Homogeneous Enzyme Immunoassay for Tobramycin Evaluated and Compared with a Radioimmunoassay

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We evaluated a commercially available homogeneous enzyme immunoassay (EMIT, Syva Co.) for tobramycin against a reference radioimmunoassay (RIA) method. Between-assay precision (CV) was 2.9% at 6.2 mg/L and 3.0% for values in the range of 1.0–7.6 mg/L. Accuracy based on a recovery experiment (1.0–13.0 mg/L) yielded an analytical recovery of 88–112%. A correlation study with 75 sera from patients on tobramycin therapy showed that EMIT = 0.984 RIA – 0.0608, \( r = 0.993 \). Neither the EMIT nor the RIA procedure was affected by the presence of gentamicin, amikacin, and vancomycin. Absorbance data from the EMIT system calculated with the conventional RIA logit-log algorithm correlate well with results generated by the Syva data-handling system (logit-log = 1.077 Syva – 0.318, \( r = 0.998 \)). A reagent stability study indicated that the EMIT reagents, once reconstituted, remain stable for at least 17 days when stored at refrigerated temperatures, or 11 days if stored at room temperature, thus enabling frequent "stat" assays without the need to prepare a calibration curve each time.

Additional Keyphrases: drug assay • antibiotics • aminoglycosides • data handling

Tobramycin is an aminoglycoside antibiotic used to treat infections caused by a wide spectrum of Gram-negative organisms, including those of the lower respiratory tract, central nervous system, urinary tract, skin, and bone (1). Studies on humans as well as animals indicate that both nephrotoxicity and ototoxicity associated with aminoglycosides are less with tobramycin than with gentamicin (2, 3). Furthermore, some microorganisms, including Pseudomonas species, are less prone to develop resistance to tobramycin (4, 5).

There are at least three reasons for measuring the serum concentration of tobramycin. Like most other aminoglycosides, tobramycin is excreted primarily by the kidneys (6, 7). The concentration of tobramycin in serum is therefore very much dependent on renal function, and patients with impaired glomerular filtration rate require appropriate dosage adjustments. Secondly, several studies indicate that the estimation of the half-life of aminoglycosides on the basis of sex, age, body weight, serum creatinine, urea nitrogen, and hematocrit is far from satisfactory (1). Hence optimal dose-regimen and dosage intervals must be determined individually to account for variations in drug clearance. Lastly, because of the narrow therapeutic range (peak–trough = 10–2 mg/L) (8), determination of tobramycin concentrations in serum is necessary to assess the risks of nephrotoxicity and ototoxicity.

Techniques commonly used for monitoring serum concentrations of tobramycin include microbiological assay (9), radioenzymatic assay (10), radioimmunoassay (RIA) (11, 12), gas chromatography (13), and "high-pressure" liquid chromatography (14). Of these, the method of choice has been RIA because of its greater sensitivity and specificity (15). Enzyme immunoassay (16, 17), in which the radioisotope is replaced by an enzyme, has been widely applied to drug analysis in the last few years. This technique is particularly useful in therapeutic drug monitoring, where exquisite sensitivity is ordinarily not a stringent prerequisite and speed of reporting is essential. We have evaluated a homogeneous enzyme immu-
noassay (EMIT\textsuperscript{1}) for tobramycin, which has recently become commercially available, vs a well-established RIA. The latter procedure, routinely used in our laboratory since 1977, has proven clinical reliability in correlating analytical results with the clinical status of patients on tobramycin therapy. Here, we report (a) the results of our comparison of the EMIT procedure vs the RIA method, (b) a possible alternative data-handling algorithm for the EMIT procedure with the use of logit-log transformation, and (c) the stability of the EMIT reagents for the tobramycin assay.

Materials and Methods

Reagents

EMIT procedure. All reagents necessary for the assay of serum tobramycin were packaged as an EMIT kit for tobramycin (Syva Co., Palo Alto, CA 94304). Each kit consists of two reagents, a buffer, and six calibrators. Reagent A is a lyophilized preparation of antibodies against tobramycin and the substrates for the enzyme glucose-6-phosphate dehydrogenase (EC 1.1.1.49). When reconstituted as directed, it contains a standardized preparation of the immunized sheep gamma-globulin, glucose-6-phosphate, the enzyme substrate, NAD\textsuperscript{+}, and preservatives in Tris - HCl buffer, 55 mmol/L, pH 5.2. Reagent B is a lyophilized preparation of glucose-6-phosphate dehydrogenase coupled to tobramycin. When reconstituted, it contains the enzyme-labeled drug and preservatives in Tris - HCl buffer, 55 mmol/L, pH 8.0. This Tris buffer solution also contains a surfactant. The six calibrators are lyophilized serum-based preparations containing 0.0, 1.0, 2.0, 4.0, 8.0, and 16.0 mg of tobramycin per liter (18).

Comparison RIA procedure. Phosphate-buffered saline (PBS), 10 mmol/L, was prepared by dissolving 10 mmol of Na\textsubscript{2}HPO\textsubscript{4}, 150 mmol of NaCl, and 15 mmol of Na\textsubscript{3}PO\textsubscript{4} (1.4 g, 8.8 g, and 1.0 g, respectively) in 900 mL of reagent-grade water; the pH was adjusted to 7.5, and the solution was diluted to 1 L. The assay buffer was gelatin (Knox), 0.1 g, dissolved in 100 mL of PBS. Normal goat serum solution was goat serum diluted with an equal volume of PBS. Polyethylene glycol solution was Carbowax PEG-6000 (Fisher Scientific, Houston, TX 77001), 25 g, dissolved in 100 mL of PBS.

A stock tobramycin solution, 1 g/L, obtained from Eli Lilly and Co., Indianapolis, IN 46206, was stored at −20 °C in 60-μL aliquots. Working standards, prepared freshly before each assay, were made by diluting 50 μL of an aliquot of the stock solution with assay buffer to a concentration of 200 μg/L and then making serial twofold dilutions with assay buffer to yield solutions containing 10, 5, 2.5, 1.25, 0.63, 0.32, and 0.16 ng of tobramycin per 50 μL.

Antiserum specific to tobramycin was a gift from Dr. A. Broughton, formerly of this institution (11). The working titer of this antiserum was assessed by performing an antibody-dilution study with 0 and 1.25 ng of tobramycin, according to the procedure to be described later. An optimal binding of 50% at zero tobramycin concentration, (B/T)\textsubscript{0}, and 25% at 1.25 ng of tobramycin was usually obtained when the antiserum dilution was between the range of 1800- and 2200-fold.

Apparatus

EMIT procedure. The EMIT procedure was performed with a Staser III Spectrophotometer (Gilford Instrument Lab., Oberlin, OH 44704) coupled to a CP-5000 Clinical Processor (Syva Co.). A Syva Model 1500 pipettor-dilutor was used for sample and reagent pipetting.

RIA procedure. A gamma-scintillation spectrometer (Model 5230; Packard Instrument Co., Downers Grove, IL, 60515) was used to measure radioactivity. A desktop computer with graphic capability (Model 9845B; Hewlett-Packard, Fort Collins, CO 80521) was used in data handling.

Procedures

EMIT procedure. All standard calibrators and samples were assayed in duplicate. Reagents A and B, reconstituted just before use, were allowed to reach room temperature. Reaction mixtures were prepared with use of the pipettor dilutor as follows: Pipet into a 2-mL beaker with 250 μL of buffer solution 50 μL of Reagent A and 50 μL of sample or standard previously diluted sixfold with buffer solution. Add 50 μL of Reagent B in 250 μL of buffer solution, then without delay aspirate the mixture into a previously purged spectrometer flow cell; this activates recording of the absorbances at 15-s (A\textsubscript{15}) and 45-s (A\textsubscript{45}) intervals for each sample mixture. The change in absorbance (ΔA = A\textsubscript{15} - A\textsubscript{0}) for the zero calibrator (ΔA\textsubscript{0}), representing the absorbance of tobramycin-free serum, is subtracted from the absorbance changes of all other calibrators, samples, and control (ΔA - ΔA\textsubscript{0}). We plotted the net increase of absorbance against the respective calibrator concentrations (milligrams per liter) manually on special graph papers supplied by Syva Co., and drew the "best-fit" straight line (estimated visually) through the points. Alternatively, we processed the EMIT data with the HP-9845B desktop computer, using the logit-log transformation program written for the data handling of the RIA method.

RIA procedure. Iodinated tobramycin was prepared by a modified procedure (11) of Bolton and Hunter (19) with the use of an acylating agent, 3-(4-hydroxyphenyl)propionic acid-N-hydroxysuccinimide ester (Tagit; Calbiochem, La Jolla, CA 92037). After column purification of the iodinated preparation, an aliquot, diluted to 12 000 cpm/100 μL, was checked for immunoreactivity according to the procedure to be described later. Bindings at 0 and 1.25 ng of tobramycin were tested at antisera dilutions of 1800-, 2000-, and 2200-fold. The antisera dilution that yielded bindings closest to 50% at (B/T)\textsubscript{0} and 25% at (B/T)\textsubscript{1.25} was the titer we used with this particular iodination.

Standard and sample incubation mixtures were made up of 100 μL of tobramycin antisum (1800- to 2200-fold dilution), 50 μL of standard solution (0.16–10.0 ng of tobramycin) or appropriately diluted serum, 100 μL of 125I-labeled tobramycin (12 000 cpm), and enough assay buffer to total 550 μL. All standards and samples were assayed in duplicate. The contents of these tubes were well mixed and allowed to incubate for 30 min at 4 °C. Then 100 μL of normal goat serum solution and 500 μL of polyethylene glycol solution were added to each tube, and incubated for 20 min longer at 4 °C. Antibody-bound and free tobramycin fractions were separated by centrifugation for 15 min at 2000 x g in a refrigerated centrifuge. After the supernatant fluid was drained from each tube, the radioactivity of the precipitate, which contained the bound fraction, was counted for 1 min with the gamma-scintillation spectrometer.

In processing the data from the bound fraction, the dose-response variables were subjected to logit-log transformation and iterative regression analysis to yield a linear slope, an 80% intercept, and a 50% intercept, all of which serve as quality-control data for monitoring the assay (20).

Specimens

Serum samples for precision and therapy correlation studies were collected from hospitalized patients on tobramycin therapy. Fifteen serum samples containing endogenous aminoglycosides other than tobramycin were obtained from Bio-Science Laboratories, Van Nuys, CA 91405, for determination of the concentrations of these aminoglycosides also. Lyophilized serum samples containing known amounts of

\textsuperscript{1} EMIT is the registered trademark of Syva Co.
tobramycin, used for recovery study, were prepared by Syva Co.

Regent Stability Study
Reagents and calibrators from the same lot number of EMIT kits for tobramycin, sufficient to assay 18 sets of calibration curves each with two controls, were reconstituted according to package instructions, combined, and then divided into two equal aliquots to be stored at 4 °C or 25 °C. One calibration curve, control 1, and control 2 were run on each of nine days within a 17-day period with reagents stored at each temperature. The absorbance data for the calibrators were plotted on Syva graph paper, and the tobramycin concentrations of the control samples were calculated accordingly.

Results
Standard Curves

EMIT procedure. The detection limit, or sensitivity, defined as the mean ± 3SD of the absorbance of tobramycin-free serum or of the zero calibrator, corresponds to 0.5 mg/L. Consequently, 1 mg/L, the lowest calibrator concentration, is taken as the limit of sensitivity for this assay.

The result of processing the EMIT data with the desktop computer and the logit-log algorithm is shown in Figure 1. The slope is linear over the range of 1–16 mg/L. A regression analysis of all absorbance data obtained with the EMIT procedure as calculated by both the manual graphic method (Syva’s power function algorithm) and by the logit-log transformation technique is shown in Figure 2. By Student’s paired t-test there was no significant difference (p = 0.5) between these two sets of calculations.

RIA procedure. Figure 3 shows a representative dose-response curve after logit-log transformation and iterative weighted regression analysis. The relationship is linear with tobramycin over an absolute range of 0.16–10.0 ng. For 100-fold diluted samples, the assay sensitivity, defined by B/B0 = 85%, was 0.3 mg/L.

Assay Characteristics

Precision. To assess the within-run precision of the EMIT procedure, we assayed the 4.0 mg/L tobramycin calibrator in replicates; the CV was 1.9% (mean = 4.2 mg/L, n = 20). For between-run precision of the EMIT procedure, we assayed

20 serum samples, with values ranging from 1.0 to 7.6 mg/L, on each of two days; the CV was 3.0%. The same precision was also obtained in another study in which the 6.0 mg/L tobramycin calibrator was assayed in 14 individual runs. The between-run precision for the RIA procedure in 30 serum samples, with tobramycin concentrations ranging from 1.0 to 17.3 mg/L, was 4.3%.

Accuracy. The accuracy of both methods was evaluated by assaying a set of lyophilized samples of human sera with known concentrations of tobramycin (1.0–13.0 mg/L). These sets of samples were assayed in duplicate on two separate occasions. The analytical recovery was 92–116% and 88–112% with the EMIT procedure, and 100–118% and 111–124% with the RIA procedure. In another accuracy study we assayed samples from 75 patients on tobramycin therapy by both procedures. A regression analysis of these two sets of data is shown in Figure 4.

Specificity. The antisera in both the EMIT and the RIA systems were assessed for cross reactivity to antibiotics other than tobramycin. Serum samples from 15 patients, of whom five each were being treated with gentamicin, amikacin, or
Table 1. Antiserum Cross Reactivity to Gentamicin, Amikacin, and Vancomycin with the EMIT and the RIA Procedures

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc. mg/L</th>
<th>EMIT Conc. mg/L</th>
<th>RIA Conc. mg/L</th>
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</thead>
<tbody>
<tr>
<td>Gentamicin</td>
<td>7.2–17.0</td>
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<td>&lt;0.3*</td>
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<tr>
<td>Amikacin</td>
<td>19–40</td>
<td>&lt;1.0</td>
<td>&lt;0.3</td>
</tr>
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<td>Vancomycin</td>
<td>20–640</td>
<td>&lt;1.0</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td></td>
<td>1280</td>
<td>1.8</td>
<td>1.6</td>
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</table>

* Limits of sensitivity for the respective procedures.

vancomycin, were assayed for tobramycin by both assays. The results (Table 1) indicated that the cross reactivity of either antiserum with these other drugs is insignificant.

Variability between Reagent Lots

A second lot of reagents for tobramycin prepared for clinical trial with antiserum from different animals (lot no. X03, Syva) was evaluated for assay performance with the reagent lot used in this study (no. X01). Forty-two samples from patients on tobramycin therapy were assayed with both lots of reagent. A regression analysis of these two sets of data (which ranged from 1.0 to 7.6 mg/L), yielded a slope of 0.987, an intercept of 0.201, and a coefficient of correlation of 0.993. No significant difference was demonstrated with Student's t-test (p = 0.06).

Reagent Stability

The precision data (CV) for control 1 (~6.5 mg/L) were 3.1% at 4 °C reagent storage and 2.5% at 25 °C reagent storage; for control 2 (~12 mg/L), respective CVs were 2.3% and 5.5%. Over the entire course of the stability study, the absorbance change for the zero calibrator (ΔA0) with reagents stored at either temperature remained relatively constant. The ΔA – ΔA0 values for both the calibrators and controls appeared stable over the 17 days of 4 °C storage, but drifts were evident with the absorbance data for the 25 °C storage, most noticeably with calibrator 8, calibrator 16, and control 2. Table 2 shows the absorbance data for these calibrators at both temperatures.

Figure 5 verifies this reagent stability at refrigerated temperatures by showing control values obtained both from individual calibration curves on the day of assay and from the curve prepared on day 1 of the stability study with reagents stored at both temperatures. Both sets of control values were stable with the reagents stored at 4 °C, but control 2, when assayed with reagents stored at 25 °C, shows a significant downward drift beginning on day 11. The decrease was greater for values read from the calibration curve of day 1.

Discussion

In our study, the assay precision of the EMIT procedure well exceeds that of the RIA method (CV = 3.0% vs 4.3%). Although assay sensitivity of the EMIT procedure did not match that of the RIA (detection limit = 1.0 mg/L vs 0.3 mg/L), its usefulness is by no means impaired, because the lower limit of the therapeutic range for tobramycin, 2 mg/L, far exceeds the limit of assay sensitivity. However, for therapeutic monitoring of drug concentration for optimizing therapy and avoiding toxicity, the EMIT procedure is easily the method of choice because of its extremely short assay time (approximately 1 min per sample) and ease of performance. Moreover, the performance characteristics of two reagent lots are extremely compatible, even though the antibodies packaged in each reagent lot were obtained from different animals. To facilitate EMIT data handling, we have used the logit-log algorithm prepared for RIA data computation. As shown in Figure 2, the values correlate well with those generated with the Syva data-handling system.

In an effort to offset the high reagent cost of the EMIT sys-

<table>
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<tr>
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<th>Cal 16</th>
<th>Cal 2</th>
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<tr>
<td>17</td>
<td>487</td>
<td>158</td>
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Cal, calibrator; ctl, control.

Table 2. Stability of Reagents Stored at Room Temperature (25 °C) or Refrigerated (4 °C)

Fig. 4. Comparison of tobramycin values obtained from the EMIT procedure with those from the RIA procedure.

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Fig. 5. Tobramycin concentrations in Control 1 and Control 2, assayed with EMT reagents used for assay after intervals of storage up to 17 days at 4 and 25 °C.

- - - - - - , tobramycin values read from the corresponding dose–response curve prepared on the same day; - - - - - - , tobramycin values from the same absorbance data but read from the dose–response curve prepared on day 1.

...tion and to determine the life expectancy of the calibration curve for the tobramycin assay, we assessed the stability of reagents A and B after reconstitution. We did not see an overall increase of ΔA₀ and ΔA – ΔA₀ as has been reported for several of the EMT kits for gentamicin, theophylline, and procainamide (21). The simultaneous decrease in ΔA – ΔA₀ for both the high calibrator (8 and 16) and control 2 stored at 25 °C is probably attributed to a deterioration of reagents A and (or) B, but not the calibrators, which may account for the much less severe downward drift for control 2 when the values were read from the calibration curves performed on the same day. We conclude from our data that reagents A and B from the EMT kit for tobramycin, once reconstituted, remain stable for at least 17 days when stored refrigerated, and up to 11 days when stored at room temperature. Furthermore, the stability of the calibration curve indicates that frequent “stat” tobramycin assays can be carried out without preparing a calibration curve each time, reducing both assay cost and technical labor as well as expediting reporting time.

We have elected to describe our own reference RIA procedure in detail here because we believe that a carefully developed RIA procedure of proven clinical utility can be useful to laboratories that wish to set up this assay to take advantage of its substantially lower cost and greater sensitivity. An ideal situation for large clinical laboratories, in our opinion, would be the implementation of the low-cost, precise RIA procedure for routine assays in conjunction with the EMT procedure for “stat” determinations.

References


18. EMT® tobramycin kit insert, no. 65314-4, Syva, Palo Alto, CA, April, 1981.

