Determination of Vancomycin in Human Serum by High-Pressure Liquid Chromatography

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A rapid, accurate, reverse-phase high-pressure liquid chromatographic procedure for vancomycin quantitation in human serum, cerebrospinal fluid, and peritoneal fluid was developed. This procedure involves a simple chemical extraction of the antibiotic and is suitable for each of these body fluids. The column and mobile phase used provided a good resolution of the vancomycin peak with a retention time of 6.1 min. The precision of the assay was within the requirement for a daily routine clinical application. Coefficients of variation for within-day reproducibility were 5.80 and 6.28%, respectively, for samples at 50 and 25 μg/ml, and for between-day reproducibility they were 11.4 and 11.1%, respectively. No interference was found with respect to beta-lactam and aminoglycoside antibiotics and many other currently used drugs, indicating a good specificity for the procedure. The detection limit of 100 ng/ml has proven to be sufficient for monitoring drug levels in serum obtained after usual dosages. Drug levels in 112 clinical serum specimens assayed by high-pressure liquid chromatography were regressed against the levels obtained for the same samples by radioimmunoassay and fluorescent polarization immunoassay. Correlation coefficients were 0.945 and 0.967, respectively, and were highly significant (α < 0.001).

Vancomycin is a glycopeptide antibiotic being isolated from both Streptomyces orientalis and Nocardia lurida (23). It was introduced in 1956 because of its strong bactericidal activity against many gram-positive bacteria, particularly Staphylococcus aureus. Because of its toxicity, vancomycin was relegated to the role of alternate therapy when antibiotics such as methicillin became available (5, 11, 14).

The increasing number of methicillin-resistant isolates of S. aureus, Staphylococcus epidermidis (6, 8, 10, 22), and Streptococcus pneumoniae (2), similar to problems of treating patients allergic to beta-lactam antibiotics, led to the rehabilitation of vancomycin (4, 6, 9, 10). In addition, it would seem that much of the toxicity was due to impure preparations (10). Farber and Moelling (10) recently carried out a retrospective examination of the toxicity of modern preparations of vancomycin. Their study revealed few side effects; phlebitis, rash, neutropenia, and fever were detected in some patients. However, auditory toxicity was not seen. With regard to nephrotoxicity, the concomitant administration of an aminoglycoside antibiotic did not allow any conclusion to be drawn. Nevertheless, pharmacokinetic studies have proven the existence of a prolonged half-life, particularly in cases of renal insufficiency (3, 4, 8, 21), and otoxicity remains a side effect of modern preparations when drug levels in serum remain over 80 μg/ml (3, 4). Thus, it is necessary to monitor levels of vancomycin in blood to prevent this toxicity.

Many microbiological assay procedures lasting from 24 to 48 h have been developed for monitoring vancomycin (2, 7, 18). Radioimmunoassay (RIA) and fluorescent polarization immunoassay (FPIA) have been developed (1, 13), and two chromatographic procedures have also been described (19, 26). The present report describes a simple, accurate, and very sensitive high-pressure liquid chromatographic assay (HPLC) suitable for routine use in hospitals.

MATERIALS AND METHODS

Chemicals. Vancomycin hydrochloride (500 mg of vancomycin in 554 mg of titrated powder) was obtained from Eli-Lilly, Strasbourg, France. All measurements were determined relative to that potency. Stock solutions of vancomycin (10 mg/ml) were prepared in double-distilled water and were found to be stable for 1 month at −80°C. Acetonitrile, methylene chloride, and isopropanol (E. Merck AG, Darmstadt, Federal Republic of Germany) were of analytical grade. Water was distilled daily in quartz. Calibration standards of 1, 2, 3, 4, 5, 10, 20, and 50 μg of vancomycin per ml were prepared in sheep serum and may be conserved for 1 month at −80°C.

Chromatographic equipment and mobile phase. The isocratic liquid chromatograph was constituted from a 112 Solvent Delivery Module (Beckman Instruments Inc., Fullerton, Calif.), a model 210 sample injection valve with a 20-μl loop (Beckman), a model 160 selectable wavelength detector (Beckman), and a model ICR 1-B recording data processor (Intersmat Instruments, Courry, France). A 15-cm-long analytical octadecylsilane column (UltraspHERE, 4-mm internal diameter; Becton Dickinson & Co.) and 5-μm particle size were used. The mobile phase was composed of acetonitrile, 0.2 M ammonium acetate (15.41 g/liter), and double-distilled water (9, 10, and 81%). Glacial acetic acid was added to adjust the pH to 5.4, and the entire mixture was then filtered through a 0.22-μm-pore-size filter. The flow rate was set at 1 ml/min and resulted in a pressure of 1,500 lb/ln². The detector range was set at 0.1 absorbance unit full scale (AUFs) for a wavelength of 214 nm (zinc lamp).

Extraction procedure. A 500-μl volume of serum was combined with a 500-μl volume of an isopropanol-acetonitrile mixture (vol/vol) in a 7-ml screw-capped glass tube and mixed thoroughly on a Vortex mixer (The Vortex Manufacturing Co., Cleveland, Ohio). The tube was kept for 10 minutes on ice to facilitate protein precipitation and then gently shaken by rotation for 10 min (20 rpm). The resulting suspension was centrifuged for 10 min at 1,000 × g. The supernatant was transferred with a Pasteur pipette to an
other 7-ml screw-capped glass tube, and a 3.5-ml volume of methylene chloride was added. The mixture was allowed to equilibrate for 10 min by rotation and then centrifuged again for 20 min at 1,000 × g. A 20-μl portion of the upper aqueous layer was then injected into the column.

Quantitation and standardization. Quantitation was based on vancomycin peak height or area under the curve as measured by the integrator. A standard curve was prepared with normal sera spiked with known amounts of vancomycin, yielding concentrations of 1, 2, 3, 4, 5, 10, 20, and 50 μg/ml, to ascertain linearity of the procedure. The spiked serum samples together with the unknown serum samples to be assayed were then processed simultaneously.

The vancomycin extraction recovery was defined as the ratio of the peak height (or area under the curve) resulting from a spiked serum to the peak height (or area under the curve) resulting from an aqueous solution at the same vancomycin concentration.

Reproducibility. Within-day reproducibility was tested by assaying 10 samples of the same serum containing 50 or 25 μg of vancomycin per ml. These sera samples were randomly distributed in the different series of routine assays. Between-day reproducibility was tested with the same sera by assaying one of each concentration (50 and 25 μg/ml) each day during 10 days in a series of routine analyses.

Specificity. Interference studies were carried out with many substances which could be coadministered to patients with vancomycin. We spiked sera containing 10 μg of vancomycin per ml with one of the following antibiotics: gentamicin (8 μg/ml), tobramycin (8 μg/ml), amikacin (15 μg/ml), netilmicin (8 μg/ml), penicillin (150 μg/ml), ampicillin (50 μg/ml), mezlocillin (250 μg/ml), cloxacillin (100 μg/ml), ticarcillin (250 μg/ml), cefotaxime (80 μg/ml), and cefoperazone (80 μg/ml). Each of these sera were extracted and chromatographed five times, and their mean concentrations of vancomycin were statistically compared to the mean concentration of five sera containing only 10 μg of vancomycin per ml (Student's t test for the comparison of two means). Specificity was also assessed with 112 clinical specimens derived from patients taking drugs other than antibiotics, such as analgesics, salicylate, phenobarbital, carbamazepine, phenytion, primidone, valproic acid, digoxin, quinidine, procainamide, lidocaine, theophylline, digitoxin, and furosemide. All of the chromatograms obtained were carefully checked for atypical vancomycin peaks, shouldering of vancomycin peak, skewed peaks, and tailing of vancomycin peak. These 112 sera were also included in the comparison made with vancomycin peaks.

Accuracy. Validation was demonstrated by a correlation study between HPLC and both RIA and FPIA for 112 sera of patients from intensive care units. RIA was performed on an LKB 1260 Multigamma gamma counter with a commercially available kit (American Diagnostic Corp., Newport Beach, Calif.). The standard curve was constructed by measuring six calibrations, 1, 2, 4, 8, 16, and 32 μg/ml. Each serum was measured twice, and the mean level was calculated for the orthogonal regression analysis with RIA and HPLC. FPIA was performed with a Therapeutic Drug Monitoring System TDx (Abbott Laboratories, North Chicago, Ill.). The standard curve was constructed by measuring six commercially available calibrations, 0.5, 10, 24, 50, and 100 μg/ml. Three vancomycin controls were intended for verification of calibration of the TDx fluorescence polarization analyzer. The three controls were 7.0, 35.0, and 75.0 μg/ml. Both methods (RIA and FPIA) are known to have good daily and intraassay coefficients of variation (1).

Statistical analysis. Vancomycin concentrations in serum obtained by RIA and HPLC and by FPIA and HPLC were compared by using orthogonal regression analysis. The absolute differences for the 112 paired values between HPLC and RIA and HPLC and FPIA were compared with a difference of zero, using Student's t test. An α value of <0.05 was considered as statistically significant.

RESULTS

Methylene chloride extraction, which was done after protein precipitation, removed acetonitrile and lipids from the aqueous phase and allowed a significant concentration of vancomycin in the aqueous layer. Typical chromatograms of vancomycin are showed in Fig. 1. The retention time of the antibiotic was 6.1 min. No additional peaks were seen after 14 min of chromatography. Extraction recovery was found to be 115%, indicating a slight concentration of vancomycin in the aqueous supernatant. This concentration comes from back extraction of the acetonitrile by methylene chloride during the last step of sample preparation. With our detector setting of 0.1 AUFS, the detection limit was 0.5 μg/ml. It could be lowered to 100 ng/ml at 0.01 AUFS.

The extraction procedure is simple, and a few peptides may not be precipitated by the acetonitrile-isopropanol mixture. Vancomycin has a larger absorbance at 214 nm than at 254 or 280 nm. As the remaining peptides showed less absorbance at 214 nm than the two other wavelengths, this resulted in cleaner chromatograms during the first minutes of chromatography. Nevertheless, with a rapid extraction procedure, the major risk of interferences at 214 nm comes from common drugs which may be coadministered to the patients receiving vancomycin. In our study, there were no statistically significant differences (0.5 < α < 0.9 each time) between mean vancomycin concentration in the five sera containing only vancomycin and mean vancomycin concentration from any other series containing either beta-lactam or aminoglycoside antibiotics.
Clincial specimens coming from patients in intensive care units were also extracted and assayed. These patients were known to receive many other medications (see above). No chromatogram looked unusual. Each of the 112 vancomycin peaks was sufficiently separated from the other peaks and no shoulderling, tailing of the vancomycin peak, or any other symptom of interferences could be detected. The retention time, which may vary if substances coelute, was very stable; the coefficient of variation on the retention time of the 112 peaks was 0.04%.

The good resolution of the vancomycin peak allowed its quantitation by measuring either peak height or peak area. We did not find it necessary to use an internal standard as we found coefficients of variations suitable for within-day reproducibility (Table 1). A good linear relationship was obtained between the peak heights (measured in integrator units) and the drug concentrations ranging from 1 to 50 μg/ml (Fig. 2). As higher concentrations were expected, sera were diluted, or linearity was assessed higher than 50 μg/ml (75 or 100 μg/ml). A profile of drug concentration versus time is depicted in Fig. 3 for two patients, with and without renal insufficiency, after a 30-min infusion of 500 mg of vancomycin. The concentrations measured are within the linearity range.

**Precision.** Results of the reproducibility study are summarized in Table 1. Within-day reproducibility was good, as demonstrated by coefficients of variation of 5.80% for the samples at 50 μg/ml, and 6.28% for the samples at 25 μg/ml. For between-day reproducibility (Table 1), the variation coefficients were 11.1 and 11.4%, respectively, for the samples at 25 and 50 μg/ml.

**Accuracy.** (i) Comparison of RIA and HPLC. There was a high degree of correlation (r = 0.945) between drug levels in serum detected by RIA and HPLC (Fig. 4). The equation found was: y(RIA) = 1.13x (HPLC) + 2.32. This correlation is highly significant (α < 0.001). In 87% of the samples tested, RIA gave higher results than did HPLC. For the paired values of vancomycin concentrations, a mean differ-

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**TABLE 1. Precision of vancomycin assay**

<table>
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<tr>
<th>Sample no.</th>
<th>Within-day reproducibilitya of titers with vancomycin at (μg/ml):</th>
<th>Between-day reproducibility of titers with vancomycin at (μg/ml):</th>
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<tr>
<td></td>
<td>25</td>
<td>50</td>
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<tr>
<td>1</td>
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<td>125,303</td>
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Coefficient of variation:

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<th>Coefficient of variation</th>
</tr>
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<tbody>
<tr>
<td></td>
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<tr>
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</tr>
<tr>
<td>2</td>
<td>5.80%</td>
</tr>
<tr>
<td>3</td>
<td>11.1%</td>
</tr>
<tr>
<td>4</td>
<td>11.4%</td>
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</table>

* Coefficient of variation calculated with integrator area units.

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**FIG. 2.** Standard curve for vancomycin HPLC. Linear regression, y = 93.5x - 40; r = 0.997. Peak height, integrator units per 10³ μg/ml.

**FIG. 3.** Vancomycin serum concentrations curves in two patients with (——) and without (——) renal insufficiency. Dose, 500 mg of vancomycin in a 30-min intravenous infusion. MCG/ML = μg/ml.

**FIG. 4.** Orthogonal regression plot of the 112 paired values of vancomycin concentration in serum determined by RIA and HPLC. y(RIA) = 1.13x (HPLC) + 2.32. r = 0.945. Units for RIA and HPLC are micrograms per milliliter.
y(FPIA) = 1.11x (HPLC) + 2.06, r = 0.967. Units for FPIA and HPLC are micrograms per millilitre.

The precision obtained for this assay was within the requirement for a daily routine clinical application. The higher coefficients of variation (11.1 and 11.4%) for the between-day reproducibility may be explained by the fact that mobile phase was freshly prepared daily. Thus, there may have been slight variations of its composition from one day to the next, involving slight variations of vancomycin absorbance.

Use of ion-exchange chromatography to prepare samples, as previously described (26), was not necessary. Our sample preparation step ensured a good practicability of this method. A complete assay was performed in 1 h. Analysis of a series of samples did not require much more time, as we operated under isocratic chromatographic conditions, which do not require reequilibration of the column after each injection, unlike gradient chromatography. Another previously reported procedure (19) differs from ours in several respects, including the composition of the mobile phase, the extraction step, and a detection limit of 5 μg/ml at 0.005 AUFS. Of the 112 sera we assayed, 13 had vancomycin levels below 5 μg/ml (128 ng/ml could be detected in one case with a signal/noise ratio of 5), showing that a detection limit of 5 μg/ml may sometimes be insufficient.

Our detection limit was not only compatible with levels obtained in blood after the usual dosages (Fig. 3) (3, 6, 8, 18, 20) but also with lower levels obtained in peritoneal fluids during chronic intermittent peritoneal dialysis (15) or cerebrospinal fluids (16). We made sure that this procedure was suitable for cerebrospinal fluids and peritoneal fluids as we received a lot of these fluids since vancomycin has been measured by HPLC in our laboratory.

Although we developed a procedure with a nonspecific wavelength, its specificity was good. Beta-lactam antibiotics, which are known to be well extracted by our sample preparation procedure (17) were chromatographically resolved and did not interfere with vancomycin. Aminoglycoside antibiotics, which may be associated with vancomycin therapy, did not interfere either. Many other commonly used drugs were known to be present in the 112 clinical samples but did not interfere. Regression analysis of the described method with two modern procedures, RIA and FPIA, showed a highly significant correlation, but in general, values obtained by RIA or FPIA were slightly higher than those obtained by HPLC. The tendency of RIA to overestimate drug concentrations has been observed (24, 25), sometimes involving displacement of the regression lines. In a recent study (1), RIA also gave higher results when compared to FPIA for measuring vancomycin levels. We found our HPLC method to be specific. HPLC has been reported to be more specific than RIA in monitoring vancomycin (13). This lower specificity of RIA may in part explain the overestimation, as many samples used for the correlation study were known to contain high concentrations of many drugs, including beta-lactam antibiotics.

Our calibration samples prepared for HPLC were assayed by the FPIA procedure. The values obtained for all of these samples were higher than the expected values. A little deterioration due to storage of the FPIA standards could explain these facts and thus explain the somewhat higher value obtained by FPIA for the clinical samples. A loss of sensitivity in the upper assay range for a gentamicin fluorescent immunoassay has been reported (27), and a similar phenomenon as well as interference with other antibiotics (24) cannot be excluded for vancomycin.

Vancomycin may be used more frequently in the future for many reasons, including the increasing number of methicillin-resistant Staphylococcus aureus infections, streptococcal endocarditis in patients intolerant to beta-lactam antibiotics, infections associated with prosthetic devices, and antibiotic-induced enterocolitis associated with Clostridium difficile (12, 14). Despite the greater purity of modern preparations of vancomycin, the persistence of ototoxicity when blood levels reach 50 to 80 μg/ml involves regular and rapid therapeutic monitoring to improve the safety of the treatment. The major advantage of RIA and FPIA over HPLC is that they are faster when assaying a large number of samples (about 1/4 of the time required for HPLC). Nevertheless, HPLC requires much less time than microbiological assay procedures. Additional advantages are the avoidance of handling of radioactive chemicals and the relatively low cost of equipment and reagents for isocratic chromatography, when compared with equipment and reagents for immunoassays.

RIA and FPIA are reliable procedures for monitoring vancomycin levels in serum; our chromatographic procedure offers attractive features relative to the other modern techniques.
ACKNOWLEDGMENTS

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LITERATURE CITED


