ANALYSIS

**DETERMINATION OF VANCOMYCIN CONTENT
IN DRIED BLOOD SPOTS FOR THERAPEUTIC DRUG MONITORING**

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Abstract: Blood spot collection is a simple method to obtain specimens for therapeutic monitoring of drugs, assessing drug adherence, and preventing any potential drug toxicity. A simple chromatographic method for the determination of vancomycin in dried blood spots (DBS) has been developed and validated. Intra and inter-day precisions (measured by CV%) were less than 11.5% and the accuracy deviation was less than 11%. The limits of detection (LOD) and quantification (LOQ) were 1 and 10 ng/mL, respectively. Interestingly, LOQ is smaller than the smallest vancomycin monitoring level. Stability analysis shows that vancomycin is stable for at least three weeks when stored at 4 to 8°C. The high sensitivity of detection and short time of analysis (less than 8 min), combining with the simplicity of the procedure made the method applicable for monitoring vancomycin in dried blood spots, which might be implemented in routine clinical settings.

Keywords: determination of vancomycin, blood spot, therapeutic drug monitoring and chromatography

Vancomycin is an amphoteric glycopeptide antibiotic that has a broad application in the treatment of serious, life-threatening gram-positive infections where resistance and/or allergy has banned the use of other antibiotics. It has a strong bactericidal activity through inhibition of cell wall synthesis in gram-positive bacteria (1). It is absorbed minimally from the gastrointestinal tract due to its large hydrophilic structure (2). In the first 24 h after intravenous administration, about 90% of vancomycin excrete unchanged by kidneys. The mean half-life in patients with normal renal function is about 6 h. Approximately, 55% of vancomycin is bound to plasma proteins. It is worthwhile to mention that therapeutic serum levels vary depending on the microorganism involved and the patient's tolerance to the drug (3, 4).

The major adverse effect of vancomycin is ototoxicity which has been reported when blood levels

exceed 80 µg/mL range (5). Moreover, vancomycin dose adjustment for kidney ill patients (i.e. renal insufficiency and hemodynamic instability) is quite complicated. Thus, the monitoring of the drug levels in the blood is a necessity to reduce the frequency of serious toxic events (6).

Currently, the quantitative measurement method for vancomycin in human blood is an *in vitro* chemiluminescent microparticle immunoassay (CMIA). It requires relatively large volumes of blood, extensive sample preparation, and combining the sample with expensive reagents. Reagents used to create a reaction mixture utilize expensive kits. The latter requires a long assay time (ca 45 min). Moreover, CMIA has a limited range of detection 3-100 µg/mL and a high potential of interference in presence of a wide range of drugs and/or proteins (7, 8). Accordingly, CMIA is less practical for therapeutic

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Table 1. Recently reported analytical methods for measuring blood and/or serum vancomycin concentration.

Name of analytical method	Sample preparation	Linearity	Limit of Quantification (LOQ) and Limit of Detection (LOD)	Precision CV%	Accuracy (Recovery)	Ref
Spectrophotometric method						
Ultracentrifuge Protein Precipitation and UV Spectrophotometer	Plasma extraction using mixture of methanol and acetonitrile to salting-out vancomycin.	Range: 12.5-200 µg/mL. (R ²): 0.99997	LOD: 0.99 µg/mL LOQ: 3.02 µg/mL	Inter and intra-day precision: ≤ 2.2%.	Accuracy (CV%): ≤ 2.2%.	(12)
Chromatographic method						
Liquid chromatography -tandem mass spectrometry (LC-MS/MS) method for vancomycin measurement in DBS	DBS with 8 mm extracted in mixture of methanol and water (1:1, v/v), containing 0.1% of formic acid and the internal standards; teicoplanin and CRE-D3	Range: 1-100 µg/mL. (R ²): ≥ 0.99	LOQ: 1 µg/mL	Inter and intra-day precision: ≤ 7%	Accuracy: 94.4-102.6%	(14)
LC-MS/MS method for the quantification of vancomycin in human whole blood employing volumetric absorptive microsampling (VAMS®) devices (20 µL)	Whole blood using Volumetric absorptive microsampling (VAMS®) Loaded according to the instructions from Neoteryx which need almost 4 h to be ready for LC-MS/MS analysis	Range: 1-100 µg/mL. (R ²): 0.997	LOD: 1 µg/mL	Inter and intra-day precision: 2.40-14.0%	Accuracy (CV%): 5.25-7.92% For 2.5, 40 and 80 µg/mL; n = 6	(13)
LC-MS/MS method for plasma vancomycin	Lithium heparin plasma was extracted by adding a acetonitrile	Range: 0.6-100 mg/L	LOQ: 0.3 mg/L	Inter and intra-day precision: 2.6-8.5%	Accuracy: 101.4 to 111.2%.	(8)

monitoring. Additionally, several liquid chromatographic methods are described in the literature; none of them is suitable for tiny volumes of blood such as those taken from dried blood spots (8-10).

Chemiluminescence, enzyme immunoassay (EIA), kinetic interaction of microparticles in solution (KIMS), enzyme-multiplied immunoassay technique (EMIT), and fluorescence polarization immunoassay (FPIA). are other commercial vancomycin tests used for therapeutic drug monitoring (TDM) (11). However, these tests and others have been recently assessed by Chen et al. (11), where they found significant differences in terms of precision between KIMS and other methods, and accuracy between EMIT, FPIA, and other techniques. Accordingly, laboratories are more likely to underestimate vancomycin concentrations. Thus, transferring vancomycin results between laboratories needs to be done with caution. Examples of other analytical methods: spectrophotometric-based methods (12) and chromatographic-based methods (8, 13, 14) were compiled in Table 1.

DBS for therapeutic drug assay can facilitate clinical studies and TDM (9, 15-17). It is a convenient and inexpensive alternative for whole blood sampling and storage (18, 19). It is simple and minimally invasive in particular for children and pregnant women as well as for small animals used in experimental research such as rats and mice. It provides a dried matrix that enhances stability for drug and metabolite (19).

However, using the DBS technique for TDM still need clinical validation, that's capillary blood from finger pricks may have different concentrations from venous blood (20). Moreover, the prediction of plasma or serum

levels from DBS measurements is affected by the distribution of the drug within the blood cells (21). In addition, obtaining of blood spot sample (5 to 50 μ L) needs very sensitive analytical methods able to measure minimum concentrations, usually below 1 μ g/mL (20, 21). Currently, there is only one report vancomycin assay based on the DBS technique by Scribel et al. (14). However, they were unable to predict plasma concentrations from DBS accurately in the clinical samples, additionally, their method is limited to vancomycin concentration above 1 μ g/mL. Therefore, the aim of this study is to develop and validate a chromatographic method able to determine plasma vancomycin concentration by using DBS and sensitive for concentrations below 1 μ g/mL.

MATERIAL AND METHOD

Material and chemicals

Vancomycin hydrochloride (Fisher Scientific®, China) was stored at -20°C in a refrigerator until use. Boron trifluoride etherate (Merck®, Darmstadt), Potassium dihydrogen phosphate monohydrate (Fisher Scientific®, India), Sodium hydroxide (Gainland® Chemical Company, UK), Phosphoric acid (Raw Mat., UK), Water HPLC Grade (Labchem®, USA), acetonitrile and methanol HPLC Grades (Fisher Scientific®, UK) were used without further purification.

Chloroform (Across Organic®, USA) and Methanol (AZ® chem for chemicals) were used in the purification of methylated vancomycin.

Trichloroacetic acid (TCA) (Riedel-deHaën®), perchloric acid (Riedel-deHaën®), and dried blood spot collection cards (Whatman, Kent, UK) were used. Blank blood and samples from vancomycin-treated patients were donated from Dr. Hadidi's lab.

Instruments

Shimadzu® HPLC-UV visible detector system (Japan) equipped with LC-20AT pump, SPD-20A detector. Chromatographic data acquired and processed using LC-solution 1.25 software. Microsoft Excel 2010 software for data analysis. The column used was Phenomenex®, PhenoSphere - NEXT™, 5 μ m particle size C18, 120 Å, 250 x 4.6 mm. The detector wavelength was set at 260 nm

Rocker® Ultrasonic Bath (Taiwan) was used for mobile phase degassing. Analytical balance (Kern®, Germany), pH-meter (Hanna® Company, Romania) coupled with Phelectroid electrode (SenTix® Company, Germany), Simple low-speed Eppendorf centrifuge (Gallenkamp®, England) and Rotavapor BUCHI® (Switzerland) were used.

Internal standard (IS) synthesis

Vancomycin hydrochloride powder (50 mg) was added to 10 mL of Boron trifluoride-methanol solution (10%) at room temperature. The reaction mixture heated at 60°C for 30 min, and then cooled to room temperature. For more purification, the TLC separation technique was performed using a mixture of methanol and chloroform in 6 : 4 ratio as a mobile phase, and then the most intense layer was scratched and extracted using 20 mL of HPLC grade methanol. Then, the solvent evaporated using Rotavapor BUCHI® at 90°C until getting a yellow powder product. The product was methylated vancomycin confirmed by the appearance of 3H at 3.16 ppm and disappearance of acid peak at 8.9 ppm by using ¹H-NMR spectra, which conducted on a Burkert 500 MHz – Avance III spectrometer NMR at The University of Jordan.

Standard preparation

Two groups of vancomycin standards were prepared: The first group prepared in HPLC- grade water to produce the following concentrations (1.376, 0.688, 0.433, 0.170, 0.130, 0.086, 0.043, 0.0215, 0.016, and 0.0086 μ g/mL) and stored at 4°C using transparent glass. The second group prepared in phosphate buffer (65 mM, pH 3) and stored at 4°C using amber glass in refrigerator to ensure highest stability conditions. The following standard concentrations were prepared (500, 400, 200, 100, 90, 85, 50, 25, 12.5, 10, 6.2, 3.125, 2, 1.5, 0.8, 0.6, 0.4, 0.2, and 0.1 μ g/mL).

QC samples and blood deproteinization:

Two QC samples were prepared: blood liquid and dried blood spot. In QC blood samples, 100 μ L of blank blood spiked with 100 μ L from each vancomycin standard and IS (almost equivalent concentrations). The samples were extracted and deproteinized by 300 μ L acetonitrile. The precipitate was removed by low-speed centrifugation for 2 min. The other QC samples were prepared by adding 100 μ L from vancomycin standard and IS to a dried blood spot card. Subsequently, the samples were extracted and deproteinized by 300 μ L acetonitrile. The precipitate was removed by low-speed centrifugation for 2 min.

Sample preparation and extraction

Patients' samples were prepared in two forms: blood and dried blood spots with 6 mm diameter equivalent to 50 μ L blood volume, which was previously tested using Architect® i1000SR immunoassay analyzer device. Each sample was mixed with 100 μ L

from IS then deproteinized using 5% v/v perchloric acid (PCA). All samples were centrifuged before they were injected.

Due to sugar moieties in vancomycin (Figure 1), part of vancomycin is bound with either proteins or hemoglobin in RBCs (3, 22, 23). Therefore, there is a demand for an efficient method to extract all vancomycin from the blood. Alzweiri et al. 2012 reported, the efficient methods to precipitate blood proteins: Acids (e.g. 5% perchloric acid and 10% trichloroacetic acid) and organic solvents (e.g. acetonitrile). It was found that perchloric acid solution is more efficient than acetonitrile and trichloroacetic acid in extracting vancomycin and salting-out most of the blood sample components. Interestingly, other authors recorded that perchloric acid is immensely able to burst RBCs and precipitate most of the biological sample proteins (24, 25).

Figure 1. Structure formula of vancomycin.

Chromatography

HPLC conditions were chosen according to optimized conditions obtained from Serri *et al* work (26). The mobile phase had an isocratic flow (1 mL/min) to keep constant (85 : 10 : 5) composition ratio between phosphate buffer (65 mM, pH 3) to organic modifiers (Acetonitrile and methanol respectively). This ratio was chosen to guarantee solubilization and deionization for the acidic group of vancomycin (analyte) and to achieve a suitable retention time. The mobile phase was filtered through a 0.45 μ m Millipore filter and degassed ultrasonically before use.

Method validation

Standard curves and linearity

For testing the linearity of the method, seven vancomycin calibration standards were prepared using IS stock solution as a diluent in the range of

0.1-10 μ g/mL (0.1, 0.2, 0.4, 0.6, 0.8, 2, and 10 μ g/mL). The calibration curve was prepared by plotting the concentrations of the analyte against their peak areas. The slope, the intercept, and determination coefficient (R^2) were determined. Each reading was the average of three trials.

Limit of quantification and limit of detection

The sensitivity of the method is evaluated by the limit of detection (LOD) and limit of quantification (LOQ). The LOD is based on a peak height three times greater than the largest baseline fluctuation (signal to noise ratio : S/N around 3), and The LOQ was established at a signal to noise ratio (S/N) of 10. LOD and LOQ values were experimentally verified by three injections of vancomycin standard solutions.

Precision

To evaluate the intra- (repeatability), inter-day (reproducibility) precision, four different concentrations were chosen from standard solutions. Precision was expressed as the coefficient of variation (CV%) for the response (vancomycin peak area), Precision (CV%) $\leq$ 15% is usually accepted (8).

Accuracy (Recovery)

The recovery of analytes was calculated by comparing the peak areas (AUC) obtained after extraction of vancomycin from 100 μ L blood spots spiked with known concentrations of vancomycin and IS and comparing them to those in the standard solution. The following formula was used:

$$\text{Recovery}\% = \left(\frac{\text{Recovery}\% \text{ of analyte}}{\text{Recovery}\% \text{ of IS}} \right)$$

The recovery of vancomycin was determined at six concentrations (400, 200, 0.8, 0.6, 0.4, 0.3 μ g/mL) ($n = 3$ at each concentration)

Stability

The stability test was performed for both blood and dried blood spot (QC) samples for 30 days. Three different vancomycin concentrations for spiked dried blood spot samples were used; 400, 200, and 0.8 μ g/mL ($n = 3$). Additionally, Three different vancomycin concentration for blood spiked samples were used; 400, 0.8 and 0.5 μ g/mL ($n = 3$). All samples were stored at 4-8°C for 4 weeks and analyzed at different points in time (1, 2, 7, 21, and 28 days), where each sample thaw at each point then refrozen after injection.

The results were compared with the results of freshly prepared blood samples. Stability was

considered acceptable if the mean value is within 15% of the original value at each concentration.

Specificity (selectivity)

Method specificity was demonstrated through analyzing blank blood spots and patient blood spots who were taking vancomycin. Representative chromatograms are generated to show that other components presented in the sample matrix are resolved from the parent analyte as well as a tiny constant interference with internal standard resolved by subtracting its area from the IS one.

Representativeness of the dried blood spot samples

To demonstrate that one dried blood spot is representative, two samples type from each patient, blood and dried blood spot samples for each were collected, then prepared and analyzed in duplicate using both the developed method and Architect® i1000SR immunoassay analyzer device for comparison.

RESULTS AND DISCUSSION

Method development

Under the applied chromatographic conditions, a group of vancomycin standards, with a wide dynamic range of concentrations, were injected. Several degradation peaks were remarked in standard chromatograms even in low concentrations of vancomycin standard (Figure 2), implying the light sensitivity and/or minimal stability of vancomycin in water.

The stability of vancomycin standards was tested in aqueous solutions with different pH values. The best stability of vancomycin was obtained in a light-protected container having a phosphate buffer,

pH3 as a solvent. It showed a clean chromatogram with a single peak for low concentration standards; lower than 1 µg/mL. However, a new peak for vancomycin dimer was observed at higher concentrations; above 2 µg/mL. Interestingly, Schäfer et al. 1996 confirmed that vancomycin exists as an asymmetric dimer resulted from hydrogen bond formation, and this dimer is important to its microbiological activity (22).

Unexpectedly, methylated vancomycin, used as internal standard (IS), eluted earlier than vancomycin (Figure 3); Methylation of vancomycin may reduce intra hydrogen bonding of the molecule rendering it to be more hydrophilic.

Figure 3. Example of chromatograms generated after injection of IS alone (a) and spiked IS with 100 ng/mL vancomycin (b).

Method validation

Linearity and sensitivity (LOD and LOQ)

Linearity within the range of 0.1-10 µg/mL was demonstrated by calculating the R^2 . Each point is established from an average of three determinations. A good linear relationship between detector signal and spiked concentrations was attained, as described by the following linear equation: $Y = 84.157x - 1.0253$ ($R^2 0.9934$), where Y is the concentration (µg/mL) and X is the area ratio (AUC drug/AUC IS). The sensitivity of the method was

Figure 2. Chromatograms for three concentrations of vancomycin standard in water. Several degradation peaks were noticed indicating instability of vancomycin.

decided through the measurement of the limit of detection (LOD). The detection limit was determined as the concentration of components giving a signal-to-noise ratio 3 : 1. The LOD was found to be 1 ng/mL with CV% < 3%. The LOQ of vancomycin was determined as the concentrations of 10 ng/mL. Interestingly, LOQ is smaller than the smallest vancomycin monitoring trough level (27-29). Therefore, this method can be used to estimate very little amounts of vancomycin in blood.

Precision and accuracy

Precision was expressed as the coefficient of variation (CV%) for the response (vancomycin peak area), Precision (CV%) ≤ 15% is accepted (8). The precision revealed coefficients of variation ranging from 2.69% to 11.45% for intra- precision and from 3.06% to 9.06% for inter- precision (Table 2).

The accuracy of the method was explored *via* calculating overall recovery% (Recovery% of vancomycin / Recovery% of IS). The recovery test was performed by comparing the analytical results for the extracted 100 µL blood spot samples previously spiked with vancomycin with unextracted standards that represent 100% recovery. The recovery of vancomycin hydrochloride was demonstrated by comparing the AUC obtained from an amount added to and extracted from blood spot with the AUC obtained for the true concentration of the pure authentic standard. Recovery of vancomycin and IS was found to be consistent, precise, and reproducible with CV% < 11% which is lower than the acceptable limit of 15% (Table 3) (8, 30).

Stability

The stability of both blood and dried blood spot (QC) samples, previously spiked with vancomycin at different concentration levels was determined by monitoring the AUC responses over a period of 30 days for samples stored at 4-8°C.

The results showed that retention time and peak area for both vancomycin and IS remained almost unchanged (within acceptable range; ca 15%). Also, no significant degradation was observed for up to three weeks. However, in the fourth week, the AUC significantly decreased by almost 75 to 50 percentage specifically in the blood samples and spot samples that have < 1 µg/mL vancomycin concentration. (Table 4). In conclusion, the results indicated that the dried blood spot samples could be safely stored at 4-8°C for three weeks without observing the degradation of products.

Specificity (selectivity)

Specificity or selectivity with respect to interferences from endogenous substances in biological fluids was established through analyzing blank blood spots, and blood spots from vancomycin-treated patients (Figure 4). Under assay conditions described above, vancomycin was well resolved with retention times between 6-7 min. The method exhibits selectivity for vancomycin with no interfering peaks was observed in the same chromatographic windows in the blank chromatogram, while a constant minute interference with IS at 3 min retention time has been observed.

Table 2. Inter and intra-day precision.

Cons (µg/mL)	Intra-day		Inter-day	
	Mean determined Cons ± SD	CV%	Mean determined Cons ± SD	CV%
	n = 3		n = 3	
400	395.78 ± 11.03	2.79%	378.96 ± 23.78	6.04%
200	190.12 ± 12.54	6.60%	198.83 ± 12.32	5.77%
10	9.39 ± 1.07	11.45%	8.88 ± 0.72	3.06%
0.8	0.79 ± 0.02	2.69%	0.77 ± 0.04	9.06%

Table 3. Recovery of vancomycin.

Standard cons (µg/mL)	400	200	0.8	0.6	0.4	0.3
Mean ± SD n = 3	115.38% ± 9.39	113.15% ± 5.67	106.34% ± 4.23	101.64% ± 4.23	114.68% ± 12.39	118.25% ± 6.67
CV %	5.64%	6.81%	3.16%	4.16%	10.81%	5.64%

Figure 4. Example chromatogram generated after injection of blank blood spot (a) and sample dried blood spot (b). The method exhibits selectivity for vancomycin with retention time between 6-7 min, and constant tiny interference with IS at 3 min.

Representativeness of the dried blood spot samples

Coefficients of variation for the three samples that were obtained for each volunteer at different times showed that the coefficient of variation was always less than 0.05% for direct blood and < 1.5% for samples of dried blood spots. This clearly indicates that one dried blood spot sample is in fact representing the actual concentration.

CONCLUSION

Recently, a DBS analysis method is reported (14), the group developed the first DBS analysis method for vancomycin, but their work was limited to vancomycin concentration above 1 μg/mL and the method has a low accuracy to predict clinical data of plasma concentrations from DBS. On other hand, this current work overcomes these limitations by introducing an easily applied method with higher sensitivity (LOD; 1 ng/mL) and accuracy.

A chromatographic method for the quantification of vancomycin in the DBS sample was developed and validated. All validation parameters met the acceptance criteria i.e. accuracy, precision and selective, etc. The blood spots proved to be stable for at least 21 days at 4-8°C.

Small blood volume, short analysis time, combined with the simplicity of the analytical technique make this method useful for monitoring vancomycin concentrations. Our preliminary experience with developing the method indicates that it can be employed in a routine clinical setting.

Acknowledgments

We wish to thank The University of Jordan represented by the Deanship of Academic Research for supporting and funding the project under financial grant number [1642].

Conflicts of interest

The authors declare no conflict of interest.

Sample conc. (μg/mL)	Day 0		Day 2		Day 7		Day 21		Day 28	
	Determined conc. (n = 3)	Relative Recovery %	Determined conc. (n = 3)	Relative Recovery %	Determined conc. (n = 3)	Relative Recovery %	Determined conc. (n = 3)	Relative Recovery %	Determined conc. (n = 3)	Relative Recovery %
100s	400	399.81	99.95%	342.14	85.54%	330.55	82.64%	327.06	417.18	104.30%
	200	226.29	113.15%	197.55	98.78%	168.15	84.08%	173.81	202.72	101.36%
	0.8	0.92	115.00%	0.84	105.00%	0.72	90.00%	0.67	0.41	51.25%
blood	400	444.18	111.05%	329.31	82.33%	318.36	79.59%	386.15	103.24	25.81%
	0.8	0.91	113.75%	0.81	101.25%	0.77	96.25%	0.68	0.50	62.50%
	0.5	0.57	114.00%	0.49	98.00%	0.44	88.00%	0.40	0.28	56.00%

Table 4. Stability of vancomycin in human blood and dried blood spot.

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