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Development and Validation of a Rapid High-Performance Liquid Chromatography Method with UV Detection for the Determination of Vancomycin in Mouse Plasma

Krishna Muppidi[1], Andrew S. Pumerantz[2], Guru Betageri[1] and Jeffrey Wang[1]*

[1]*Department of Pharmaceutical Sciences, College of Pharmacy, Western University of Health Sciences, USA*
[2]*Division of Infectious Disease, Department of Internal Medicine, College of Osteopathic Medicine of the Pacific, Western University of Health Sciences, Pomona, USA*

Abstract

A rapid high-performance liquid chromatography (HPLC) method for the quantification of vancomycin in mouse plasma samples was developed and validated. Norvancomycin was used as the internal standard. Chromatographic separation was achieved on a Vydac C18 column (4.6×50 mm, 3 µm particle size) and the detection was made at 214 nm. A gradient elution was programmed with the mobile phases of 0.1% v/v trifluoroacetic acid (A) and 95:5 v/v acetonitrile: 0.1% TFA (B) and a flow rate of 1 ml/min. The total run time was 15 min. The calibration curve was linear over the range of 0.1-20 µg/ml, with a correlation coefficient (r) higher than 0.997 and the lower limit of quantitation (LLOQ) of 0.1 µg /ml. The intra-day accuracy values were between 90 and 112% and the inter-day ones ranged from 96 to 104%. Precision values ranged from 1.7 to 9.5% for intra-day and 6.3 to 9.4% for inter-day. Stability studies indicated that the mouse plasma samples containing vancomycin could be stored in freezer at -80°C and handled under normal laboratory conditions without significant loss of the drug. The assay was successfully applied to the pharmacokinetics and bio-distribution study of novel formulations of vancomycin in mice.

Keywords: Vancomycin; HPLC-UV; Mouse plasma; Validation; Stability; Pharmacokinetics

Introduction

Vancomycin (Figure 1) was first isolated as "compound 05865" in 1953 by Edmund Kornfield of Eli Lilly and Co, from a dirt sample from Borneo that contained the organism, *Streptomyces orientalis* [1]. Nearly 60 years after its first clinical introduction, vancomycin remains the most widely used antimicrobial agent in the prevention and treatment of infection due to Gram-positive bacteria and methicillin-resistant Staphylococci in particular. Vancomycin is a bactericidal agent that targets the D-alanyl-D-alanine terminal on the peptidoglycan chain in the cell wall. The vancomycin plasma concentration-time profile is complex and can be characterized as one, two, and three compartment pharmacokinetic models [2] with 80%-90% of the dose recovered unchanged in the urine within 24 h after administration of a single dose [3]. High serum levels of vancomycin lead to increase in the risk of nephrotoxicity [4]. To date a few quantification methods to determine the concentration of vancomycin in biological fluids have been developed. Among them, immunoenzymatic techniques such as FPIA, EMIT and RIA [5] and chromatographic methods are the most relevant. Although immune enzymatic techniques have been widely used clinically owing to their simplicity and high speed, several drawbacks associated with these techniques hinder their usage in research studies involving pharmacokinetics of vancomycin [5,6]. These drawbacks have led to the development of various chromatographic techniques for the accurate and precise determination of vancomycin in biological fluids. Vancomycin has been quantified in plasma and serum samples by using high-performance liquid chromatography methods with ultraviolet detection [7-9], fluorescence detection [10], electrochemical detection [11] and mass spectrometry detection [12,13]. Vancomycin has also been quantified in tissues [8,14]. Although some of these methods provide high sensitivity and specificity, tedious extraction techniques and expensive technology are a major hindrance for their practical implementation. Therefore, a simple HPLC method with a routine extraction procedure is desirable.

In the present work, we developed and validated an HPLC method for the quantification of vancomycin in mouse plasma samples.

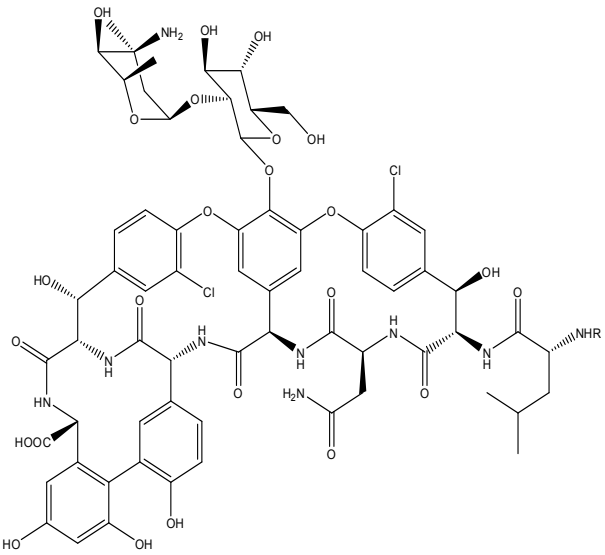

Figure 1: Chemical structures of vancomycin (R=CH_3) and norvancomycin (R=H, internal standard).

***Corresponding author:** Jeffrey Wang, Department of Pharmaceutical Sciences, College of Pharmacy, Western University of Health Sciences, 309 E. 2nd Street, Pomona, CA 91766, USA, E-mail: jwang@westernu.edu

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The method used UV detection with a structurally closely related compound norvancomycin (Figure 1) as the internal standard. The sample preparation technique employed was a one-step protein precipitation procedure. A lower limit of quantization (LLOQ) of 0.1 µg/ml was achieved. The method exhibited good sensitivity, linearity, accuracy and precision according to the International Conference on Harmonization (ICH) guidelines [15]. The method shows similar or higher sensitivity than most other complex techniques used to date. The application of the same method for quantification of tissue concentrations of vancomycin is also an advantage. Earlier methods reported for the quantification of vancomycin tissue samples has a higher LLOQ of 0.5 µg/ml [9]. Our method has been successfully used for the quantification of vancomycin in both plasma and tissue samples from mice treated with several formulations of vancomycin.

Experimental Section

Materials

1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) and MPEG-2000-DSPE were obtained from Genzyme Pharmaceuticals (Cambridge, MA). Cholesterol, vancomycin hydrochloride, sodium chloride, trifluoroacetic acid and sucrose were obtained from Sigma Chemicals (St. Louis, MO). Norvancomycin was procured from Northern China Pharmaceutical Corporation (Shijiazhuang, Hebei, China). Potassium chloride and dibasic sodium phosphate were obtained from Baker (Philipsburg, NJ). Monobasic potassium phosphate was provided by Fisher scientific (Houston, TX). HPLC grade chloroform, acetonitrile and methanol were obtained from EMD Chemicals (Gibbstown, NJ). Mouse plasma was acquired from BD Biosciences (Franklin Lakes, NJ). Nanopure water was used for all the experiments.

Preparation of Vancomycin liposomal formulations

Initially vancomycin was encapsulated into liposomes using the thin-film hydration method [16] and the ammonium sulfate gradient method [17]. As the encapsulation efficiency and the stability of the formulations prepared using the above two methods were poor, it was further decided to prepare the formulations using a modified dehydration-rehydration method [18]. Both the conventional and PEGylated vancomycin liposomal formulations were prepared using the lipids DSPC, cholesterol and PEG in 3:1:0 and 3:1:0.02 molar ratios, respectively. The detailed procedure for the preparation of vancomycin liposomal formulations has been published [19].

Instrumentation and HPLC conditions

The HPLC method for the quantification of vancomycin was developed and validated using a Hewlett-Packard 1050 HPLC system, consisting of a degasser, a quaternary pump, and an auto sampler. The system also included a diode array detector and a computer running Chemstation software (Rev. A.08.03) for data acquisition and processing. The chromatographic separation was performed on a Vydac C18 column (4.6×50 mm, 3 µm particle size, Alltech, Deerfield, IL). The detection was made at 214 nm and the acquisition time was 9 min. The flow rate was 1 ml/min and the mobile phases were composed of 0.1% v/v trifluoroacetic acid (A) and 95:5 v/v acetonitrile: 0.1% TFA (B), programmed for a gradient elution, where the mobile phase B increased from 0% to 15% in 8 min. With column cleaning and equilibrium steps, the total run time for each sample was 15 min.

Preparation of standard solutions

Stock solutions of vancomycin were prepared by dissolving 1 mg of vancomycin in 1 ml of water (1 mg/ml). The vancomycin stock solution was diluted with water to working solutions ranging from 1-100 µg/ml. Norvancomycin stock solution was also prepared by dissolving 1 mg of the drug in 1 ml of water. Internal standard working solution (100 µg/ml) was prepared by diluting the stock solution with water. All samples were freshly prepared.

Preparation of calibration and quality control samples

Calibration samples at 0.1, 0.5, 1, 5, 10 and 20 µg/ml concentrations were prepared by spiking respective amounts of each from the working solutions into 200 µl of blank plasma. These standards were used to construct calibration curves for the quantization of vancomycin in plasma at concentrations ranging from 0.1-20 µg/ml. Quality control (QC) samples were independently prepared at three level concentrations of 0.2, 1 and 15 µg/ml. All the quality control samples were prepared fresh from the working solutions.

Plasma extraction procedure

A protein precipitation procedure was used to extract vancomycin and norvancomycin from mouse plasma. Approximately 20 ml of mouse plasma was removed from storage and allowed to thaw at room temperature. For making calibration standards and dilution QCs, 200 µl of blank plasma was taken into 1.5 ml microcentrifuge polypropylene tubes. Required amounts of vancomycin were spiked into the blank plasma from the working solutions to make fresh calibration standards in the range of 0.1-20 µg /ml. 10 µl of norvancomycin (100 µg/ml) was added to each tube followed by vortex mixing for 1 min. To each tube, 250 µl of acetonitrile and 250 µl of methanol were added to precipitate out the proteins, vortex mixed for 1 min and centrifuged at 14,000 rpm (4°C) for 10 min. The supernatant (400 µl) was transferred into a new tube and subjected to evaporation for dryness using a stream of filtered air for 1-2 h. The dried residue was then reconstituted with 200 µl of water and 20 µl of the filtered reconstituted sample was injected into the HPLC system.

Method validation

The method was validated according to the International Conference on Harmonization (ICH) guidelines [15] for validation of analytical procedures with respect to specificity, linearity, lower limit of detection (LLOD), LLOQ, accuracy and precision, recovery and stability. The LLOD was selected at a signal/noise (S/N) ratio of 5. The LLOQ was selected at an S/N ratio of 10.

Linearity: Linearity of the method was evaluated by a calibration curve in the range of 0.1 to 20 µg/ml of vancomycin. The calibration curve was obtained by least-squares linear regression analysis.

Precision and accuracy: Calibration curves, with triplicates of QCs at each concentration level (0.2, 1 and 20 µg/ml) were performed in three consecutive days to determine intra-day and inter-day precision and accuracy. The intra-day variation was determined by evaluating triplicate measurements of low, medium and high QC samples on one single day whereas the inter-day variation was assayed for triplicate measurements of each QC sample for three consecutive days. The percent co-efficient of variation (% CV) of the regressed (measured) concentrations was used to report precision. Precision for all concentrations was accepted if the % CV fell within ± 15%. The accuracy was determined by comparing the calculated concentration from the standard curves to the theoretical concentration. The limits for the accuracy values were set as the range of 85-115%. Intra-day

and inter-day precision were assessed on the same samples used to determine intra- and inter-day accuracy.

Recovery: The recovery of vancomycin hydrochloride was demonstrated by comparing the detector response obtained from an extracted plasma quality control sample with the detector response obtained for the true concentration of the pure authentic standard representing 100% recovery. Recovery experiments were performed by comparing the analytical results for extracted samples at low, medium and high concentrations (0.2, 1 and 15 µg/ml) with un-extracted standards. The recovery of internal standard from plasma was determined at a concentration of 5 µg/ml by the same method.

Stability studies: The stability of vancomycin in plasma was evaluated using low, medium and high concentration QC samples for freeze-thaw, bench top, long-term, short-term and post-preparative stabilities. The freeze-thaw stability of vancomycin as determined over three cycles within 3 days. In each cycle, three aliquots each of the low, medium and high concentrations of QC samples, stored at -80°C for 24 h were thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h at -80°C. Bench top stability was evaluated after thawing three aliquots each of the low, medium and high concentrations of QC samples at room temperature for 6 h and then the samples were analyzed. For the short-term stability, plasma samples were stored at -80°C for 24 h and then analyzed. The long-term stability was evaluated after keeping the plasma samples frozen at -80°C for 1, 2 and 3 months. For the post-preparative stability, the stability of the prepared plasma samples was tested after keeping the samples at room temperature for 24 h. The samples were analyzed and the results were compared with those obtained for freshly prepared samples. For the acceptance criterion of stability, the deviation compared to the freshly prepared standard should be within ± 15%.

Pharmacokinetic application

The validated method was applied for the pharmacokinetics and bio-distribution study of novel liposomal formulations of vancomycin in male CF-1 mice. Free vancomycin, conventional vancomycin liposomes and PEGylated vancomycin liposomes at a dose of 5 mg/kg vancomycin were administered intravenously. At predetermined time points, the animals were sacrificed and major organs such as liver, kidneys, spleen, lungs, muscle and blood were collected. The plasma samples processed and analyzed by the HPLC method described above. Tissue samples were weighed and homogenized in PBS (0.5 mg/ml for liver, kidneys, lungs and muscle; 0.2 mg/ml for spleen). After homogenization, 300 µl of the homogenate was taken into a 1.5 ml microcentrifuge tube. 10 µL of a 100 µg/mL solution of norvancomycin was added to each tube and the mixture was vortex mixed for 1 min. Then, 250 µL of acetonitrile and 250 µL of methanol were added to the mixture, which was subsequently vortex mixed for 1 min and centrifuged at 14,000 rpm, 0°C for 10 min. 500 µl of supernatant was transferred into a test tube and subjected to evaporation to dryness. The residue was reconstituted with 200 µl of water and 20 µl of the reconstituted sample was injected to HPLC analysis using a method similar to the one described above. The details of the pharmacokinetics and bio-distribution were published recently [20].

Results and Discussion

Specificity and sensitivity

Blank plasma was extracted and analyzed for the assessment of potential interferences due to endogenous substances. No significant interfering peaks from endogenous substances in blank plasma were seen in drug-free mouse plasma at the retention time of the analyte (vancomycin) and the internal standard (norvancomycin). The peaks of vancomycin and norvancomycin were close but well resolved and the observed retention times were 7.9 and 7.5 min respectively (Figure 2). Representative chromatograms of mouse blank plasma, mouse blank plasma spiked with 5 µg/ml concentration of norvancomycin and mouse plasma spiked with 10 µg/ml vancomycin and 5 µg/ml norvancomycin were shown in (Figures 2a-c), respectively. The lower limit of detection (LLOD) of vancomycin was 0.05 µg/ml and the lower limit of quantitation (LLOQ) was 0.1 µg/ml. Thus the HPLC-UV method was sensitive to detect vancomycin in mouse plasma. Given the fact that the minimum inhibitory concentration (MIC) of vancomycin is in the 1-4 µg/ml range, the sensitivity of our method is satisfactory for experimental and clinical pharmacokinetic investigations.

Linearity

Calibration curves were obtained by plotting the peak area ratio of vancomycin to that of norvancomycin against plasma vancomycin concentrations. The standard calibration curve was linear in the concentration range of 0.1-20 µg/ml, with a correlation coefficient (r) higher than 0.997, which indicates a good linearity according to the ICH guidelines.

Precision and accuracy

The inter-day and intra-day precision and accuracy were determined by the analysis of triplicates of QC samples at 3 different concentrations

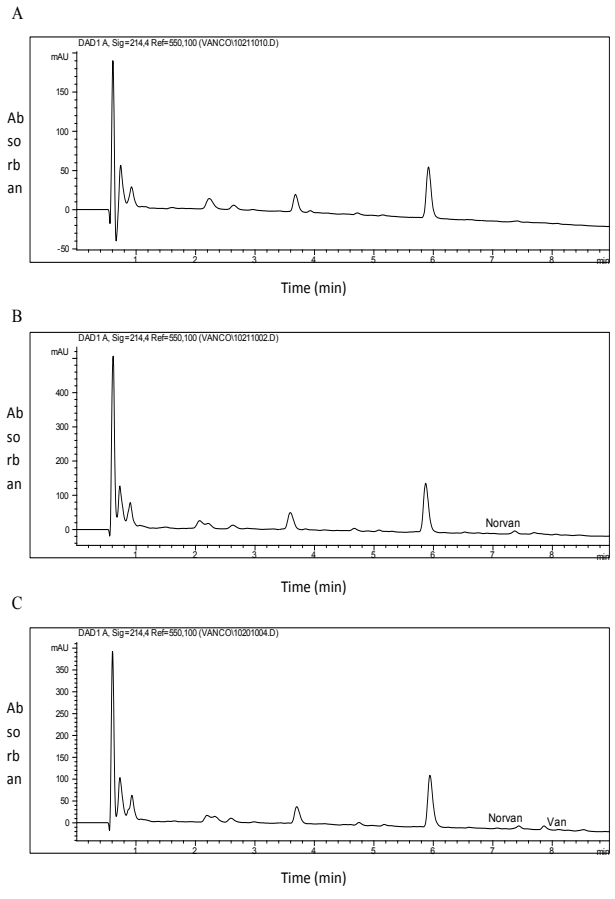

Figure 2: Representative HPLC chromatograms of blank mouse plasma (A), blank mouse plasma spiked with 5 µg/ml of norvancomycin (B), and blank mouse plasma spiked with of 10 µg/ml of vancomycin and 5 µg/ml norvancomycin (C).

(0.2, 1 and 15 µg/ml) of vancomycin. The results are reported in table 1. The intra-day accuracy values were between 90 and 112% and the inter-day accuracy values ranged from 96 to 104%. Precision values were based on the calculation of CV%. CV% ranged from 1.7 to 9.5% for intra-day and 6.3 to 9.4% for inter-day precision (Table 2).

Recovery

Table 3 shows the recovery of vancomycin at 3 different concentrations (0.2, 1 and 15 µg/ml) and of norvancomycin at 5 µg/ml concentrations. The recovery of vancomycin from plasma samples ranged from 41.6 to 71.5%, with a CV between 2.1 to 11.3%. The relatively lower recovery at higher concentrations might due to insufficient extraction. For the internal standard norvancomycin, a recovery of 55% was obtained with a CV% value of 4.7%.

	Target Concentration (µg/ml)	Measured concentration (µg/ml)	Accuracy %	Precision % CV
Intra-day	0.2	0.20 ± 0.02	98.7	9.5
	1	0.91 ± 0.03	90.9	3.5
	15	16.88 ± 0.29	112.5	1.7
Inter-day	0.2	0.19 ± 0.01	96.4	7.2
	1	0.98 ± 0.06	98.0	6.3
	15	15.66 ± 1.47	104.4	9.4

Table 1: Precision and accuracy of analysis of vancomycin in mouse plasma samples (n=3).

Compound	Concentration (µg/ml)	Recovery (%)	Precision (% CV)
Vancomycin	0.2	71.47 ± 1.32	1.9
	1	50.29 ± 5.66	11.3
	15	41.56 ± 0.86	2.1
Norvancomycin	5	54.99 ± 2.55	4.7

Table 2: Recovery of vancomycin and norvancomycin from mouse plasma samples (n=3).

Test Condition	Nominal Concentration (µg/ml)	Calculated Concentration (µg/ml)	Accuracy %	Precision (% CV)
Bench top stability	0.2	0.22 ± 0.03	111.0	14.2
	1	0.97 ± 0.05	96.6	5.1
	15	14.65 ± 0.38	97.7	2.6
Freeze-thaw stability	0.2	0.22 ± 0.01	111.8	6.1
	1	0.96 ± 0.03	96.0	3.3
	15	15.18 ± 0.11	101.2	0.7
Post-preparative stability	0.2	0.21 ± 0.02	102.7	9.6
	1	1.02 ± 0.04	102.5	3.7
	15	14.78 ± 0.34	98.6	2.3
Short-term stability	0.2	0.22 ± 0.02	108.8	10.1
	1	0.96 ± 0.01	95.7	1.0
	15	14.49 ± 0.10	96.6	0.7
Long-term stability				
1 Mon	0.2	0.21 ± 0.02	102.8	9.9
	1	1.03 ± 0.07	103.3	6.9
	15	14.65 ± 0.26	97.7	1.8
2 Mon	0.2	0.21 ± 0.01	105.0	1.4
	1	1.08 ±0.06	107.5	5.6
	15	14.60 ± 0.77	97.4	5.3
3 Mon	0.2	0.19 ± 0.01	96.7	3.4
	1	0.94 ± 0.07	94.0	7.6
	15	13.86 ± 0.25	92.4	1.8

Table 3: Stability of vancomycin in mouse plasma samples (n=3).

Stability

Plasma QC samples at 0.2, 1 and 15 µg/ml of vancomycin were subjected to bench top, post-preparative (24 h at 24°C), freeze-thaw, short-term storage and long-term storage stability tests. The deviation of the mean test responses was within ± 15% of appropriate controls in all stability tests of vancomycin in mouse plasma as shown in table 3. Bench top storage at room temperature for 6 h, three freeze-thaw cycles, short-term storage stored at -80°C for 24 h, long-term storage at -80°C for 3 months had no effect on quantification of vancomycin. The extracted samples were analyzed after being kept at room temperature for 24 h. All the values obtained were within the acceptance criteria. All these stability studies suggested that the mouse plasma samples containing vancomycin can be stored at -80°C and handled under normal laboratory conditions without significant loss of the drug.

Pharmacokinetic application

The validated analytical method was successfully applied for the pharmacokinetics and bio-distribution study of vancomycin liposomal formulations in mice at 5 mg/kg [20]. The representative chromatograms of samples from mice injected with 5 mg/kg dose of free vancomycin, conventional liposomal vancomycin and PEGylated liposomal vancomycin at 5 min post-injection were shown in (Figures 3a-c), respectively. This method, following minor modifications, also allowed for the determination of vancomycin concentrations in

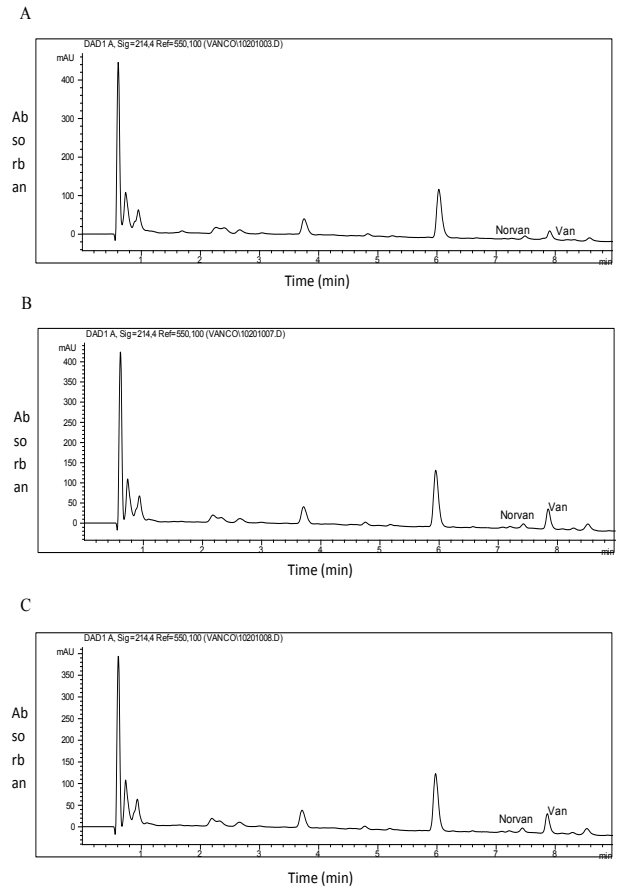

Figure 3: Representative chromatograms of mouse plasma from a mouse 5 min post injection of 5 mg/kg dose of free vancomycin (A), conventional liposomal vancomycin (B), or PEGylated liposomal vancomycin (C), each spiked with 5 µg/ml norvancomycin.

various tissue samples, which is a further advantage to determine the bio-distribution patterns of different vancomycin formulations. Our results showed that PEGylated liposomal vancomycin, compared to standard and conventional formulations, significantly prolonged blood circulation time, increased deposition in lung, liver, and spleen yet reduced accumulation in kidney tissue, an target organ for vancomycin toxicity [20].

Conclusion

We have developed and validated a simple, sensitive and accurate HPLC-UV method for the quantification of vancomycin concentration in mouse plasma. The novelty is largely upon the employment of a structurally closed related analog norvancomycin as the internal standard, a simple mixed solvent extraction procedure, and a very short C18 column which allows an acquisition time of 9 min. Vancomycin is stable in mouse plasma samples stored in freezer at -80°C and under normal laboratory handling conditions without significant loss of the drug. Compared to other previously reported assays, this method employs more common instrumentation with UV detection, simpler extraction procedure, and faster run time, without compromising assay specificity and sensitivity. It could also be easily modified for tissue samples. This assay has been successfully applied for the pharmacokinetics and bio-distribution study of novel formulations of vancomycin in mice.

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