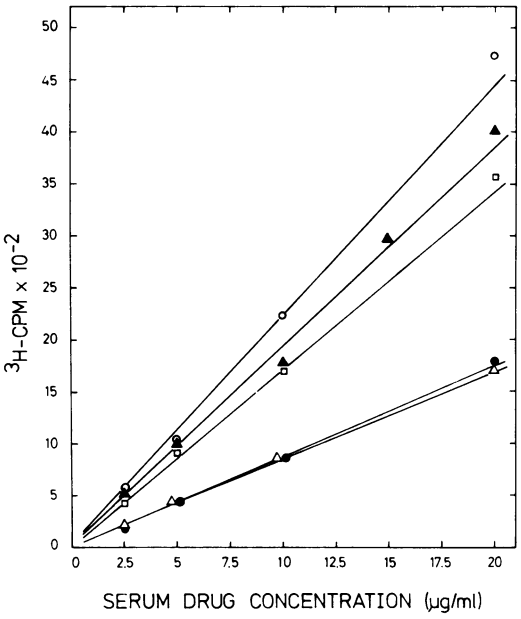

FIG. 1. *Enzymatic adenylylation by aminoglycoside 4'-adenylyltransferase of various aminoglycoside antibiotics in human blood sera (standard curves). Experimental details of the assay are given in the text. Count rates are corrected for background activity. Symbols:* (●) *amikacin,* (□) *kanamycin,* (△) *butirosin,* (○) *neomycin,* (▲) *paromomycin.*

TABLE 1. *Efficiency of the assay for amikacin*

Amikacin assayed (μg/ml)	Amt of amikacin in assay tubes (mmol × 10^{-8})	Amt of amikacin determined by enzymatic assay (mmol × 10^{-8})	Adenylylation[a] (%)
3	5.13	4.87	95
3.5	5.98	5.3	88.6
4	6 84	7.35	107.5
4.5	7.69	7.01	91.1
6	10.26	9.58	93.3
7	11.97	12.14	101.4
8	13.68	12.14	88.8
9	15.39	14.8	96.1
12	20.52	20.7	100.8
14	23.94	23.1	96.4
16	27.36	26.3	96.3
18	30.78	30.78	100

[a] The average adenylylation was 96.3%, and the standard deviation was 5.5%.

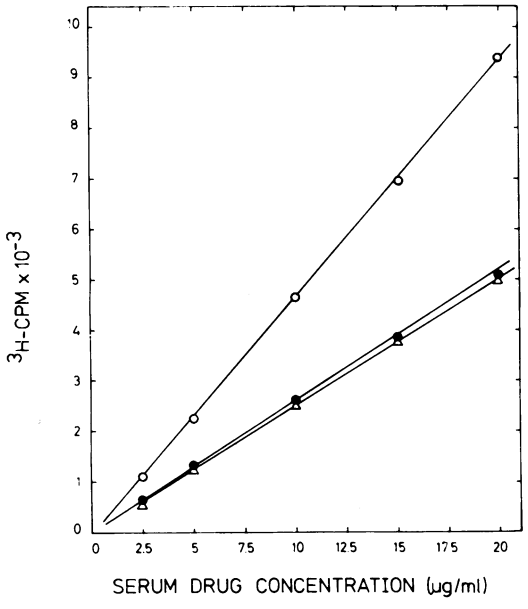

FIG. 2. *Enzymatic adenylylation by aminoglycoside 2'adenylyltransferase of different aminoglycoside antibiotics in human blood sera (standard curves). Experimental details of the assay are given in the text. Count rates are corrected for background activity. Symbols:* (●) *gentamicin,* (△) *tobramycin,* (○) *sisomicin.*

TABLE 2. *Efficiency of the assay for gentamicin*

Gentamicin assayed (µg/ml)	Amt of gentamicin in assay tubes (mmol × 10^{-7})	Amt of gentamicin determined by enzymatic assay (mmol × 10^{-7})	Adenylylation[a] (%)
0.25	0.09	0.08	88.9
0.4	0.15	0.16	106.6
0.7	0.26	0.22	88.0
1.4	0.52	0.44	84.6
2.1	0.77	0.7	91.0
2.8	1.03	0.92	89.3
3.5	1.29	1.24	96.1
4.2	1.55	1.47	94.8
5.6	2.06	2.09	101.4
6.2	2.28	2.39	104.8
6.8	2.51	2.43	96.8
7.5	2.76	2.77	100.3
8.4	3.09	3.06	99.0
10.2	3.75	3.68	98.1
12.4	4.56	4.52	99.1
15.0	5.5	5.32	96.7

[a] The average adenylylation was 96%, and the standard deviation was 6.2%.

prepared from 20-µg/ml stocks and assayed by AAD(2″) as described. The linear relationship between adenylylation and the amount of drug is demonstrated in Fig. 2. Table 2 summarizes efficiency of the assay for gentamicin. The average adenylylation was 96%, and the relative standard deviation was 6.2%.

DISCUSSION

In the last few years, a number of rapid methods have been developed for measuring the concentration of aminoglycoside antibiotics in serum and other body fluids. These methods include microbiological assays (1, 13), radioimmunoassays (4, 10), and enzymatic assays (3, 5, 7–10, 14–17). Each type of assay has its own advantages. Radioimmunoassays and enzymatic assays are highly specific and are useful in assaying aminoglycosides, particularly in the presence of other antibiotics. However, workers having experience with both assay systems prefer enzymatic assays to radioimmunoassays because the reagents are easier to prepare, the experimental protocols are somewhat simpler, and the results are less affected by minor variations in experimental procedures (10).

AAD(2″) was the first enzyme proposed for enzymatic determination of gentamicin (16). This enzyme has been satisfactorily used in our laboratory for more than 3 years. Because of production of new aminoglycoside antibiotics, which are not modified by this enzyme, and because of demands by clinicians to determine in certain patients concentrations of neomycin and paromomycin, the suitability of AAD(4′) was examined. The ability of AAD(4′) to adenylylate the aminoglycoside 4′-OH group and perhaps also the 4″-OH group, because 4′-deoxykanamycin was found to be a substrate but not 4′deoxybutirosin, makes it unique in having a very broad substrate spectrum (Santanam and Kayser, in press). It surpasses AAC(6′) in this respect and eliminates the requirement for [^{14}C]acetyl coenzyme A as a cosubstrate, thus reducing the cost per assay to less than half. It is also more stable at room temperature than AAC(6′) (8), and the activity does not appreciably change during storage at −40°C over a period of at least 2 months (Santanam and Kayser, in press). AAD(2″), on the other hand, adenylylates the three components of commercially available gentamicin, whereas AAC(6′) does not acetylate gentamicin C_1. Since this component is present in varying amounts from batch to batch (professional brochure, Schering Corp., 1974) and since gentamicin C_1 shows a somewhat different pharmacokinetic behavior than the gentamicin complex (11), it would be appropriate to use AAD(2″) for assaying gentamicin.

Thus, AAD(4′) in conjunction with AAD(2″)

is ideally suited to assay, with identical reagents, many of the aminoglycoside antibiotics that are used currently in chemotherapy. Both the enzymes are easily extractable in the form of osmotic shockate, which can be used directly without purification.

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