

Substrate-Labeled Fluorescent Immunoassay for Amikacin in Human Serum

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A homogeneous substrate-labeled fluorescent immunoassay has been developed to measure amikacin levels in human serum. Amikacin is covalently labeled with the fluorogenic enzyme substrate β -galactosyl-umbelliferone. This β -galactosyl-umbelliferone-amikacin conjugate is nonfluorescent under assay conditions until it is hydrolyzed by β -galactosidase to yield a fluorescent product. When antiserum to amikacin binds the substrate-labeled drug, the antibody complex formation inhibits hydrolysis of the fluorogenic substrate. Reaction mixtures containing a constant level of substrate-labeled amikacin and a limiting amount of antiserum enable labeled and unlabeled amikacin to compete for the antibody-binding sites. Unbound substrate-labeled drug is hydrolyzed by the enzyme to release a fluorescent product that is proportional to the unlabeled amikacin concentration. The amikacin levels found in clinical serum samples with this method were comparable ($r = 0.987$) to those obtained by radioimmunoassay. The fluorescent immunoassay is rapid and simple to perform and requires only 2 μ l of serum.

Amikacin is a semisynthetic aminoglycoside antibiotic currently administered for the treatment of severe infections of gram-negative bacteria. Like gentamicin and tobramycin, amikacin is effective within a narrow therapeutic range of serum concentrations above which the drug can be considered potentially toxic (1, 5). Peak levels of amikacin in serum are generally expected to be between 20 and 25 μ g/ml, whereas the trough level should be 5 to 10 μ g/ml (1). Monitoring of the serum amikacin concentration during the course of treatment is usually justified for maintenance of a therapeutic yet nontoxic level of the drug.

Various assays for amikacin in serum have been reported in recent years. These methods include microbiological assays (9), radioimmunoassay (7, 11), and high-performance liquid chromatographic (8) and spectrophotometric techniques (10). We have previously developed substrate-labeled fluorescent immunoassays (SLFIA) to measure the therapeutic levels of tobramycin (2), gentamicin (4), and phenytoin (12). We now report the extension of the SLFIA to the determination of amikacin levels in human serum.

The SLFIA for amikacin requires that the drug be labeled with β -galactosyl-umbelliferone, a fluorogenic substrate for the enzyme β -galactosidase (EC 3.2.1.33), to form a β -galactosyl-umbelliferone-amikacin conjugate, hereafter denoted as the fluorogenic amikacin reagent (FAR). FAR is nonfluorescent under normal assay conditions unless it is hydrolyzed by the

enzyme to yield a fluorescent product. When FAR is bound by an antibody to amikacin, enzymatic hydrolysis is inhibited. This inhibition is relieved upon the addition of amikacin to the reaction since it competes with FAR for the limited number of antibody-binding sites. The amount of unbound FAR that is available for reaction with the enzyme is therefore proportional to the concentration of amikacin in the sample being assayed. Separation of the free FAR from the FAR bound to antibody is not required, since only the free FAR is available for reaction with the enzyme to produce fluorescence. Thus, the assay is performed simply and rapidly.

MATERIALS AND METHODS

Instruments. Fluorescence was measured with an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, Md.) with excitation and emission wavelengths set at 400 and 450 nm, respectively. Corrected fluorescence spectra were determined with a SLM model 8000 spectrophotometer (SLM Instruments, Inc., Urbana, Ill.). All fluorescence determinations were performed at room temperature in disposable polystyrene cuvettes (Evergreen Scientific, Los Angeles, Calif.). Radioimmunoassays were counted in a Gammacord II gamma counter (Ames Co., Elkhart, Ind.). Absorbance was measured with a model 2000 spectrophotometer (Gilford Instrument Labs, Inc., Oberlin, Ohio) or a model 16 spectrophotometer (Cary Instruments, Monrovia, Calif.).

Enzyme. β -Galactosidase from *Escherichia coli* (Worthington Biochemical Corp., Freehold, N.J.) was assayed at 25°C in 50 mM Bicine-0.1% azide, pH 8.5, containing 3 mM *o*-nitrophenyl- β -D-galactoside. Un-

der these conditions, the millimolar extinction coefficient for the product of this reaction, *o*-nitrophenol, is 4.27 at 415 nm. One unit of enzyme activity hydrolyzes 1.0 μ mol of substrate per min.

Chemicals. Bicine buffer, *N,N*-bis(2-hydroxyethyl)glycine (grade A, Calbiochem, LaJolla, Calif.), 50 mM, was used at pH 8.5. Sodium azide was purchased from Fisher Scientific Co., Fairlawn, N.J.

N-Hydroxysuccinimide, dicyclohexyl carbodiimide, dimethylformamide, and *o*-nitrophenyl- β -D-galactoside were purchased from Aldrich Chemical Co., Milwaukee, Wis. CM-Sephadex C-25 was purchased from Pharmacia, Uppsala, Sweden. Ammonium formate was purchased from Mallinckrodt, St. Louis, Mo. Normal human serum was purchased from Nolan Enterprises, Dallas, Tex. and amikacin was purchased from Bristol Laboratories, Syracuse, N.Y. Other drugs were obtained from their respective manufacturers.

Clinical serum samples were provided by Robert Betts, University of Rochester Medical Center, Rochester, N.Y.; Maryanne McGuckin, Antimicrobial Testing Laboratory, University of Pennsylvania, Philadelphia, Pa.; and Paul Stevens, Department of Medicine, University of California, Los Angeles, Calif.

Synthesis of the FAR. The synthetic scheme for preparation of the substrate-labeled amikacin derivative is presented in Fig. 1. A 1.0-mmol amount (370 mg) of 7- β -D-galactosyl-coumarin-3-carboxylic acid (Fig. 1, I; reference 4) and 1.0 mmol (120 mg) of *N*-hydroxysuccinimide were dissolved in 20 ml of dimethylformamide, and the solution was cooled to -6°C . After the addition of 1.0 mmol (210 mg) of *N,N*-dicyclohexylcarbodiimide, the reaction was stirred for 24 h at -6°C . The reaction was then filtered to remove precipitated dicyclohexyl urea. The filtrate containing the activated ester (Fig. 1, II) was added dropwise over a 2-h period to 1.3 mmol (615 mg) of amikacin (Fig. 1, III) in 20 ml of distilled water at -3°C . The reaction was subsequently stirred for 24 h at 0°C , brought to room temperature, and stirred for an additional 3 to 4 h after adding 20 ml of distilled water. The solution was evaporated to dryness under reduced pressure (0.1 mm of Hg at 37°C) to yield a solid yellow residue. This was dissolved in 20 ml of distilled water and applied to

a column (5.5 by 60 cm) of CM-Sephadex C-25 which was previously equilibrated with 50 mM ammonium formate. The column was first eluted with 2 liters of 50 mM ammonium formate to remove the unreacted 7- β -D-galactosyl-3-carboxylic acid and other side products. FAR (Fig. 1, IV) was then eluted with 1.5 M ammonium formate. Fractions (20 ml) were collected, and the absorbance at 343 nm was monitored. The appropriate fractions were pooled, concentrated on a rotary evaporator at 40°C , and sublimed (0.10 mm of Hg at 40°C) to remove the ammonium formate.

Antiserum. Antiserum to amikacin was produced in rabbits as previously described (6), with an amikacin-bovine serum albumin conjugate.

Radioimmunoassay for amikacin. Amikacin concentrations were determined by radioimmunoassay with the Monitor Science Amikacin RIA Kit (Monitor Science Corp., Newport Beach, Calif.).

SLFIA for determination of serum amikacin levels. To perform the SLFIA, 3.0 ml of a reagent containing the antibody and enzyme was added to a series of reaction cuvettes. (The antibody-enzyme reagent prepared in 50 mM Bicine-0.1% azide, pH 8.5, contained 50 mU of β -galactosidase per ml and sufficient antiserum to decrease the fluorescence output to 13% of that in the absence of antiserum.) Samples of 100 μ l of amikacin standards, controls, and unknowns (previously diluted 50-fold in buffer) were then added to the respective cuvettes. The reaction was initiated with the addition of 100 μ l of FAR (0.007 absorbance units at 343 nm per ml in 5 mM sodium formate-0.1% sodium azide buffer, pH 3.5) followed by immediate mixing. The FAR was added to each cuvette at 15-s intervals until all of the reactions in the run were initiated. After the first reaction mixture had incubated for 20 min, the fluorescence intensity for this and subsequent cuvettes was measured at 15-s intervals. The unknown amikacin concentrations were determined from a standard curve of fluorescence versus amikacin concentration.

RESULTS

Absorbance and fluorescence spectra of FAR. The absorbance spectrum of FAR is es-

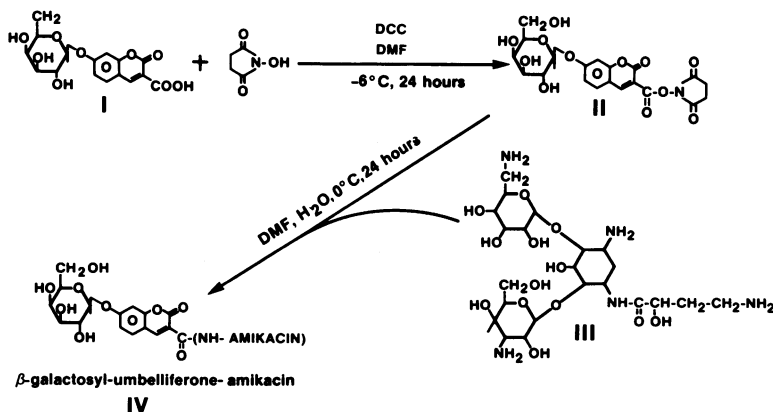


FIG. 1. Reaction sequence for the synthesis of the fluorogenic amikacin reagent, β -D-galactosyl-umbelliferone-amikacin.

essentially the same as that previously reported for the substrate-labeled fluorogenic drug reagents for the tobramycin and gentamicin SLFIAs (2, 4). The FAR absorbs maximally at 343 nm, but upon hydrolysis by β -galactosidase, the absorbance at 343 nm decreases and a new maximum appears at 405 nm. The absorbance of FAR at 405 nm after hydrolysis is 1.6 times that at 343 nm before hydrolysis.

The fluorescence spectrum for the FAR is also identical to those reported earlier for the tobramycin and gentamicin fluorogenic substrates (2, 4). Corrected fluorescence spectra showed that the excitation and emission maxima shifted upon enzymatic hydrolysis from 352 to 398 nm and 397 to 448 nm, respectively, whereas the fluorescent intensity concomitantly increased eightfold. For the SLFIA, the excitation and emission wavelengths are set at 400 and 450 nm, respectively. Under these conditions, the fluorescence of unhydrolyzed FAR is negligible.

Antibody-binding reactions. Inhibition of enzymatic hydrolysis of FAR by rabbit antiserum to amikacin is illustrated in Fig. 2. Normal rabbit serum has little effect. When a 50-fold dilution of a 40- μ g/ml amikacin standard was added to antiserum-containing reaction mixtures before the addition of FAR, the inhibition was partially removed by competition of the drug with FAR for antibody-binding sites. The percent fluorescence difference in the presence and absence of standard at any one level of antiserum approximates the relative fluorescence range for an assay standard curve.

Competitive binding reactions. Optimum concentrations of β -galactosidase, antiserum, and FAR were determined as described earlier (2, 12). Levels of antiserum that inhibited the hydrolysis of FAR 80 to 90% after a 20-min incubation period resulted in the best standard curves. Reactions containing varying levels of amikacin, 0.15 U of β -galactosidase, and 7.1 μ l of antiserum to amikacin in a volume of 3.1 ml were initiated by the addition of 0.0007 absorbance units of FAR at 343 nm. The fluorescence was measured at various incubation times, and the results are presented in Fig. 3. The curves on the left side of the figure show the percentage of the maximum attainable fluorescence (com-

pletely hydrolyzed FAR). The curves on the right (Fig. 3) are the respective standard curves as they appeared when the cuvette containing the highest amikacin standard was set to 90

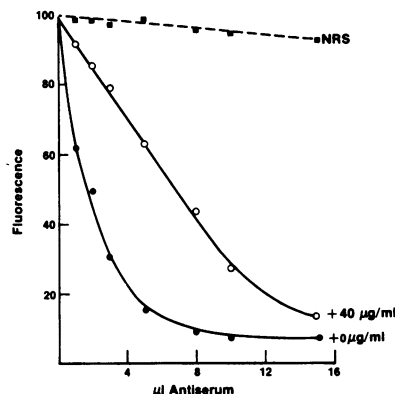


FIG. 2. Effect of normal rabbit serum (dashed line) and rabbit antiserum to amikacin (solid line) on the hydrolysis of FAR by β -galactosidase. Reactions containing antiserum were conducted in the absence (●) or presence (○) of the 40- μ g/ml amikacin standard.

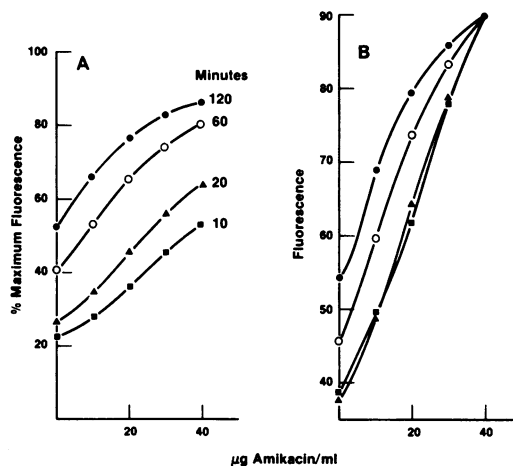


FIG. 3. Standard curves for the amikacin SLFIA observed after various reaction times, (A) plotted as a function of the maximum attainable fluorescence and (B) normalized to the fluorescence observed in the cuvette containing the highest drug standard.

TABLE 1. Precision of the amikacin SLFIA

Amikacin concn (μ g/ml)	No. of runs	No. of tests	Mean concn (μ g/ml)	Within-run precision		Between-run precision	
				SD ^a (μ g/ml)	% CV ^b	SD (μ g/ml)	% CV
5	10	50	4.7	0.3	6.9	0.2	4.7
15	10	50	15.8	0.4	2.4	0.2	1.2
30	10	50	31.7	0.7	2.3	1.0	3.2

^a SD, Standard deviation.

^b CV, Coefficient of variation.

fluorescence units. Longer incubation times decreased the slope of the curve at amikacin concentrations greater than 20 µg/ml. Figure 3 also shows that acceptable curves were generated with incubation times between 10 min and 2 h; however, a 20-min incubation time is routine and was used for the studies described below.

Performance characteristics of the amikacin SLFIA. The precision of the amikacin SLFIA was evaluated by assaying amikacin serum controls at three levels in five replicates each for 10 runs. The data were pooled ($N = 50$) for determination of the intra- and interassay precision that is shown in Table 1. The variation is comparable to that which can be obtained by radioimmunoassay.

Recovery experiments were performed by mixing equal volumes of standard and clinical

specimens and then measuring the amikacin concentration of the mixtures. The recovery varied from 93 to 113% of that expected between 5.2 and 30.7 µg of amikacin per ml. Since some clinical samples may have amikacin concentrations greater than 40 µg/ml, these specimens would require an appropriate dilution to bring the concentration within the range of the standard curve. We found that when the original dilutions of serum samples with concentrations greater than 30 µg/ml were mixed 1:1 with the diluted 0-µg/ml standard, the corrected results were equivalent to those diluted 1:50 with buffer.

The amikacin concentrations of clinical samples determined by the SLFIA were compared with those measured by radioimmunoassay (Fig. 4). As shown, the two methods correlate very well ($r = 0.987$).

Specificity of the amikacin SLFIA. The cross-reactivity of the antiserum was examined by measuring the dose-response of other aminoglycosides in the amikacin assay (Fig. 5). Drugs structurally similar to amikacin, namely kanamycin and tobramycin, were the most cross-reactive. Other aminoglycosides such as gentamicin, netilmicin, sisomicin, and streptomycin had essentially no cross-reactivity. Furthermore, other antibiotics which could be used in combination with amikacin therapy (carbenicillin, cephalothin, chloramphenicol, erythromycin, methicillin, neomycin, and tetracycline) did not cross-react or interfere in the assay when therapeutic levels were mixed with amikacin.

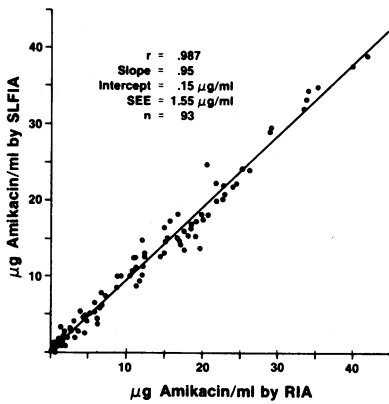


FIG. 4. Correlation between SLFIA and radioimmunoassay determinations of amikacin in clinical serum samples. SEE denotes the standard error of estimate.

DISCUSSION

We have shown that the determination of amikacin serum levels by SLFIA is a rapid and valid method. The same simple SLFIA assay

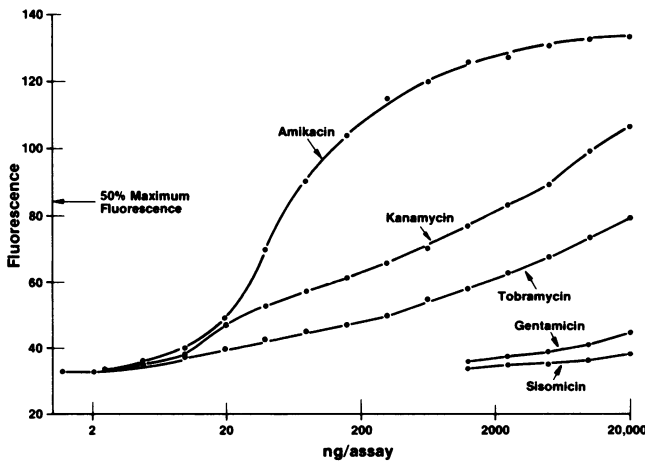


FIG. 5. Cross-reactivity of various aminoglycosides in the amikacin SLFIA. The cross-reactivity of each was determined by measuring the level of drug required to restore 50% of the maximum fluorescence.

format has now been demonstrated for several therapeutic drugs (J. F. Burd, *Abstr. Annu. Meet. Am. Assoc. Clin. Chem.* 1979, *Clin. Chem.* 25:1077) in addition to the major aminoglycoside antibiotics gentamicin, tobramycin, kanamycin, sisomicin, and netilmicin (3).

With one exception, all of the clinical serum specimens that we examined were below the concentration of the highest standard (40 $\mu\text{g}/\text{ml}$). When a specimen contains amikacin exceeding 40 $\mu\text{g}/\text{ml}$, it can be diluted to a concentration that is within the range of the standard curve. The precision, recovery, and correlation studies indicate that the SLFIA for amikacin is comparable to radioimmunoassay.

Simultaneous administration of combinations of aminoglycosides is not routinely practiced; therefore, cross-reactivity with kanamycin or tobramycin does not present a problem when measuring amikacin serum levels.

The amikacin SLFIA offers many advantages including a non-radioactive label which does not require separation of the bound and free label. The assay is easily performed, rapid, and quantitative, and several drug assays are now possible with the same assay format.

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