Evaluation of an Automated Fluorescence Polarization Immunoassay for Vancomycin

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An automated fluorescence polarization immunoassay for the determination of vancomycin levels in serum was evaluated. The vancomycin assay is a homogeneous competitive inhibition immunoassay based on changes in fluorescence polarization that occur with antibody binding. This assay was compared with a liquid chromatographic assay and an agar well diffusion bioassay method by using clinical serum specimens and controls. Linear regression analysis of the data obtained on clinical specimens by the three methods resulted in correlation coefficients of 0.97 for the fluorescence polarization immunoassay versus the liquid chromatographic assay (n = 60), 0.90 for the fluorescence polarization immunoassay versus the bioassay (n = 57), and 0.90 for the liquid chromatographic assay versus the bioassay (n = 57). Repetitive analysis of control sera containing 7, 35, and 75 μg of vancomycin per ml by the fluorescence polarization immunoassay yielded coefficients of variation of 3.0, 1.7, and 2.3, respectively. No interference was demonstrated in spiked hemolytic, lipemic, or icteric sera, and the assay was free of matrix effects. The automated fluorescence polarization immunoassay system offers a rapid, efficient, and accurate method for monitoring vancomycin serum levels for both toxicity and efficacy.

Vancomycin is a glycopeptide antibiotic that was isolated from Streptomyces orientalis in 1956. It acts by inhibiting the synthesis of the pentaglycine cross-link in the bacterial cell wall, a step that occurs before the step that is inhibited by penicillin. The use of vancomycin, which is a nephrotoxic, narrow-spectrum, intravenous antibiotic, has been increasing in recent years.

Vancomycin is highly effective in the treatment of infections caused by gram-positive cocci. It has been used effectively in intravenous treatment of resistant staphylococcal or streptococcal septicemia, endocarditis, and osteomyelitis (10, 12, 16). It is active against gram-positive bacteria that are resistant to the penicillins, including multiply resistant corynebacteria (1, 4, 6), and Clostridium difficile. More recently, its use has increased because it is the drug of choice for the treatment of methicillin-resistant staphylococci and for the treatment of serious gram-positive infections in patients who are allergic to penicillin or cephalosporins (or both).

In adult patients with normal renal function, the usual dosage of vancomycin is 1 g every 12 h or 0.5 g every 6 h, a dose that gives peak levels of 25 to 35 and 10 to 15 μg/ml, respectively (5). Vancomycin is approximately 10% protein bound and is excreted by glomerular filtration. In renal failure, the vancomycin dose must be reduced, and monitoring of blood levels is recommended (5). In anuric patients, Eykyn et al. (2) found sufficient blood levels on a dose of 1 g every 7 days. Vancomycin levels are not reduced by hemodialysis (11).

Vancomycin can be ototoxic and nephrotoxic at serum concentrations of greater than 30 μg/ml. Other toxicity problems include febrile reactions and thrombophlebitis. When vancomycin is used in combination with an aminoglycoside, ototoxicity and nephrotoxicity may be additive (1).

Current methods for monitoring vancomycin include bioassay (9, 15), high-pressure liquid chromatographic (HPLC) assay (14), and radioimmunoassay (RIA) (3). Samples for the HPLC vancomycin assay must be extracted on an ion-exchange column before being subjected to chromatography, and the sample size required is relatively large (400 μl). The bioassay requires overnight incubation, and interference by other antibiotics can be a problem. A new fluorescence polarization immunoassay (FPIA) (7, 8) has been developed with a totally automated bench top fluorescence polarization analyzer.

This study was undertaken to compare two currently available nonsitopic assays, HPLC
and bioassay, with the fluorescence polarization immunoassay for the determination of vancomycin levels in patient samples.

**MATERIALS AND METHODS**

**FPIA.** The principles of FPIA, a homogeneous competitive inhibition assay, have been described in detail (7, 8). Briefly, unlabeled vancomycin in the patient serum competes with fluorescein-labeled vancomycin (tracer) for limited antibody sites. The binding of tracer to specific antibody results in an increase in the polarization of fluorescent light when compared with that of unbound tracer. The polarization of fluorescent light is inversely proportional to the amount of unlabeled antibiotic in the sample. The relationship between concentration of unlabeled drug and polarization is established with calibrators containing 0, 5, 10, 25, 50, and 100 µg of vancomycin per ml. Obtained values are subjected to a nonlinear least-squares curve fit, which is stored and used to determine the concentration of vancomycin in patient samples.

The reagents and automated, bench-top fluorescence polarization analyzer (TDX) were obtained from Abbott Laboratories, North Chicago, Ill. The reagent preparation and apparatus have been previously described (8). Briefly, samples (>50 µl), which can include standards, controls, or patient specimens, are each placed in the sample side of a dual-chamber sample cup. The sample is then automatically diluted in a 0.1 M phosphate buffer containing protein stabilizer and preservative (TDX dilution buffer) and read for background fluorescence. All initial values are stored in the instrument memory. Antibody to vancomycin (25 µl), and tracer (fluorescein-labeled vancomycin) (25 µl) are automatically dispensed into each cuvette containing the diluted sample, bringing the total volume to 1.95 ml. After a 3 min incubation, a final polarization measurement is made on each sample.

The resulting polarization values are blank corrected, and either a standard curve is computed, by using a weighted log-logit-type curve fit, or the concentrations of specimens are determined by reference to a stored curve.

**HPLC.** Samples were analyzed for vancomycin by a modification of the HPLC method of Uhl and Anhalt (14). Equal portions of serum and the internal standard solution containing ristocetin (75 µg/ml) were mixed. Vancomycin and ristocetin were separated from other serum components by ion-exchange gel chromatography. Carboxymethyl-Sephadex column preparation and elution solvents used have been previously described (14). Vancomycin and ristocetin were eluted from the column by using 800 µl of borate buffer. The first 300 µl of eluate was discarded, and the remainder was collected for analysis. A 25-µl sample of the eluate was injected into a Waters HPLC system (Waters Associates, Milford, Mass.) consisting of a model U6K injection value, a model 6000A solvent pump, a C18 u-Bondapak column, and a model 450 variable wavelength detector set at 210 nm. The mobile phase and operating conditions used are as previously described by Uhl and Anhalt (14).

**Bioassay.** The bioassay for the concentration of vancomycin in serum was performed by an agar well diffusion method. Antibiotic medium no. 5 (Difco Laboratories, Detroit, Mich.) was seeded with an appropriately standardized (no. 1 McFarland standard) fresh culture of a multiply resistant *Staphylococcus aureus* (D078) and poured into a 15- by 150-mm petri dish. Vancomycin standards were prepared in pooled human serum (Flow Laboratories, Inc., Rockville, Md.) from standard powder of stated potency provided by Eli Lilly Co., Indianapolis, Ind. A 5-µl sample of each standard and patient serum was pipetted in triplicate into 3-mm wells punched in the seeded plates. A separate standard curve with vancomycin concentrations of 10, 20, 40, and 80 µg/ml and a control serum (20 µg/ml) was run concurrently in triplicate on each plate with patient specimens, each in triplicate. After a 5-h preincubation period at 25°C and overnight incubation at 35°C, the diameter of each zone of inhibition was measured with calipers. A standard curve was constructed by utilizing a log-linear least-squares regression of zone size (millimeters) versus concentration (micrograms per milliliter). The vancomycin concentrations of patient sera were calculated from their concurrent standard curve, which met the statistic control criteria (*r* = 0.990 and control serum ± 2 standard deviations of determined value).

In the presence of rifampin, cefotaxime, and amikacin, antibiotics known to interfere with the assay, the following procedures were utilized. *S. aureus* D078 "trained" for rifampin resistance was used in determining the vancomycin concentration in sera of patients receiving this drug. Cefotaxime was inactivated by diluting patient sera 1:2 with β-lactamase (Watman Biochemicals, Ltd., Clifton, N.J.). Amikacin was removed by treatment of sera with cellulose phosphate before the assay (13). Control samples spiked with rifampin, cefotaxime, and amikacin have been shown to give accurate vancomycin levels when these methods of inactivation of the interfering antibiotics were employed.

**Specimens.** Patient sera utilized in this evaluation were those on which vancomycin levels had been requested as part of the patient’s clinical management. After the standard bioassay of the vancomycin level, sera were frozen at −20°C until assayed by HPLC and FPIA. Before treatment of specimens requiring either β-lactamase or cellulose phosphate, sera were split, providing untreated samples for both HPLC and FPIA.

**Data analysis.** Linear least-squares regression analysis was used to determine the correlation between the three methods.

**RESULTS**

The precision of the FPIA was determined by assaying a high, midrange, and low control each 5 times daily for 10 days. The means, standard deviations, and coefficients of variation for within-run and between-run assays of the three control sera are shown in Table 1.

Dilution effects were evaluated by taking samples with high vancomycin levels and diluting them 1:2, 1:4, and 1:8 in pooled human serum or TDX dilution buffer and assaying each by FPIA. The result was multiplied by the appropriate dilution factor to obtain the vancomycin level. The values demonstrate considerable variation,
perhaps due to the manual dilution of very small quantities (<0.1 ml) of serum. Recovery from the serum dilutions ranged from 103 to 112%. Recovery from TDX buffer dilutions ranged from 103 to 126%. The TDX instrument can be programmed to automatically dilute samples that contain high levels of antibiotic.

Recovery from icteric, lipemic, and hemolyzed samples was assessed by spiking these serum samples with 20 μg of vancomycin per ml and assaying by FPIA after 1 to 3 h. The percent recovery ranged from 88.5 to 109% (Table 2). Average recovery was 104% from hemolytic samples, 95% from icteric samples, and 104% from lipemic samples.

Throughout the study period (4 weeks) the same standard curve for FPIA was used, and the three controls remained well within acceptable limits—10% of the assigned value for the high and midrange controls and 15% for the low control. The control sera were assayed at least once on each assay carousel of ≈20 samples. The coefficients of variation for the control sera of each assay method are compared in Table 3.

The calibrators at 5, 10, 25, 50, and 100 μg/ml for the FPIA standard curve were assayed as unknowns six times in the 4-week study period. The standard deviation range was 0.68 for the calibrator at 5 μg/ml to 3.35 for the calibrator at 100 μg/ml. The coefficients of variation ranged from 13.8% for the calibrator at 5 μg/ml to 3.53% for the calibrator at 100 μg/ml.

**Table 3. Precision of control sera for the FPIA, HPLC, and bioassay**

<table>
<thead>
<tr>
<th>Assay</th>
<th>n</th>
<th>Target value</th>
<th>Mean</th>
<th>SD</th>
<th>CV%</th>
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<tbody>
<tr>
<td>FPIA</td>
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<td>7</td>
<td>7.5</td>
<td>0.46</td>
<td>6.13</td>
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<td>35</td>
<td>36.8</td>
<td>1.03</td>
<td>2.81</td>
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<td>18.01</td>
<td>1.39</td>
<td>7.74</td>
</tr>
<tr>
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<td>20</td>
<td>19.19</td>
<td>1.45</td>
<td>7.57</td>
</tr>
</tbody>
</table>

*CV, Coefficient of variation.*

**Table 2. Recovery of vancomycin from hemolytic, icteric, and lipemic samples by FPIA***

<table>
<thead>
<tr>
<th>Sample type</th>
<th>No.</th>
<th>Recovery of vancomycin (μg/ml)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before spike</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>20 μg/ml</td>
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<td>Hemolytic</td>
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</tr>
<tr>
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<td>2</td>
<td>1.2</td>
<td>22.0</td>
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<td></td>
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<td>1.3</td>
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<td>Icteric</td>
<td>1</td>
<td>0</td>
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<tr>
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<td>2</td>
<td>0</td>
<td>19.3</td>
</tr>
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<td></td>
<td>3</td>
<td>0</td>
<td>17.7</td>
</tr>
<tr>
<td></td>
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<tr>
<td>Lipemic</td>
<td>1</td>
<td>1.3</td>
<td>21.9</td>
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<tr>
<td></td>
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<td>2.0</td>
<td>23.5</td>
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*Serum samples were spiked with 20 μg of vancomycin per ml.*

**FIG. 1. Correlation of the FPIA and HPLC assay of vancomycin on 60 clinical specimens.**
bioassay (10 μg/ml) were not used in the correlations.

**DISCUSSION**

The increased use of vancomycin for treatment of methicillin-resistant *S. aureus*, multiply resistant corynebacteria, and multiply resistant *Staphylococcus epidermidis* sepsicemia in oncology patients already on aminoglycosides has prompted and underscored the importance of monitoring drug levels in these patients. The most significant adverse effect of vancomycin treatment, ototoxicity, is rarely seen when serum levels are maintained below 30 μg/ml. To ensure the presence of adequate levels of antibiotic the timely performance of vancomycin levels is of value, particularly for patients with relatively resistant organisms or renal impairment or for those receiving concomitant aminoglycoside therapy.

The FPIA for vancomycin is fully automated and fast. Samples can be batched, and results can be made available on the same day. Stat samples, when required, can be performed. Automatic dilution of samples, timing of the assay, and reading and calculation of results dramatically reduced technologist hands-on time required to perform the vancomycin assay compared with HPLC or the bioassay. The precision of the stored standard curve is confirmed for, but not limited to, one month in this study. Interference of hemolysis, lipemia, and icteremia is not significant since background readings are automatically taken on each serum sample.

Our bioassay for vancomycin is relatively easy and inexpensive to perform. A bioassay can be developed which has a high degree of precision (Table 3). In bioassays, vancomycin has the disadvantage of being a large molecule with a relatively slow diffusion rate. Consequently we allow a 5-h prediffusion time before incubation of the plate, and turnaround time is after overnight incubation. Although our assay organism, a methicillin-resistant staphylococcus, is relatively resistant to antibiotics in general, the patient population in which vancomycin levels are most often requested (oncology patients) are often receiving 3 to 6 antibiotics. Antibiotics known to interfere with the assay are inactivated, but additive and synergistic effects of multiple drug combinations and dosages against our assay organism cannot be controlled. Discrepancies between the bioassay versus FPIA and bioassay versus HPLC results did not show the bioassay to give consistently higher results, but did show higher variation when compared with either FPIA or HPLC.

Vancomycin determination by HPLC is a more complex procedure than both the bioassay and FPIA procedures. The sample size required is much larger than that needed for the bioassay and FPIA. HPLC and FPIA do provide results on the same day, whereas the bioassay requires an overnight incubation. Sample preparation, including the column preparation and serum extraction, is lengthy. Variability may exist in the column preparation and extraction, which could potentially lead to discrepant results. Interference by other antibiotics is less of a problem in the HPLC assay than in the bioassay, but
there is the potential for interference by any number of drugs used in clinical treatment or by serum components unique to a particular patient which may not be removed during the extraction procedure. Discrepancies between the HPLC assay and the bioassay and the FPIA could possibly reflect the presence of interfering peaks with retention times identical to those of vancomycin or the internal standard.

The renewed interest in vancomycin as an effective antibiotic in several difficult clinical situations and its potential for toxicity in those settings make it important to keep the serum concentration at a proper level. The FPIA in the TDX system is an efficient and accurate method for the timely determination of vancomycin serum levels.

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LITERATURE CITED