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Enzymatic Acetylation as a Means of Determining Serum Aminoglycoside Concentrations

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A rapid and accurate enzymatic assay has been developed for the determination of blood serum levels of BB-K8 and other suitable aminoglycoside antibiotics.

Extensive clinical use of aminoglycoside antibiotics has prompted the development of rapid assay methods to determine the concentration of these antibiotics in the serum of patients being treated with the drug. Microbiological methods are normally used to determine serum drug concentrations, but since such assays often require long time periods, more rapid and precise assay methods are desirable. By using enzymatic methods, efficient monitoring of the concentration of an aminoglycoside in blood serum can be accomplished at frequent intervals during treatment. This allows the therapeutic adjustments necessary to maintain a desired serum drug concentration, particularly in patients who excrete the drug at a more rapid rate than is normal.

Such enzymological assays employ drug-modifying enzymes isolated from R-factor (R^+)-containing strains of *Escherichia coli*. The general principles of the assay procedure rely on the facts that (i) most aminoglycoside antibiotics are strongly cationic and bind to phosphocellulose paper, (ii) the functional group which is enzymatically transferred to the aminoglycoside drug can be obtained in a radiolabeled form, and (iii) unreacted radiolabeled cofactor does not adhere to phosphocellulose paper. Thus, determination of the amount of radioactivity bound to phosphocellulose paper in the presence of drug and enzyme allows determination of the amount of drug present. Such a technique has been described (4, 5; P. S. Lietman et al., Abstr. Annu. Meet. Amer. Soc. Microbiol., 72nd, Philadelphia, p. 121, 1972) and successfully applied for the determination of gentamicin serum concentrations via the transfer of a ^{14}C-adenylic acid moiety to gentamicin by using gentamicin adenylylate synthetase isolated from a gentamicin-resistant R^+ *E. coli*. However, this assay cannot be used to assay for aminoglycosides which are inert to this modification. For example, neomycin, butirosin, and BB-K8 cannot be adenylylated, and their clinical use may require a rapid assay. The availability of an enzymatic assay with wide applicability is thus desirable. The only enzyme known to modify a large number of aminoglycoside antibiotics is kanamycin acetyltransferase (KAT) first described by Okamoto and Suzuki (3) and Umezawa et al. (6) and subsequently characterized by Benveniste and Davies (1). This enzyme acetylates all aminoglycosides with a free 6'-amino group. We have developed a rapid, enzymatic assay for various aminoglycosides, including BB-K8 and butirosin, by using KAT with 1-^{14}C-acetyl coenzyme A-*1*-^{14}C (AcCoA-*1*-^{14}C) as a cosubstrate.

The enzyme is readily obtained by osmotic-shock treatment (1) of *E. coli* strains harboring the R-factor R5 (*E. coli* W677/R5 was used in our studies). The "osmotic shockate" obtained by this procedure serves directly as the enzyme source without further purification. The acetylating activity is unstable, and the preparation must be maintained at 4 C at all times during use. However, the enzyme may be stored at −71 C for several months without appreciable loss of activity. It is convenient to prepare as much as 200 to 400 ml of enzyme at one time, which is then frozen in small portions sufficient for one day's assay. Repeated freezing and thawing of the enzyme results in loss of activity.

Figure 1 presents the results obtained when serum preparations of known drug concentration are incubated with KAT and AcCoA-*1*-^{14}C.

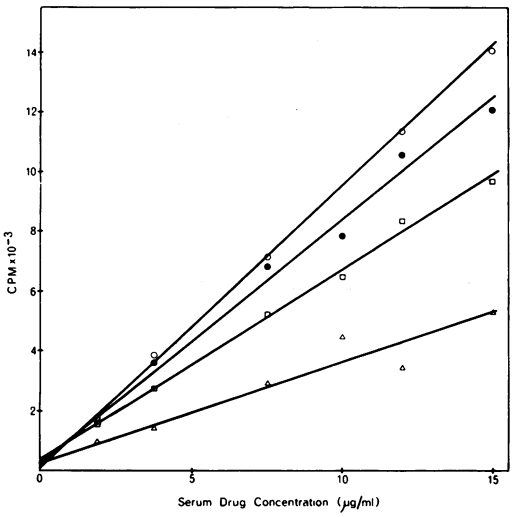

FIG. 1. *Enzymatic acetylation of various aminoglycosides in blood serum. E. coli W677/R5 were grown to late log phase in rich medium containing kanamycin A (20 μg/ml) to prevent R-factor segregation. Osmotic shockate was prepared as noted in text. Experimental details are given in the text. Count rates are corrected for background activity.* ○, *Kanamycin A;* □, *BB-K8;* ●, *tobramycin;* △, *gentamicin* C_{1A}.

The standards were prepared by adding the appropriate amounts of drug to serum. We have found little variation in results by using a variety of serum specimens. The reaction mixtures used in these experiments contained 20 μliters of serum (containing antibiotic from 0 to 50 μg/ml), 10 μliters of "shockate," 2.75 nmol of AcCoA-*1*-^{14}C (specific activity 36.3 Ci/mol), 3.0 μmol of sodium citrate buffer (pH 5.7), 0.3 μmol of $MgCl_2$, and 0.1 μmol of dithiothreitol in a total volume of 45 μliters. The reaction mixtures were prepared in tubes in an ice bath, and the enzyme was added last. Citrate, $MgCl_2$, and dithiothreitol may be premixed as a "cocktail" prior to addition to the reaction vessels. Such a "cocktail" is stable if stored at −71 C. The reactants were mixed by gentle shaking and incubated for 15 min at 30 C, at which time the tubes were returned to the ice bath, and 20-μliter samples of each were applied to separate 2.25-cm^2 phosphocellulose paper squares (Whatman P-81). The papers were immersed in deionized water at 70 C, 4 min later they were rinsed three times with distilled water (300 ml per rinse) and dried, and radioactivity was determined in a scintillation counter by using a toluene-based scintillant. After use, the paper squares may be removed and the fluid may be reused. A control reaction mixture containing drug-free serum was included, because the use of high-specific-activity AcCoA-*1*-^{14}C results in a substantial blank reading which must be subtracted from other values. The data of Fig. 1 indicate a linear relationship between drug concentration and radioactivity transferred to the paper for all drugs studied.

Because some aminoglycosides, notably kanamycin and BB-K8, are used at serum concentrations higher than 20 μg/ml, it is desirable to have a linear standard curve for the determination of serum concentrations of these drugs above this level. Fig. 2 illustrates that the desired linear relationship can be obtained. The high molar ratio of AcCoA to drug (14.5 for the assay of the 50 μg/ml drug sample) and extended period of incubation are necessary to obtain accurate values at the higher concentrations assayed. The data of Fig. 2 represent duplicate assays at all drug concentrations.

On the basis of the known drug concentrations in the serum samples and the specific activity of the ^{14}C-AcCoA employed in the assay, it is possible to calculate the efficiency of the assay for BB-K8. Table 1 shows the results of such a calculation by employing the data and experimental conditions of Fig. 2.

These data indicate that the degree of acetylation of the BB-K8 added to samples from different sources is nearly quantitative and relatively constant from sample to sample over a wide range of drug concentrations. Assays of

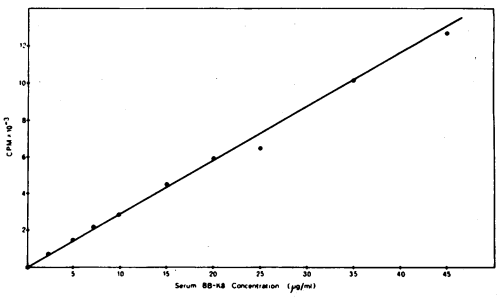

FIG. 2. *Enzymatic acetylation of a range of concentrations of BB-K8 in serum. Drug-free serums from several sources were adjusted to the desired BB-K8 concentrations by using concentrated aqueous BB-K8 solution. Samples (10 μliters) were then mixed with 12.1 nmol of AcCoA-1-*^{14}C *(specific activity 24.8 Ci/mol), 3.0 μmol of pH 5.7 citrate buffer, 0.3 μmol of* $MgCl_2$, *0.1 μmol of dithiothreitol, and 10 μliters of KAT shockate preparation in a total volume of 40 μliters. After incubation for 20 min at 30 C, 25-μliter samples of the reaction mixtures were applied to 2.25* cm^2 *squares of phosphocellulose paper (Whatman P-81), washed, dried, and counted as described in the text.*

TABLE 1. *Efficiency of the assay for BB-K8*

BB-K8 assayed (μg/ml)	Amt of BB-K8 in Rx tubes (mmoles × 10^8)	Amt of BB-K8 determined by acetylation assay (mmoles × 10^8)	Acetylation (%)[a]
2	3.42	3.48	102
5	8.56	7.81	91.2
7	12.0	11.3	94.2
10	17.1	15.3	89.5
15	25.7	24.0	93.4
20	34.2	31.1	90.9
25	42.8	34.2	79.9
35	59.9	53.5	89.3
45	77.1	65.1	84.4

[a] The average percentage of acetylation was 90.5%, and the standard deviation was 6.2.

23 different drug-free serum samples showed that the blank values were constant to within ±6%. We anticipate that it will be possible to adapt the KAT assay for use in determining drug concentrations in other bodily fluids.

The KAT assay was used to determine serum BB-K8 concentrations in several "unknown" samples kindly provided by K. E. Price of Bristol Laboratories. Table 2 shows a comparison of the concentrations determined by microbiological and enzymatic assay methods. Fig. 2 served as the standard curve for the determination of the BB-K8 concentration in these unknowns. The standard drug serum samples and the unknowns were assayed simultaneously. The values for the concentration of BB-K8, as indicated by the two assay methods, are in good agreement, especially at the lower drug concentration.

The KAT assay is simple, rapid, accurate, and inexpensive (less than $1 [40 pence] per assay, the cost of the ^{14}C-AcCoA being the main factor). Furthermore, it has a wide range of applicability, because the only aminoglycosides which cannot be assayed in this fashion are kanamycin C, lividomycin, paromomycin, and gentamicins A and C_1. All others can be assayed satisfactorily provided that standard curves are constructed for each drug (Fig. 1) and for each series of assays.

In similar fashion, an assay for gentamicin in the presence of kanamycin has been developed in our laboratory by using a gentamicin acetyltransferase isolated from certain strains of *P. aeruginosa* and R$^+$ *E. coli* (S. Biddlecome, unpublished data).

TABLE 2. *BB-K8 concentrations determined by two assay methods*

BB-K8 sample no.	KAT assay (μg/ml)	Microbiological assay (μg/ml)
1	23.3	18.5
2	20.6	20
3	23.2	19.5
4	27.7	23
5	18.4	15.5
6	22.9	20
7	14.7	10
8	13.3	10
9	3.8	3.4
10	2.9	2.4

The accuracy and sensitivity of these enzymatic methods may also prove of some value in biochemical studies with the aminoglycoside antibiotics. Because radioactively labeled aminoglycosides are not readily available, it should be possible to study the binding of such drugs to ribosomes and other macromolecules by enzymatic assay methods.

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