# Rapid, Reproducible Enzyme Immunoassay for Tobramycin

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An enzyme immunoassay for tobramycin utilizing glucose 6-phosphate dehydrogenase was compared with the radioimmunoassay. The enzyme immunoassay for tobramycin was accurate, specific, and easily performed. It offers an alternative method for assaying aminoglycosides and could be used in institutions that use the enzyme immunoassay to assay other drugs.

Tobramycin has proved to be a useful agent in the chemotherapy of gram-negative aerobic infections (2, 11). However, studies in animals and humans have revealed a narrow toxic/therapeutic ratio (2). Furthermore, serum levels adequate to cure major infections must be achieved (11). Although serum levels are predictable in healthy, male volunteers used to evaluate the initial clinical pharmacology of aminoglycosides, the long terminal phase of excretion and patient variation cause serum levels to be less predictable during multiple-dose therapy of infection (7, 16). Thus, as with gentamicin, there is a need to monitor serum levels of tobramycin (13).

Bioassays of serum levels of aminoglycosides are reliable if careful attention is given to problems inherent in these assays (15). However, the results are usually not available as rapidly as needed to make dosage adjustments. Other assays, including radioimmunoassay (RIA), radioenzymatic assays (utilizing adenylating or acetylating enzymes), spectrofluorometry, gasliquid chromatography, and high-pressure liquid chromatography, have been developed (1, 3, 6, 8–10, 17). These techniques, however, have problems attendant with their use (1, 3, 6, 8–10, 17).

Homogenous enzyme immunoassay (EIA) was introduced in 1972 (14). The technique has been utilized widely to monitor serum levels of antiepileptic agents, asthma medications, and cardiac drugs and to monitor thyroid function (4, 5). Recently, a study in our laboratory demonstrated the accuracy and usefulness of the EIA technique in assaying serum gentamicin levels (12). In addition, the equipment to perform EIA is available in many hospitals. We therefore decided to evaluate this technique to assay tobramycin serum levels and to compare the technique with the RIA technique now in widespread use.

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## MATERIALS AND METHODS

Serum samples were collected from patients hospitalized at Columbia-Presbyterian Medical Center who were receiving tobramycin. The dose, route of administration, and time of administration were obtained in all cases. Other medications were noted. Sera were separated by centrifugation and assayed immediately by EIA and by RIA. Samples were stored frozen at $-20°C$ for later assay to determine changes and to compare lots of standards with each other and with RIA simultaneously. Samples also were prepared from tobramycin provided by Eli Lilly & Co., Indianapolis, Ind.

Reagents for the EIA study were obtained from Syva Corp., Palo Alto, Calif. The method employs a bacterial glucose 6-phosphate dehydrogenase enzyme to which tobramycin has been linked. The active site of the enzyme is adjacent to the bound aminoglycoside. Antitobramycin antibody will bind to the tobramycin adjacent to the enzyme, thereby decreasing enzymatic activity. The assay follows the conversion of nicotinamide adenine dinucleotide to reduced nicotinamide adenine dinucleotide, which occurs when the enzyme acts on a substrate. Free tobramycin in standards or from a sample competes for antibodies with drug bound to the enzyme. When less antibody blocks the active site of the glucose 6-phosphate dehydrogenase, the activity of the enzyme is increased. Standards of tobramycin were prepared in serum.

With a pipetter-diluter, 50 µl of a sample or standard was diluted with 250 µl of tris(hydroxymethyl)-aminomethane-hydrochloride buffer, pH 8, 0.055 M; 50 µl of the first dilution was diluted again with 250 µl of buffer. To this second dilution was added 50 µl of substrate and antibody (reagent A) and 250 µl of buffer. Finally, 50 µl of tobramycin-enzyme preparation (reagent B) and 250 µl of buffer were added. A sample was taken into the cuvette.

Absorbance of the reaction mixture was measured at 340 nm with a Gilford 300-N microsample spectrophotometer equipped with a thermally regulated flow cell set at 30°C. Readings were taken at 15 and 45 s and recorded with a CP-1000 EMIT timer printer. A standard curve was prepared by using tobramycin standards of 1, 2, 4, 8, and 16 µg/ml. Data were applied to specially designed, modified log-function graph pa-

per to yield a straight line within the range of the assay.

## RESULTS

A standard curve produced by assay of standards containing 1, 2, 4, 8, and 16 μg of tobramycin per ml is shown in Fig. 1. The data yielded a sigmoid-shaped curve, but with the use of probit paper this was converted to a straight line from which unknown concentrations could be calculated. The precision of the EIA method was determined by assaying samples 20 times on the same day (4.0-μg/ml sample) and subsequently assaying 29 times on 29 successive days (6.0-μg/ml sample). By utilizing a tobramycin standard of 4 μg/ml for the EIA, an assay mean of 3.98 μg/ml with a standard deviation of 0.29 (coefficient of variation, 7.4%) was obtained. By utilizing a tobramycin standard of 6.0 μg/ml in the RIA, a mean of 6.06 μg/ml with a standard deviation of 0.22 (coefficient of variation, 3.6%) was found. Over a 29-day period, the standard deviation of the EIA was 0.19 μg/ml (coefficient of variation, 3.0%).

The specificity of the assay was determined by assaying prepared serum samples containing amikacin, sisomicin, netilmicin, kanamycin, and tobramycin. Only with kanamycin and amikacin did the test show a cross-reaction. This would be expected since tobramycin contains the basic kanamycin nucleus (11). A number of β-lactam compounds—ampicillin, azlocillin, methicillin, carbenicillin, ticarcillin, and mecillinam—and chloramphenicol added to the serum at concentrations up to 200 μg/ml did not affect the assay.

Sera (100 samples) from 54 patients were analyzed by EIA and RIA. Figure 2 shows the 57 samples with tobramycin levels greater than 0.5 μg/ml by either assay. The correlation coefficient for the methods was 0.92 with a standard error of the estimate of 0.79. No sera less than 0.5 μg/ml by one method were greater than or equal to 0.5 μg/ml by the other method. Subsequently, 35 stored samples were assayed simultaneously by using two different lots of reagents and standards, and the results were compared with the results of the RIA from the same day (Fig. 3). The correlation coefficient comparing the two EIA lots to each other was 0.98, with a standard error of the estimate of 0.14. Comparing one lot of results with those of the RIA revealed a correlation coefficient of 0.99 with a standard error of 0.09, whereas comparison of the results of the other EIA lot with those of the RIA revealed a correlation coefficient of 0.98 with a standard error of 0.16. No significant difference was found between values originally obtained by EIA and values obtained after storage for more than 1 month. The differences in correlation coefficient and standard error of the estimate could be accounted for by differences in the technique of the operators and by the recalibration and replacement of parts of the spectrophotometer during the initial assays. In an accuracy study comparing two lots of EIA reagents to RIA of known standard with a single operator, the measurement revealed a variation of 25% with confidence limits of 95% versus RIA variation of 32% with confidence limits of 95% (Table 1).

## DISCUSSION

Clinically, the measurement of serum tobramycin levels is of value if the result is returned to the physician rapidly (11, 17). Many hospitals using the RIA set up the test once a day. In this
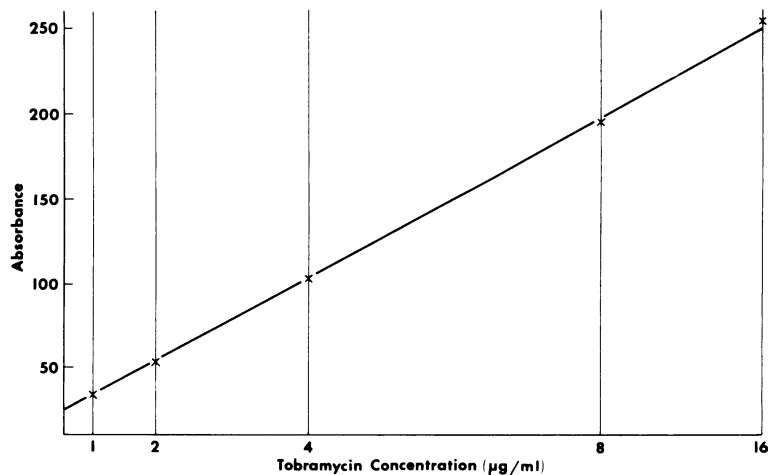


FIG. 1. *Standard curve of tobramycin serum standards of 1, 2, 4, 8, and 16 μg/ml.*
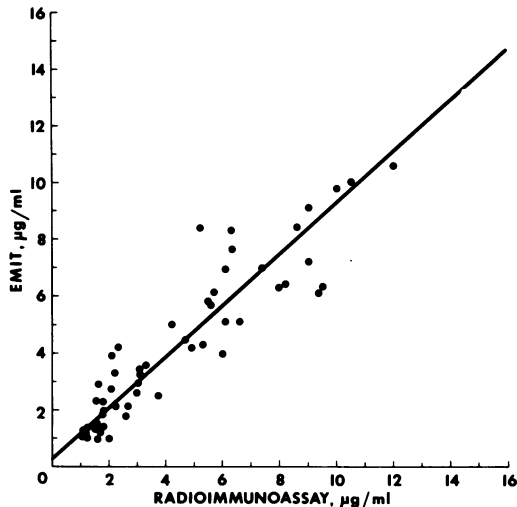
FIG. 2. *Results of samples assayed for tobramycin by the EIA (=EMIT, enzyme-mediated immunoassay for tobramycin) and RIA methods showing agreement of results.*
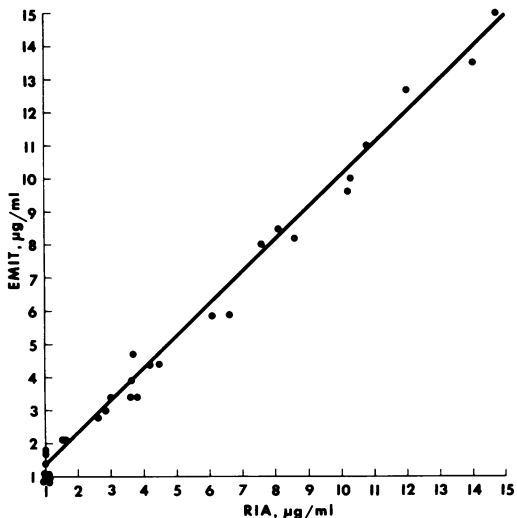


FIG. 3. *Comparison of results of serum assays for tobramycin using different lots of EIA (=EMIT, enzyme-mediated immunoassay for tobramycin) reagents with the results from RIA method.*

TABLE 1. *Accuracy study*[a]

| Test | Correlation coefficient | Standard error of estimate | Variation within 95% confidence limits (%) |
|---|---|---|---|
| Lot A EIA | 0.9857 | 0.1872 | 29 |
| Lot B EIA | 0.9899 | 0.1369 | 22 |
| RIA | 0.9812 | 0.2033 | 32 |

[a] Standard solutions prepared in normal human serum were compared for 4 and 8 $\mu$g of tobramycin. A total of 100 tests were done over a 1-month period. The assay was performed by three of the authors.

and a prior study, we found that setup time for the first sample by RIA is 1 h and 35 min, with 5 to 10 min needed for each additional test (12). In addition, the RIA requires special skill and equipment and yields radioactive waste. Although a rapid biological method for measurement of tobramycin has been developed, it also requires some time to set up and then at least 2.5 h before reading (17). Other methods, including radioenzymatic assay and spectrofluorome-

tric assays, also require special equipment and skill, and all but the RIA require horse or human serum for dilution of test sera and standards in addition to special reagents (1, 3, 6, 8, 9, 10, 17). Finally, commercially available reagent kits for RIA of tobramycin have a shelf life of only about 1 month.

The EIA method for determining tobramycin levels required a set up time of 25 to 30 min, with 2 min needed for each additional sample. The standard curve was reproducible during the working day, so that samples could be run at any time during the day. Delay time between obtaining the sample and return of assay results to the physician may be 30 min, very acceptable for decision making. In addition, the test could be performed on standard equipment available in many laboratories, with little special skill required of the operator. No diluting sera are required, and samples and standards are stable at 4°C and −20°C, respectively, for periods considerably longer than 1 month (up to 6 months in our study). No radioactive wastes are produced, and only 50 $\mu$l of serum is required per assay. This latter fact makes the assay particularly useful in pediatric situations. Precision and specificity of the EIA and RIA were similar. Accuracy of the EIA was better than RIA in this study, however. Variation of 25% within 95% confidence limits has been considered acceptable and is probably more so in face of variation of 32% with the commerically available RIA method used in this study (13). Thus, for laboratories already using the same instrumentation to measure other drugs, EIA would be a preferable method for determining tobramycin levels. It has already been shown to be so for determining gentamicin levels (12). For other laboratories EIA offers a viable alternative to currently available methods for determining tobramycin serum levels. The greatest value of the EIA is to provide a means to rapidly adjust dosage programs for critically ill patients. Neither the RIA nor the microbiological method, albeit accurate and

precise, has aided our group in adjusting the dosage programs of aminoglycosides in patients with rapidly changing renal function, whereas the EIA has.

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