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Article

Development of HPLC Methods for the Determination of Vancomycin in Human Plasma, Mouse Serum and Bronchoalveolar Lavage Fluid

Mao Hagihara[1,2], Christina Sutherland[1] and David P. Nicolau[1*]

[1]Center for Anti-Infective Research and Development, Hartford Hospital, Hartford, CT, and [2]Aichi Medical University, Aichi, Japan

*Author to whom correspondence should be addressed. Email: dnicola@harthosp.org

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Two high-performance liquid chromatography methods utilizing a protein precipitation technique were developed to analyze vancomycin in human plasma, mouse serum and bronchoalveolar lavage (BAL) fluid. The mobile phase consisted of ammonium phosphate buffer with acetonitrile. A cross-matrix validation was performed to ensure that the mouse serum was comparable to the original biological matrix of human plasma. Murine BAL samples were run on a saline standard curve. For saline samples, the mobile phase from the human plasma study was used with the addition of 1M sodium hydroxide (0.2%) to avoid interfering peaks. A reversed-phase column was used with an ultraviolet detector set at 240 nm for human plasma and 198 nm for saline to increase peak size. The standard curves were liner over the ranges of 1 to 80 μg/mL for human plasma and 0.1 to 10 μg/mL for saline. These assays are simple, reproducible and accurate. These analytical techniques were successfully applied to analyze vancomycin concentrations in mouse serum and BAL samples.

Introduction

Vancomycin, a glycopeptides antibiotic, is almost universally accepted as the drug of choice for the treatment of most methicillin-resistant *Staphylococcus aureus* (MRSA) infections; the ratio of the area under the serum drug concentration-versus-time curve (AUC) to the minimum inhibitory concentration (MIC) appears to be a predictor of the response based on data from limited clinical studies (1, 2). Furthermore, the relationship between antibiotic efficacy and drug concentration at the site of infection has been shown to be highly important to more precisely predict the therapeutic outcome (3). For the treatment of patients with pneumonia, valuable information may be provided by determination of the epithelial lining fluid (ELF) concentration from bronchoalveolar lavage (BAL) samples, but the poor penetration of vancomycin renders this assessment difficult when using radioimmunoassay (RIA) techniques (4). As BAL studies increase regarding humans and animals, a more sensitive method for the detection of vancomycin is needed in a variety of matrixes.

To date, RIA and fluorescent polarization immunoassay (FPIA) have been the most frequently employed for the routine analysis of vancomycin in serum samples in the clinical setting (5–7). These procedures offer sensitivity and selectivity. However, because these methods were developed and validated for the anticipated serum concentration–time profile in humans, they have insufficient specificity and sensitivity for other biological matrices.

Although traditional methods to determine the bronchopulmonary distribution of compounds of interest involved invasive sampling of whole lung tissue (8, 9), with the advent of the BAL technique, it is now possible to directly obtain a sample of the fluid and cells lining the epithelial surface of the lower respiratory tract to detect drug concentrations in both the intracellular and extracellular spaces. This approach provides a useful and less invasive means of monitoring drug concentration in the spaces commonly associated with infecting pathogens.

Thus, the high-performance liquid chromatography (HPLC) method is powerful tool for the determination of antibiotics in biological fluids. However, an HPLC assay suitable for the detection of vancomycin in the BAL fluid matrix has not been established, although the some HPLC assays for plasma and serum samples have been reported (10, 11, 12). Hence, the purpose of the present study was to develop a simple, very sensitive, and reproducible HPLC method for human plasma, mouse serum and BAL samples.

Experiment

Chemicals

Vancomycin standard powder with purity of 980 μg/mg (Lot 050M1312, exp. June 2012), ammonium phosphate monobasic and caffeine standard powder with purity of 100% (Lot 021M0091V, exp. Feb. 2015), were used as the internal standard (IS), and were purchased from Sigma (St. Louis, MO). HPLC-grade acetonitrile (Mallinckrodt Baker, Phillipsburg, NJ) was used without further purification. Deionized water was obtained from a Milli-Q analytical deionization system (Bedford, MA).

Instrumentation and chromatographic conditions

An HPLC system consisting of a Waters 510 pump (Waters, Milford, MA) and 717 plus autosampler (Waters) was equipped with a 5-μm Spherisorb C18 ODS column (4.6 × 150 mm, Waters) coupled to a μBondapak C18 10 μm Guard-pak precolumn (Waters). The autosampler was cooled to 10°C. The column was maintained at room temperature. EZChrom Elite chromatography data system (Scientific Software, Pleasanton, CA) was used to quantify the peak heights. The flow rate was 1.2 mL/min. The running time for one sample was 20 min. Chromatographic procedures were performed at room temperature.

For human plasma, the mobile phase consisted of a mixture of 0.05M ammonium phosphate buffer with 11% acetonitrile. A programmable ultraviolet (UV) detection set at 240 nm (Model 526; ESA, Chemsfold, MA) was used. For the saline standard curve, the mobile phase consisted of the solution that was used as mobile phase for human plasma sample, with an additional 1M sodium hydroxide (0.2 mL/100 mL mobile phase) used to avoid interfering peaks (pH 5.5). Based on the lower limit of quantification, the UV detector was lowered to 198 nm to increase peak size, because there were no interfering peaks in saline.

Standard solutions and controls

Vancomycin stock solution of 2000 μg/mL was made in a volumetric flask using 0.85% physiological saline to dissolve and dilute the stock standard. The IS, caffeine in concentrations of 50 and 10 μg/mL, was prepared in water for use in the human plasma and BAL procedures, respectively. Caffeine was selected for this purpose because the retention time of the compound was unaltered over the utilized pH range (10).

Drug free human plasma and mouse serum were purchased from Bioreclamation (Hicksville, NY). The pooled blank plasma was stored at −10°C before use. Vancomycin was spiked into the human plasma to make six standard solutions (1, 3, 10, 20, 40 and 80 μg/mL) and two quality controls (2 and 60 μg/mL). The murine BAL samples were run on the saline curve, and vancomycin was spiked into saline to make six standard solutions (0.1, 0.3, 0.8, 2, 5 and 10 μg/mL) and two quality controls (0.2 and 8 μg/ml). These ranges of the standard curves were based on the anticipated concentration-versus-time profile for vancomycin. Aliquots of the standards and IS were stored at −80°C until analysis.

A cross-matrix validation was performed to ensure that the mouse serum was comparable with the original biological matrix of human plasma. Vancomycin was spiked into mouse serum to make two quality controls (2 and 60 μg/mL). Quality controls in mouse serum at low and high concentrations were used to evaluate the precision and accuracy against the human plasma standard curve with a range of 1 to 80 μg/mL.

Sample extraction

A 200-μL sample of standard, quality control or unknown sample, along with a 50-μL aliquot of IS was pipetted into a polyethylene tube. The samples were extracted with the addition of 0.5 mL of acetonitrile. The sample was vortexed for 30 s and centrifuged for 10 min at 1,000 g. The supernate was placed into another polyethylene tube and dried under a stream of nitrogen for 60 min at 35°C. The residuals were reconstituted with 200 μL of mobile phase, vortexed for 30 s, and placed into an autosampler vial for injection.

Assay validation

Calibration curves were generated by plotting the peak height ratio of vancomycin to that of the IS. Weighted (1/concentration) least-squares regression analysis was applied to generate the liner regression equation. This equation was used to calculate the concentrations of the quality controls and unknown samples. Linearity of the standard curve was assessed with the correlation coefficient.

A full validation ($n = 6$ runs) was performed. Quality controls at low and high concentrations were used to evaluate the precision and accuracy. The precision was determined by the relative standard deviation (RSD) and the accuracy was determined by the relative error from the theoretical concentrations. The lower limit of quantification (LLQ) for the assay was evaluated on five samples with 1 μg/mL for human plasma and 0.1 μg/mL for saline. Recovery experiments were performed in triplicate by comparing the analytical results for extracted samples at each of the quality control concentrations with unexpected controls prepared in saline that represent 100% recovery.

The room temperature, autosampler and freeze and thaw stability of vancomycin were determined for each quality control sample. The freeze and thaw stability was performed by completely thawing the quality controls at room temperature and freezing them at −80°C for 24 h. The freeze-thaw cycle was repeated two more times, and then analyzed on the third cycle. The stability of IS was also assessed at room temperature.

Animal studies

To confirm the reliability of these assays for mouse serum and BAL, samples from pharmacokinetic and bronchopulmonary penetration studies were analyzed with the current HPLC method. The study was approved by the Hartford Hospital Institutional Animal Care and Use Committee (IACUC) for animal use. In brief, BALB/c mice were infected with MRSA to produce pneumonia. Vancomycin was given subcutaneously to simulate the observed ELF exposure in humans (13). On the day of BAL, blood samples were collected from each mouse at 0, 1, 2, 4 and 8 h after the last dose. Blood samples were centrifuged (1,000 g at 4°C for 10 min) immediately after collection. Separated serum was stored at −80°C until analysis by HPLC.

For BAL sample, a catheter was inserted into the trachea of the mice and lungs, lavaged with four 0.4-mL aliquots of normal saline. Hence, these samples were mostly saline. The concentration of vancomycin in the BAL fluid combined with the urea concentrations in BAL and serum was used to determine vancomycin ELF concentrations at each of the four time points.

The volume of ELF within the BAL was calculated by the urea dilution method (14). The urea concentrations in serum and BAL fluid collected simultaneously at the time of bronchoscopy were analyzed by a colorimetric enzymatic assay (Teco Diagnostics, Anaheim, CA) by a spectrophotometer detection method (Cary 50 series; Varian, Walnut Creek, CA). Vancomycin concentrations in the ELF ($\text{Vancomycin}_{\text{ELF}}$) were calculated using the following formula: $\text{Vancomycin}_{\text{ELF}} = \text{Vancomycin}_{\text{lavage}} \times (\text{urea}_{\text{serum}} / \text{urea}_{\text{lavage}})$, where $\text{urea}_{\text{serum}}$ and $\text{urea}_{\text{lavage}}$ are the concentrations of urea in the serum and lavage fluid, respectively, and $\text{Vancomycin}_{\text{lavage}}$ is the concentration of vancomycin in the lavage fluid. The AUC was calculated using the trapezoidal rule. The degree of penetration into the ELF was determined by comparing the AUC of ELF with the AUC of serum.

Correlation of RIA and HPLC for human plasma

Correlation of RIA with HPLC was assessed in 62 clinical samples obtained from patients receiving vancomycin over the period of August 31, 2011 to November 8, 2011. All samples were obtained in accordance with the institution's IRB review and approval of a protocol to collect such information.

Results

Chromatography

Several mobile phases containing various concentrations of acetonitrile and phosphate buffer with differing pH were investigated with the extraction methodology to determine optimal assay conditions. Lowering the acetonitrile concentration increased the retention time of both vancomycin and caffeine. The retention times of vancomycin and caffeine were 15.0 and 27.0 min using a mobile phase that consisted of a mixture of 0.05M ammonium phosphate buffer with 10% acetonitrile, and 3.0 and 11.0 min were the retention times when 15% acetonitrile was utilized. Additionally, increasing the pH with 1M sodium hydroxide resulted in negligible changes in retention time, as has been previously reported (10). Six sources of human plasma were tested for interference using the described protein precipitation technique and showed no interfering peaks with vancomycin or the IS. The retention times of vancomycin and caffeine were 8.5 and 13.7 min for human plasma, and 14.4 min and 17.4 min for saline, respectively (Figures 1 and 2). The retention times and peak shapes were similar through the assay period.

Linearity

For the human plasma, the peak height ratio of vancomycin and the IS versus the theoretical concentration coefficient for each calibration curve was ≥ 0.998 ($n = 6$). The slope was 0.0472 ± 0.0035 [mean $\pm$ standard deviation (SD)] and the intercept was 0.0075 ± 0.0075. For saline, the coefficient for each calibration curve was ≥ 0.999 ($n = 6$). The slope was 0.1845 ± 0.0076 (mean $\pm$ SD) and the intercept was -0.0059 ± 0.0032 (Supplementary Figure 1).

Lower limit of quantification

For human plasma, the LLQ of 1 μg/mL vancomycin was chosen as the concentration for the lowest standard sample. The precision and accuracy of LLQ ($n = 5$) were 7.0 and 1.3%. No response was observed from the blank human plasma at the vancomycin retention time. For saline, the LLQ of 0.1 μg/mL vancomycin was chosen as the concentration for the lowest standard sample. The precision and accuracy of LLQ ($n = 5$) were 9.87 and 3.6%. No response was observed from the blank murine BAL sample at the vancomycin retention time.

Precision and accuracy

The summary data for the inter-day and intra-day precision and accuracy for both assays are shown in Tables I and II. For human plasma and saline, the precision and accuracy, which were determined by the RSD of the assays, were within acceptance criteria ($\pm 10\%$).

Cross-matrix validation

The mouse serum intra-run precision of the 2 and 60 μg/mL samples was 5.09 and 0.40%, respectively. Mouse serum inter-run accuracy of the 2 and 60 μg/mL samples were 3.48 and 5.16%, respectively. The recovery of vancomycin in the 2 and 60 μg/mL mouse serum samples was 90.8 ± 0.016 and 85.1 ± 0.003%, respectively. The recovery of the IS in 50 μg/mL mouse serum sample was 90.8 ± 0.004%.

Stability

Vancomycin quality control samples (2 and 60 μg/mL) were stable at room temperature at 23°C for at least 5 h with ≤ 2 and $\leq 8\%$ degradation and were stable for three freeze and thaw cycles with ≤ 1 and $< 3\%$ degradation, respectively. The quality control samples (2 and 60 μg/mL) after extraction in the 10°C autosampler were stable for 24 h with ≤ 4 and $\leq 10\%$ degradation, respectively.

Animal study

Vancomycin concentrations in ELF and serum for mice with pulmonary infection are shown in Figure 3. These methods were successfully applied to analyze the serum and BAL samples from the pharmacokinetic study conducted in mice. The serum concentration–time profiles of the single subcutaneous dose of vancomycin at 20 and 25 mg/kg are shown. Penetration ratios of vancomycin for each regimen were 78.5 and 55.3% for the 25 mg/mL and 20 mg/kg regimen, respectively.

Comparison to RIA

As shown in Figure 4, the two methods correlated in the determination of vancomycin plasma levels ($r = 0.916$). The established equation was: y (HPLC) $= 0.651x$ (RIA) $+ 3.987$. In 91.9% (57/62 samples) of the tested samples, vancomycin concentration of RIA was higher than that of HPLC.

Discussion

The major advantage of immunoassays such as RIA is that they are faster when assaying a large number of samples. Although the calibration curve is quite robust (0 to 80 μg/ml of cobas c501, RIA; Roche, West Sussex, UK) and variation coefficients range from 2.4 to 4.4% over 7 to 60 μg/mL, the detection limit of 1.7 μg/mL often restricts the utility of the assay for biological matrices such as BAL fluid. Compared with immunoassay, HPLC is more sensitive and specific and has the ability to detect low concentrations with high precision and accuracy, although HPLC is not the simplest and or quickest method (8, 9, 15). Hence, HPLC techniques have been used to detect vancomycin concentrations below the detection limit of the immunoassay. Although FPIA and RIA analyses yield reliable results for monitoring vancomycin in blood samples, HPLC is often required when low concentrations are expected, such as

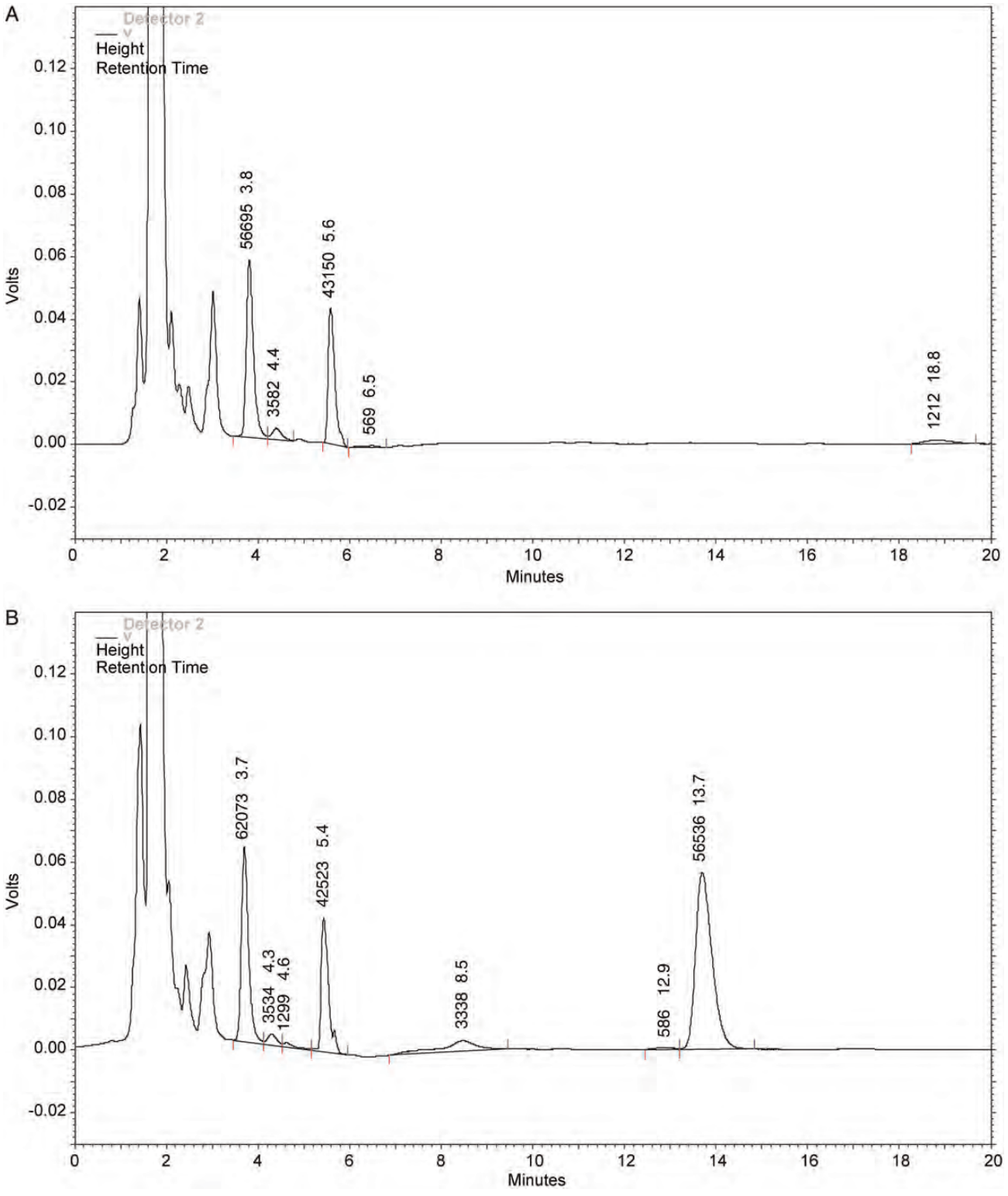

Figure 1. Chromatograms of: blank human plasma (A); LLQ (1 μg/mL) in human plasma (B). The retention times of vancomycin and the IS were 8.5 and 13.7 min, respectively.

those in tissue. Additionally, once the method is established, isocratic chromatography is associated with a relatively low cost for equipment and reagents when compared with the equipment and reagents required for immunoassay.

Previously, Jehl and colleagues reported the precision [5.80–6.28% (intra-day), 11.1–11.4% (inter-day)] and lower detection limit (0.5 μg/mL) of an HPLC assay developed for human plasma (16). Compared with their assay, the current HPLC

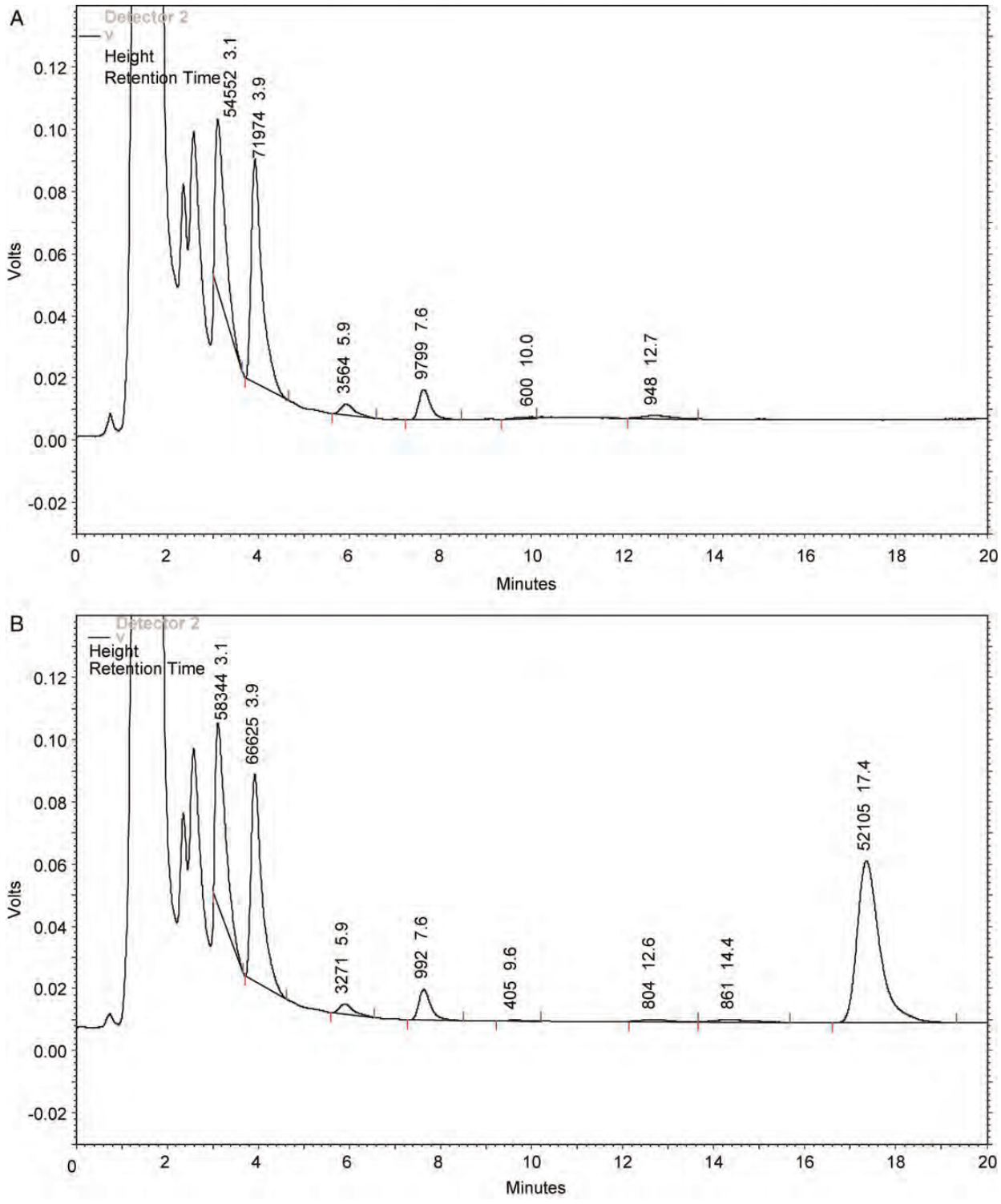

Figure 2. Chromatograms of: blank saline (A); LLQ (0.1 μg/mL) in BAL (B). The retention times of vancomycin and the IS were 14.4 and 17.4 min, respectively.

method is suitable to more precisely detect vancomycin concentrations in saline and plasma. Moreover, the current detection limit allowed the determination of concentrations in both blood after the usual dosages of vancomycin, and lower values obtained in BAL fluid (Figure 3). Additionally, although others have reported HPLC methods for the assay of vancomycin in human plasma, artificial perfusion fluid and lung tissue, the current assay is preferable due to the detection limit, simplicity and precision (8, 9, 15, 16). Thus, the method and validation of an isocratic HPLC technique for the quantification of

Table I
Precision and Accuracy of Vancomycin in Human Plasma

	Theoretical concentration (μg/mL)	
	Low (2)	High (60)
Inter-run ($n = 6$)		
Mean	2.00	61.36
SD	0.04	1.03
RSD (%)	2.00	1.68
Relative error (%)	0.00	2.27
Intra-run ($n = 10$)		
Mean	2.06	63.14
SD	0.05	1.42
RSD (%)	2.43	2.25
Relative error (%)	3.00	5.23

Table II
Precision and Accuracy of Vancomycin in Saline

	Theoretical concentration (μg/mL)	
	Low (0.2)	High (8)
Inter-run ($n = 6$)		
Mean	0.20	7.77
SD	0.01	0.30
RSD (%)	5.55	3.86
Relative error (%)	0.00	2.88
Intra-run ($n = 10$)		
Mean	0.21	7.43
SD	0.01	0.21
RSD (%)	4.76	2.83
Relative error (%)	5.00	7.13

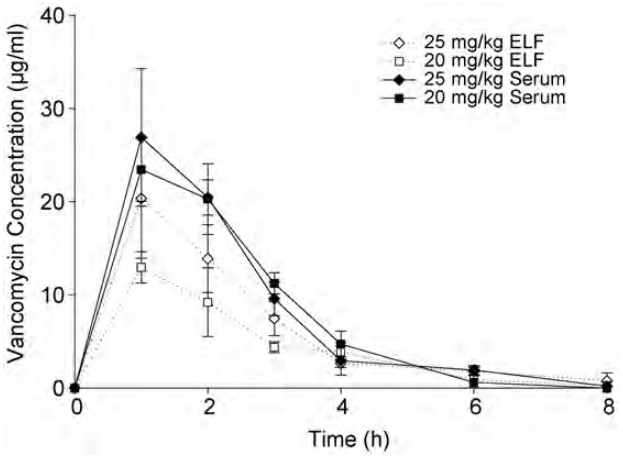

Figure 3. Mouse serum concentration and ELF-concentration-time profiles of vancomycin following a single dose of 20 mg/kg (serum, closed square; ELF, open square) and 25 mg/kg (serum, closed diamond; ELF, open diamond) (mean ± SD, $n = 6$ for each time point).

vancomycin concentrations in BAL fluids show that is rapid, simple, uses a basic HPLC system and can be applied to the assay of human BAL samples.

Finally, regression analysis of the described method with RIA showed a highly significant correlation ($r = 0.916$, Figure 4), but most values obtained by HPLC were slightly lower than those obtained by RIA. In the current data, if the two samples

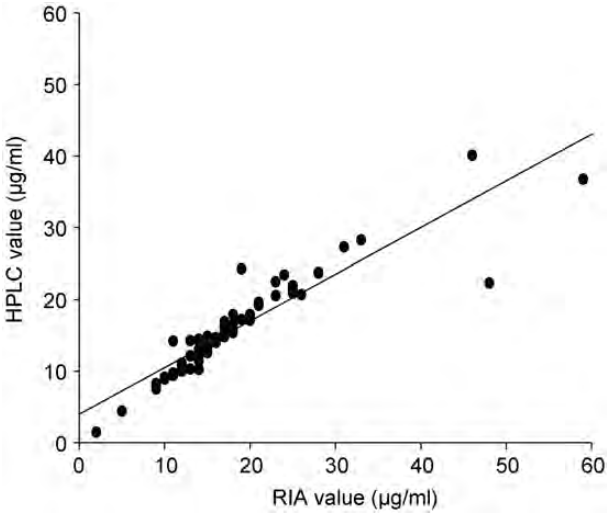

Figure 4. Regression plot of the 62 paired values of vancomycin concentrations in human plasma determined by RIA and HPLC: $y(\text{HPLC}) = 0.651x\ (\text{RIA}) + 3.987$; $r = 0.916$. Units for RIA and HPLC are μg/mL.

that showed more than 40% different values between the assays were excluded, the correlation ratio would be much better [formula: $y(\text{HPLC}) = 0.859x\ (\text{RIA}) + 0.766$, $r = 0.973$]. Some studies have shown that values obtained by RIA and FPIA were slightly higher than those obtained by HPLC, and the tendency of RIA to overestimate drug concentrations has been observed (8, 9, 15, 17–19).

Conclusions

Validated HPLC methods have been developed to assess vancomycin concentrations in human plasma, mouse serum and BAL fluid. These data support a methodology that results in a precise and accurate assay. This assay has been successfully utilized to determine vancomycin concentrations in mouse serum and BAL fluid with minimal interference of the host matrix.

References

1. Kullar, R., Davis, S.L., Levine, D.P., Rybak, M.J.; Impact of vancomycin exposure on outcomes in patients with methicillin-resistant *Staphylococcus aureus* bacteremia: Support for consensus guidelines suggested targets; *Clinical Infectious Diseases*, (2011); 52: 975–981.
2. Moise-Broder, P.A., Forrest, A., Birmingham, M.C., Schentag, J.J.; Pharmacodynamics of vancomycin and other antimicrobials in patients with *Staphylococcus aureus* lower respiratory tract infections; *Clinical Pharmacokinetics*, (2004); 43: 925–942.
3. Gould, I.M., Harvey, G., Golder, D., Reid, T.M., Watt, S.J., Friend, J.A., *et al.*; Penetration of amoxycillin/clavulanic acid into bronchial mucosa with different dosing regimens; *Thorax*, (1994); 49: 999–1001.
4. Lodise, T.P., Drusano, G.L., Butterfield, J.M., Scoville, J., Gotfried, M., Rodvold, K.A.; Penetration of vancomycin into epithelial lining fluid in healthy volunteers; *Antimicrobial Agents and Chemotherapy*, (2011); 55: 5507–5511.
5. Pfaller, M.A., Krogstad, D.J., Granich, G.G., Murray, P.R.; Laboratory evaluation of five assay methods for vancomycin: Bioassay, high-pressure liquid chromatography, fluorescence polarization

immunoassay, radioimmunoassay, and fluorescence immunoassay; *Journal of Clinical Microbiology*, (1984); 20: 311–316.
6. Filburn, B.H., Shull, V.H., Tempera, Y.M., Dick, J.D.; Evaluation of an automated fluorescence polarization immunoassay for vancomycin; *Antimicrobial Agents and Chemotherapy*, (1983); 24: 216–220.
7. Lamer, C., de Beco, V., Soler, P., Calvat, S., Fagon, J.Y., Dombret, M.C., *et al.*; Analysis of vancomycin entry into pulmonary lining fluid by bronchoalveolar lavage in critically ill patients; *Antimicrobial Agents and Chemotherapy*, (1993); 37: 281–286.
8. Valle, M.J., López, F.G., Navarro, A.S.; Development and validation of an HPLC method for vancomycin and its application to a pharmacokinetic study; *Journal of Pharmaceutical and Biomedical Analysis*, (2008); 48: 835–839.
9. Farin, D., Piva, A.G., Gozlan, I., Kitzes-Cohen, R.; A modified HPLC method for the determination of vancomycin in plasma and tissues and comparison to FPIA (TDX); *Journal of Pharmaceutical and Biomedical Analysis*, (1998); 18: 367–372.
10. Del Nozal, M.J., Bernal, J.L., Pampliega, A., Marinero, P., López, M.I., Coco, R.; High-performance liquid chromatographic determination of vancomycin in rabbit serum, vitreous and aqueous humour after intravitreal injection of the drug; *Journal of Chromatography A*, (1996); 727: 231–238.
11. Cheng, C., Liu, S., Xiao, D., Hollembaek, J., Yao, L., Lin, J., *et al.*; LC-MS/MS method development and validation for the determination of polymyxins and vancomycin in rat plasma; *Journal of Chromatography B*, (2010); 878: 2831–2838.
12. Plock, N., Buerger, C., Kloft, C.; Successful management of discovered pH dependence in vancomycin recovery studies: Novel HPLC method for microdialysis and plasma samples; *Biomedical Chromatography*, (2005); 19: 237–244.
13. Rodvold, K.A., Gotfried, M.H., Loutit, J.S., Porter, S.B.; Plasma and intrapulmonary concentrations of oritavancin and vancomycin in normal healthy adults. 14th European Society of Clinical Microbiology and Infectious Diseases, Prague, Czech Republic, (2004).
14. Rennard, S.I., Basset, G., Lecossier, D., O'Donnell, K.M., Pinkston, P., Martin, P.G., *et al.*; Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution; *Journal of Applied Physiology*, (1986); 60: 532–538.
15. Abu-Shandi, H. K.; Determination of vancomycin in human plasma using high-performance liquid chromatography with fluorescence detection; *Analytical and Bioanalytical Chemistry*, (2009); 395: 527–532.
16. Jehl, F., Gallion, C., Thierry, R.C., Monteil, H.; Determination of vancomycin in human serum by high-pressure liquid chromatography; *Antimicrobial Agents and Chemotherapy*, (1985); 27: 503–507.
17. Wilson, J.F., Davis, A.C., Tobin, C.M.; Evaluation of commercial assays for vancomycin and aminoglycosides in serum: a comparison of accuracy and precision based on external quality assessment; *Journal of Antimicrobial Chemotherapy*, (2003); 52: 78–82.
18. Ratcliff, R.M., Mirelli, C., Moran, E., O'Leary, D., White, R.; Comparison of five methods for the assay of serum gentamicin; *Antimicrobial Agents and Chemotherapy*, (1981); 19: 508–512.
19. Rotschafer, J.C., Morlock, C., Strand, L., Crossley, K.; Comparison of radioimmunoassay and enzyme immunoassay methods in determining gentamicin pharmacokinetic parameters and dosages; *Antimicrobial Agents and Chemotherapy*, (1982); 22: 648–651.