ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1986, p. 961–964
0066-4804/86/060961-04$02.00/0

Vol. 29, No. 6

Determination of Kanamycin Concentration in Serum by Substrate-Labeled Fluorescent Immunoassay

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Received 31 October 1985/Accepted 5 March 1986

A homogeneous substrate-labeled fluorescent immunoassay was developed for the measurement of kanamycin concentrations in serum. A fluorogenic drug reagent (FDR) (β-galactosyl-umbelliferone-tobramycin) was prepared that is nonfluorescent under the conditions of the assay but is hydrolyzed upon catalysis by β-galactosidase to yield a fluorescent product. Binding of the FDR to the antiserum to kanamycin prevented enzyme hydrolysis. The fixed level of FDR in the assay competed with kanamycin in the sample for a limited number of antibody-binding sites. Unbound FDR was hydrolyzed by β-galactosidase to release a fluorescent product that is proportional to the kanamycin concentration in the sample. The assay exhibited good sensitivity, precision, and accuracy and correlated well with other methods.

Kanamycin is an aminoglycoside antibiotic which is effective in the treatment of severe infections caused by gram-negative bacteria (3). It has pharmacokinetic properties very similar to amikacin (4, 8). It has been used to treat infections caused by *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella* spp., and *Proteus* spp., as well as other bacteria. Since kanamycin has a narrow therapeutic range, proper dosage administration is necessary to achieve maximum therapeutic efficacy and to avoid adverse effects, including nephrotoxicity and ototoxicity (5). Interpatient variability is also evident in response to a given dose of an aminoglycoside antibiotic. Therefore, therapeutic drug monitoring with a fast and accurate method is the most effective way of ensuring adequate therapy (6, 7, 10). We developed a homogeneous substrate-labeled fluorescent immunoassay (SLFIA) for the determination of total kanamycin levels in serum. The principle of the SLFIA, described previously (2, 12, 13), is based on the following findings. The kanamycin fluorogenic drug reagent (FDR), which is actually the cross-reacting compound β-galactosyl-umbelliferone-tobramycin, is nonfluorescent under the conditions of the assay. (The FDR compound was used for convenience, since it had previously been synthesized for use in an SLFIA for the detection of tobramycin.) However, hydrolysis catalyzed by β-galactosidase (*E. coli* βD-galactoside galactohydrolase; EC 3.2.1.23) yields a fluorescent product. When antiserum to kanamycin binds to the FDR, it is inactive as a substrate for β-galactosidase, and fluorescence is inhibited. Kanamycin in the clinical sample competes with a constant amount of FDR for a limiting number of antibody-binding sites so that a fluorescent response is generated as a function of the kanamycin concentration.

The performance of the kanamycin assay was evaluated for accuracy and precision, standard curve reproducibility, time dependence, sensitivity and cross-reactivity. Comparison of the assay with radioimmunoassays (RIA), high-performance liquid chromatography (HPLC), and gravimetric values is shown.

MATERIALS AND METHODS

Instruments. Fluorescence was measured with an Aminco-Bowman fluorescence spectrophotometer (SLM Instruments, Inc., Urbana, Ill.) or with an Ames Fluorostat (Ames Co., Elkhart, Ind.) with excitation and emission wavelengths of 405 and 450 nm, respectively. All fluorescence determinations were performed at room temperature in disposable polystyrene cuvettes (Evergreen Scientific, Los Angeles, Calif.). The fluorescence of a standard {7-hydroxycoumarin-3-[*N*-(2-hydroxyethyl)]carboxamide} (Miles Laboratories, Inc., Elkhart, Ind.) was used to relate the signal to nanomolar units where indicated. Absorbance was measured with a model 250 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Enzyme. β-Galactosidase from *E. coli* (Sigma Chemical Co., St. Louis, Mo.) was assayed at 25°C in 50 mM Bicine (*N*,*N*-bis[2-hydroxyethyl]-glycine)–0.1% sodium azide, pH 8.3, containing 3 mM *o*-nitrophenyl-β-D-galactoside. Under these conditions, the millimolar extinction coefficient for the product of this reaction, *o*-nitrophenyl, is 4.27 at 415 nm. One unit of enzyme activity hydrolyzes 1.0 μmol of substrate per min.

Chemicals. Bicine buffer, *N*, *N*-bis(2-hydroxyethyl)glycine (50 mM; grade A; Calbiochem-Behring, La Jolla, Calif.), was used at pH 8.3. Sodium azide was obtained from Fisher Scientific Co., Pittsburg, Pa.

Kanamycin, gentamicin, amikacin, and tobramycin were obtained from U.S. Pharmacopeial Convention, Inc., Rockville, Md., and dibekacin was obtained from Meiji Seika Kaisha, Ltd., Tokyo. The structural similarity of kanamycin and tobramycin is shown in Fig. 1.

The kanamycin specimens consisted of normal human serum samples spiked with kanamycin, in addition to six clinical samples (St. Joseph Mercy Hospital, Mason City, Iowa). Standard curves were produced for gravimetrically prepared standards of kanamycin in normal human serum containing 0, 5, 15, 25, and 40 μg of drug per ml. Gravimetrically prepared controls (10, 20, and 30 μg/ml) were also used throughout the study.

HPLC of kanamycin. The HPLC method of Anhalt and Brown (1) for the determination of aminoglycosides was modified to incorporate the following changes (11). The addition of the ion-pairing reagent to the sample was eliminated in an attempt to increase the usable life of the bonded-phase column matrix. The derivatizing reagent was diluted three times with a corresponding increase in the pumping rate to reduce the pump noise.

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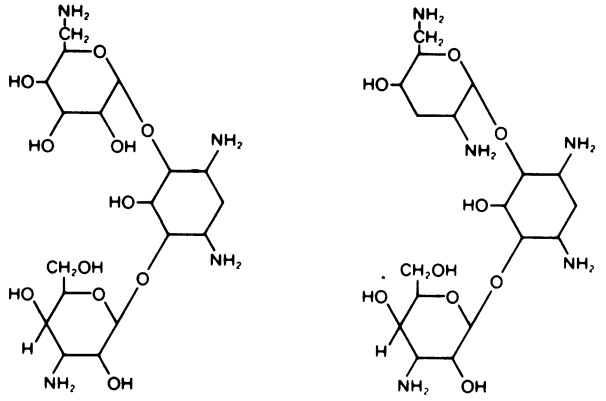

FIG. 1. Structure of the aminoglycoside antibiotics kanamycin and tobramycin.

RIA. A RIA kit (American Diagnostics Corp., Newport Beach, Calif.) for the quantitative determination of kanamycin in serum was used.

FDR. The FDR β-galactosyl-umbelliferone-tobramycin was synthesized as previously described (2).

Antiserum. Antiserum to kanamycin was produced in goats by using a kanamycin-bovine serum albumin conjugate as the immunogen (9). The antibody enzyme reagent was prepared by adding 85 μl of antiserum per ml and 1.6 U of β-galactosidase per ml to 50 mM Bicine–0.1% sodium azide, pH 8.3.

SLFIA for the determination of kanamycin concentration in serum. For the semiautomated SLFIA, the following additions were made sequentially with an Ames dilutor to a series of reaction cuvettes. (i) A 50-μl volume of antibody enzyme reagent plus 500 μl of 50 mM Bicine–0.1% NaN_3, pH 8.3, was dispensed into the cuvette. (ii) Samples (50 μl each) of kanamycin standards, controls and unknowns previously diluted 1:51 with 50 mM Bicine–0.1% sodium azide, pH 8.3, plus 500 μl of the same buffer were added to the antibody enzyme reagent. (iii) The reaction was initiated by the addition of 50 μl of FDR (0.018 A_{343}/ml) in 30 mM sodium formate buffer, pH 3.5, plus 500 μl of 50 mM Bicine–0.1% sodium azide, pH 8.3. The FDR was delivered to each cuvette at 20-s intervals until all of the reactions in the run were initiated. After the first reaction mixture had incubated at room temperature for 20 min, the fluorescence intensity for this cuvette as well as subsequent cuvettes was measured at 20-s intervals. The unknown kanamycin concentrations were determined from a standard curve of fluorescence versus the kanamycin concentrations.

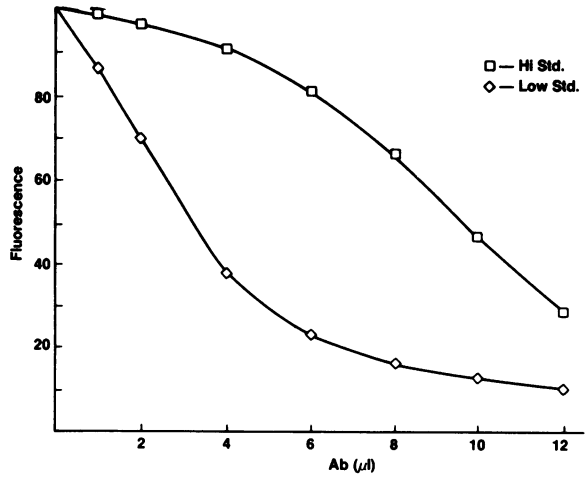

FIG. 2. Effect of kanamycin antiserum on the hydrolysis of FDR by β-galactosidase. Reactions were conducted in the presence of a diluted 40-μg/ml kanamycin standard (□) or of a diluted 0-μg/ml kanamycin standard (◇).

TABLE 1. Kanamycin SLFIA intra-assay accuracy and precision

Control value (μg/ml)	Mean (μg/ml) (10 dilutions, 1 replicate of each)	SD (μg/ml)	% CV[a]	% Relative error[b]
10	9.2	0.3	3.3	8.0
20	20.7	0.3	1.4	3.5
30	31.3	0.9	3.0	4.3

[a] CV, Coefficient of variation.
[b] (Mean − control)/control × 100%.

RESULTS

Antibody-binding reactions. The inhibition of enzymatic hydrolysis of the kanamycin FDR by antikanamycin was investigated. SLFIA reactions were carried out in the presence of a 40- or a 0-μg/ml kanamycin standard. The addition of the 40-μg/ml kanamycin standard yielded increased fluorescence due to competition of the drug with the FDR for antibody-binding sites (Fig. 2). The fluorescence difference in the presence and absence of the standard at any one level of antiserum gives an indication of the dose response at that level. The antibody level which provides the maximum difference between the two curves indicates the approximate level of antiserum to yield a standard curve with a good dose response.

Competitive binding reactions. The effect of reaction time on the shape of the standard curve was also studied (Fig. 3A and B). The curves in Fig. 3A show the measured fluorescence, whereas the curves in Fig. 3B are normalized to the fluorescence of the high standard. Although the kanamycin assay was run with a 20-min incubation, it is possible to incubate the reactions for other lengths of time, depending on the requirements of the user. Shorter incubation times facilitate quick results but limit the number of samples that can be processed per run, whereas longer incubation times permit a larger number of samples.

Performance characteristics of the kanamycin SLFIA. The intra-assay precision was evaluated by assaying 10 dilutions of each control once (Table 1). Each standard was assayed in

TABLE 2. Kanamycin SLFIA intra-assay accuracy and precision[a]

Control value (μg/ml)	Mean (μg/ml)	SD (μg/ml)	% CV[b]	% Relative error
10	9.2	0.3	3.8	8.0
20	21.0	0.2	1.1	5.0
30	32.1	0.9	2.9	7.0

[a] *n*, 27 assays; nine runs over 9 days.
[b] CV, Coefficient of variation.

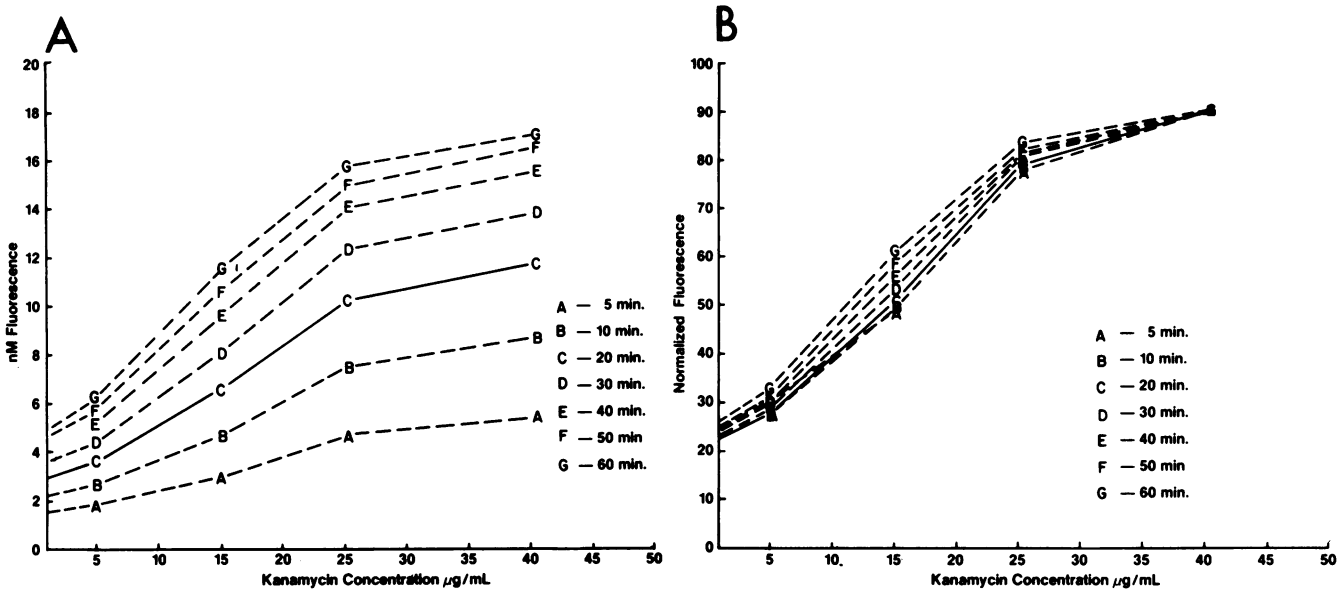

FIG. 3. Effect of incubation time on the kanamycin SLFIA standard curve. Standard curves generated between 5 and 60 min of incubation are shown. (A) Curves show the actual nanomolar fluorescence, calculated by comparison with a standard with known fluorescence. (B) Curves are normalized to the fluorescence of the high standard set to 90%.

triplicate. Interassay precision was determined from data generated in nine runs over a period of 9 days. In each run a new standard curve was generated, and 1 dilution of each control was assayed in triplicate (Table 2). Precision was good over all runs, and the standard curve was very reproducible over all runs throughout the 9-days (Fig. 4). Overrange experiments were performed by diluting contrived kanamycin serum samples (45, 55, and 65 μg/ml) 1:1 with 50 mM Bicine–0.1% sodium azide, pH 8.3, as well as with serum containing no kanamycin. The results obtained ranged from 102 to 106% of the expected value.

The sensitivity of the kanamycin assay was evaluated by assaying 10 replicates each of 0, 1.0, 2.0, 3.0, 4.0, and 5.0 μg/ml contrived by diluting the 5.0-μg/ml standard with the 0-μg/ml standard. This study indicated that 0.8 μg of kanamycin per ml could be distinguished from the 0-μg/ml standard at a 95% confidence level.

The kanamycin concentrations of spiked serum samples, prepared by adding different kanamycin concentrations to a normal human serum pool, and of six clinical samples were determined by SLFIA, RIA (Fig. 5), and HPLC (Table 3). The SLFIA results were also compared with the target gravimetric values (Table 3). Comparison of all methods to SLFIA revealed good correlation and good regression parameters.

Specificity of the kanamycin SLFIA. The cross-reactivity

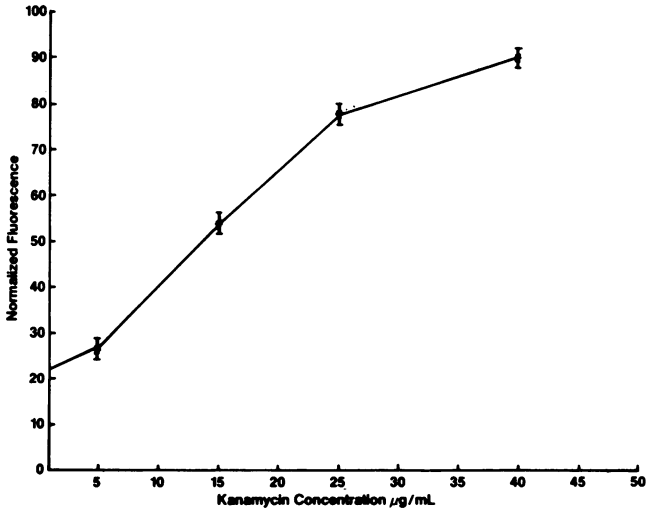

FIG. 4. Reproducibility of the standard curve over nine runs with a 20-min incubation period. Error bars indicate ±2 standard deviations from the mean value of the run.

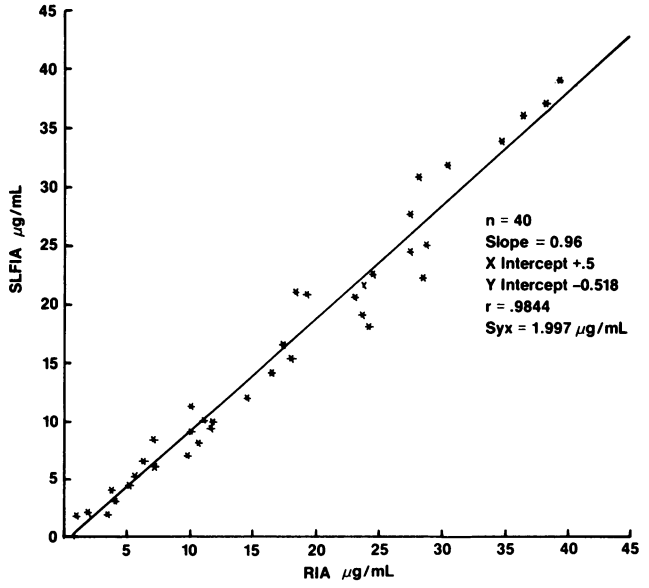

FIG. 5. Comparison of the kanamycin SLFIA with RIA. S_{yx}, Standard error of estimate.

TABLE 3. Comparison of kanamycin SLFIA to other methods

Assay x axis[a]	r	Intercept (μg/ml)	Slope	S_{yx}[b] (μg/ml)	n
RIA	0.984	−0.52	0.96	1.99	40
HPLC	0.978	−1.04	1.09	2.49	30
Gravimetric	0.998	−0.93	1.06	0.78	34

[a] y axis, SLFIA.
[b] S_{yx}, Standard error of estimate.

of the antiserum to kanamycin with other aminoglycosides was previously reported (12). These drugs are not coadministered. The cross-reactivity of the drugs was calculated at the concentration of the drug that elicits a fluorescent response equivalent to 50% of the maximum response due to kanamycin. Tobramycin and dibekacin cross-reacted 133 and 145%, respectively. Amikacin cross-reacted 1%. The unrelated aminoglycoside gentamicin did not cross-react at all. Other antibiotics that may be coadministered with kanamycin, such as neomycin, tetracycline, and chloramphenicol, did not cross-react at therapeutic levels.

DISCUSSION

The kanamycin SLFIA takes advantage of the cross-reactivity of tobramycin with the kanamycin antiserum. Thus, the assay utilizes β-galactosyl-umbelliferone-tobramycin as the FDR. The assay is similar to the tobramycin SLFIA (2), although optimal amounts of the reagents are different. The assay shows good intra- and interassay precision up to 40 μg/ml. Also, it exhibits good sensitivity and accuracy. Comparison of the SLFIA with HPLC and RIA results yields good correlation.

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