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ORIGINAL ARTICLE

Comparison of New Liquid Chromatography-Mass Spectrometry Method and Enzyme-Multiplied Immunoassay Technique for Routine Therapeutic Drug Monitoring of Vancomycin in Chinese Patients

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SUMMARY

Background: **Therapeutic drug monitoring of vancomycin is very valuable due to the good correlation between trough levels and clinical outcome. Therefore, it is important to accurately determine the concentration of vancomycin in patient plasma for adequate dose-adjustment. The objective of this study was to develop a new liquid chromatography-mass spectrometry (LC-MS) method for determination of vancomycin in patient plasma and compare the results with those obtained from enzyme-multiplied immunoassay technique (EMIT).**

Methods: **After extraction by simple protein precipitation, vancomycin and bergenin (internal standard) were separated on a C_{18} column (150×4.6 mm, 5 µm) at 40°C by gradient elution with 0.1% formic acid and acetonitrile as the mobile phase and measured by electrospray ionization source in positive selective ion monitoring mode. Seventy-nine plasma samples from patients with severe infection were analyzed by enzyme-multiplied immunoassay technique and LC-MS method. MedCalc 15.2 software with Bland-Altman analysis and Passing-Bablok regression analysis was used for statistical analysis.**

Results: **The weighted ($1/x^2$) calibration curve of the validated LC-MS was linear within the concentration range of 0.25 - 40 µg/mL. The inter- and intra-day precisions (%RSD) were less than 10.0%. No significant matrix effect was observed in the relevant time ranges. Comparison of the two methods indicated that results of the LC-MS were close to that of EMIT with a correlation coefficient of 0.957. Upon Bland-Altman analysis, the bias amounted to 2.9 µg/mL (95% confidence intervals of -3.4 - 9.2 µg/mL).**

Conclusions: **The established LC-MS method and EMIT were both suitable for routine TDM of vancomycin.**
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vancomycin, liquid chromatography-mass spectrometry, enzyme-multiplied immunoassay technique, therapeutic drug monitoring, comparison

INTRODUCTION

Vancomycin, a glycopeptide antibiotic with strong bactericidal activity, is commonly used for treatment of serious infections caused by gram-positive bacteria, including not only methicillin-resistant *Staphylococci*, but also penicillin resistant organisms, such as *Streptococci* and *Corynebacteria* [1]. Vancomycin is mainly applied

in respiratory tract infection, endocarditis, sepsis, and surgical prophylaxis during prosthetic implantations. It is also selected to treat severe gram-positive infections in patients who are hypersensitive to penicillins and cephalosporins.

Therapeutic drug monitoring (TDM) of vancomycin is recommended not only because of the correlation between trough concentrations and clinical outcome, but also the large inter- and intra-patient variability of pharmacokinetics. Low plasma concentration of vancomycin can cause therapeutic failure, while high plasma concentration may cause toxicity, such as nephrotoxicity and ototoxicity, especially in combinations with other nephrotoxic and ototoxic drugs [2]. Vancomycin in plasma or serum is measured routinely in many hospitals, and the level measured is used to guide the individualized therapy. It is recommended that the trough concentrations of vancomycin are in the range of 10 - 15 µg/mL or up to 15 - 20 µg/mL for severe infections, and the peak concentrations should be less than 50 µg/mL. Therefore, it is important to accurately determine the concentration of vancomycin in patient plasma.

Several analytical methods are available for TDM of vancomycin in serum or plasma, such as enzyme-multiplied immunoassay (EMIT), fluorescence polarization immunoassay (FPIA), and chromatographic methods. EMIT and FPIA are widely used for TDM due to their high speed and simplicity. But these methods are not specific enough as they cannot distinguish vancomycin from CDP, a degradation product of vancomycin [3], and they are less precise for higher concentrations and have lower sensitivity with an upper limit of quantification of 5 or 2 µg/mL, respectively for EMIT or FPIA. However, chromatographic methods usually are less susceptible to interferences. Vancomycin has been determined in human serum or plasma using high performance liquid chromatography with ultraviolet detection [4-10] or fluorescence detection [11]. These methods had either more plasma consumption or a higher lower limit of quantitation (LLOQ). Recently, a number of methods using mass spectrometry detection (LC-MS or LC-MS/MS) for determination of vancomycin in human plasma have been described. These methods, however, used more complicated sample preparation methods [12,13], more consumption of organic reagent for sample preparation [14], higher LLOQ [15-17], or more expensive instruments [13-17]. The overview for these methods is summarized in Table 1. LC-MS is currently regarded as the gold standard because of its superior analytical specificity compared to HPLC with conventional detection. It also has the ability of using lower amounts of the biological sample to produce higher sensitivity, which is very important, particularly for TDM in children and newborns. Moreover, the LC-MS instrument is cheaper and easier to operate than LC-MS/MS. So, the purpose of this study was to develop and validate a novel LC-MS method for determination of vancomycin and compare the results of this method with those obtained from EMIT assay. This was done in order to determine whether the EMIT assays that are currently available are suitable for determination of vancomycin in plasma as part of TDM and to provide suggestions for individualized treatment of vancomycin.

MATERIALS AND METHODS

Reagents and instruments

Vancomycin (Lot No. 130360-201302) and bergenin standard (internal standard, IS, Lot No. 101018, 99.92%) were purchased from National Institutes of Food and Drug Control (Beijing, China) and Yusen Pharmaceutical Factory (Shanhai, China), respectively. HPLC grade Formic acid (Lot No. 20110908) and acetonitrile (Lot No. 0000096305) were purchased from Tianjin Kemiou Chemical Reagent Co., Ltd (Tianjin, China) and J.T. Baker (USA), respectively. Analytical grade trichloroacetic (TCA, Lot No. 20141023) was purchased from Fine Chemical Plant of Laiyang Economic and Technological Development Zone (Laiyang, China). Pure water was purchased from Hangzhou Wahaha Group Co., Ltd. (Hangzhou, China). Vancomycin-specific calibrators, control samples, and sample preparation reagents were obtained from Siemens Healthcare Diagnostic Ltd. (Newark, NJ, USA).

The LC analyses were performed using an Agilent 1100 HPLC system, including a quaternary solvent pump, an autosampler connected to an Agilent 1946D mass spectrometer equipped with an electrospray ionization source (Agilent Technologies, USA). The Siemens Viva-E automatic biochemical analyzer (Siemens Healthcare Diagnostics, Inc.,) was used for immunological assay of vancomycin.

Plasma samples

Seventy-nine plasma samples were collected from patients receiving vancomycin for treatment at Qilu Hospital of Shandong University between March and August 2017. This study was approved by the Ethics Committee of Qilu Hospital, and signed informed consent forms were obtained from all patients who participated in this study or their caregivers according to the Declaration of Helsinki. Blood samples were collected and separated by centrifugation (5,000 rpm for 5 minutes). Plasma was transferred to a -20°C temperature-monitored freezer for storage until analysis. Prior to extraction, all plasma samples were brought to room temperature and then gently mixed.

LC-MS method

Chromatography and mass spectrometry conditions

An Inertsil ODS-SP C_{18} column (150 x 4.6 mm, 5 µm) was used for separation. The column temperature was 40°C. The mobile phase consisted of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). The flow rate was 0.5 mL/minute. Gradient elution of increasing mobile phase B was used as follows: 10% hold until 1 minute, 10% - 50% from 1 to 4 minutes, 50%

- 70% from 4 to 8 minutes, 70% hold from 8 to 10 minutes, 70% - 10% from 10 to 10.1 minutes, 10% hold until 15 minutes. The injection volume was 5 µL.
The electrospray ionization (ESI) source was used in positive ion mode. The parameters were as follows: capillary voltage 4,000 V, nebulizer pressure 50 psi, dry gas temperature 350°C, gas flow rate 11 L/minute. The fragment voltages of vancomycin and IS were 110 V and 110 V, respectively. Quantification was performed in selected ion monitoring (SIM) mode at m/z 724.7 for vancomycin and 329 for IS.

Sample preparation
Eighty microliters plasma was mixed with 20 µL water and 10 µL IS (3 µg/mL) in a plastic Eppendorf. After vortexing for 30 seconds, 50 µL precipitant consisting of 25% trichloroacetic acid and acetonitrile (1:1, v/v) was added, then centrifuged at 10,800 rpm for 5 minutes. The supernatant was transferred into a glass vial and five microliters was injected into the LC-MS system.

Method validation
Method validation was carried out following FDA guidelines in terms of specificity, matrix effect, extraction recovery, linearity, precision, accuracy and stability.

Specificity
The specificity was evaluated by comparing chromatograms of blank plasma, vancomycin and IS standard, blank plasma spiked with vancomycin and IS, and patient plasma spiked with IS, to identify the potential interference of endogenous substances in peak regions of vancomycin and IS.

Matrix effect and extraction recovery
The matrix effect (ME) was evaluated by comparing the peak area ratios (A_1) of vancomycin and IS spiked at 0.4 and 30 µg/mL in water with the peak area ratios (A_2) of vancomycin and IS spiked at the same concentrations in six different blank plasma extracts (five from patients who received other drugs, but not vancomycin, and one from a healthy volunteer). The ME was calculated by $A_2/A_1 \times 100$. The extraction recoveries were determined based on the ratio of peak areas of vancomycin spiked in blank plasma, before and after extraction at two concentration levels (0.4 and 30 µg/mL).

Linearity and LLOQ
The calibration curve was constructed using seven none zero points (0.25, 0.5, 1, 2.5, 5, 20, 40 µg/mL), typically described by the equation $y = ax + b$, where y corresponds to the peak-area ratio and x to the concentration ratio of vancomycin to IS. The linearity of the calibration curve was assessed by linear regression with a weighting factor of the reciprocal of the concentration squared ($1/x^2$). The lower limit of quantification (LLOQ) was analyzed using five replicates of spiked plasma at the concentration of 0.25 µg/mL (coefficient of variation < 20%).

Accuracy and precision
Accuracy and precision were quantified using four concentrations of quality control (QC) samples (0.25, 0.4, 4, and 30 µg/mL) in sequence on the same day (n = 5) or on three consecutive days (n = 15). Precision was expressed by coefficient of variation (CV) which was required to be within 15%, and 20% for LLOQ. The accuracy was evaluated by relative error (RE %) and should also be within 15% and 20% for LLOQ.

Stability
The stability of vancomycin was evaluated using two concentration levels (0.4 and 30 µg/mL) in three replicates. The plasma samples were stored or processed under different conditions, i.e., storage at -20°C for 7, 14 or 30 days, freezing (-20°C), and thawed (room temperature) for one or two cycles, leaving untreated plasma sample on bench top for 6 hours and leaving extracted samples in the auto-sampler for 20 hours at room temperature. All the stability mentioned above are presented as the recovery (%) relative to the nominal concentration.

EMIT assay
The EMIT assay was based on a competitive assay format using a selective vancomycin monoclonal antibody. Samples were processed according to the vancomycin-EMIT kit's instructions. Six different concentration calibrators (0, 5, 10, 20, 30, 50 µg/mL) were used to generate the calibration curve, and three QC standards (11.3, 34.3, 64.5 µg/mL) accompanied the sample test. An automated biochemical analyzer was used to measure the concentration of the analyte.

Statistics
The software of MedCalc (15.2) with Passing-Bablok regression analysis and Bland-Altman analysis was used to study variations of vancomycin concentration between the two different analytical procedures [18,19].

RESULTS

Method validation
Specificity: There was no endogenous interference in the six blank plasma samples at the retention time of vancomycin and IS. The typical chromatograms of blank plasma, vancomycin and IS standard, blank plasma spiked with vancomycin and IS, and patient plasma spiked with IS are shown in Figure 1. No significant interfering peak was observed at the retention time of vancomycin and IS.

Table 1. Overview of some chromatographic methods for determination of vancomycin in human plasma or serum.

Reference	Detector	Sample preparation	Plasma or serum volume µL	Internal standard	LLOQ µg/mL	Method comparison
4	UV	PPT	200	Caffeine	1.0	ND
5	PDA	PPT	200	p-Aminobenzoic acid	1.0	FPIA
6	UV	PPT	100	none	0.4	ND
7	UV	SPE	1000	none	0.11	ND
8	UV	PPT	200	none	1	ND
9	UV	PPT	200	none	0.25	ND
10	PDA	SPE	500	Cefuroxime	1.6	ND
11	FLD	PPT	500	Erythromycin	0.005	ND
12	MS	SPE	200	Atenolol	0.005	ND
13	MS/MS	PPT	50	Tobramicin	0.1	FPIA
14	MS/MS	PPT	40	Vancomycin-des-leucine formiate	0.3	FPIA and PETINIA
15	MS/MS	PPT	100	Polymyxin B	0.5	ND
16	MS/MS	PPT	25	Kanamycin B	1.0	ND
17	MS/MS	PPT	50	linezolid	1.0	Roche immunoassay

PPT - protein precipitation, SPE - solid phase extraction, ND - not done.

Table 2. The matrix effects and extraction recoveries of vancomycin and IS.

Matrix	Vancomycin				IS	
	0.4 µg/mL		30 µg/mL		0.3 µg/mL	
	Matrix effect/%	Extraction recovery/%	Matrix effect/%	Extraction recovery/%	Matrix effect/%	Extraction recovery
Matrix 1	91.3	95.6	105.7	91.1	109.5	109.8
Matrix 2	83.5	102.6	110.6	86.9	119.8	115.1
Matrix 3	99.3	83.7	105.3	99.0	100.7	101.0
Matrix 4	99.5	79.8	112.3	94.7	100.7	105.6
Matrix 5	104.4	91.0	97.5	100.4	95.8	99.6
Matrix 6	96.7	87.2	108.3	93.8	103.4	106.7
Mean	95.8	90.0	106.6	94.3	104.9	106.3
SD	7.4	8.3	5.2	5.0	8.6	5.7
RSD/%	7.7	9.2	4.9	5.3	8.2	5.4

Matrix effect and extraction recovery

The recoveries of vancomycin and IS were good and reproducible. The mean extraction recoveries were 90.0% to 94.3% for vancomycin and 106.3% for IS. The mean matrix effects ranged from 95.8% to 106.6% for vancomycin and 104.9% for IS. No peak signals in the retention time windows of vancomycin or IS were observed. The results for the different matrices are shown in detail in Table 2.

Linearity and LLOQ

The calibration curve was linear over the concentration range of 0.25 to 40 µg/mL with the regression equation of $y = 2.09x - 0.02$ and correlation coefficient (r^2) of 0.999. The LLOQ was 0.25 µg/mL with RSD of 1.92%.

Table 3. Precision and accuracy for vancomycin in human plasma (n = 5).

Concentration µg/mL	Intra-day			Inter-day		
	Mean ± SD µg/mL	Accuracy RE%	Precision CV%	Mean ± SD µg/mL	Accuracy RE%	Precision CV%
0.25	0.26 ± 0.03	4.4	10.0	0.24 ± 0.02	-3.6	10.0
0.4	0.38 ± 0.00	-4.0	1.2	0.37 ± 0.01	-7.8	4.0
4	3.69 ± 0.16	-7.8	4.4	3.59 ± 0.14	-10.1	3.9
30	30.00 ± 0.82	0.01	2.7	29.09 ± 1.90	-2.8	6.3

Table 4. Stability of vancomycin in human plasma at various conditions (n = 3).

Conditions	0.4 µg/mL		30 µg/mL	
	Relative recovery ± SD	RSD%	Relative recovery ± SD	RSD%
Bench top/6 hours	111.5 ± 2.9	2.6	102.2 ± 1.4	1.3
In autosampler/20 hours	95.0 ± 2.2	2.3	97.1 ± 2.8	2.9
One freeze-thaw cycle	88.9 ± 2.3	2.6	91.8 ± 2.0	2.2
Two freeze-thaw cycles	98.2 ± 2.6	2.7	105.9 ± 2.0	1.9
7 days at -20 °C	108.5 ± 4.8	4.4	103.4 ± 0.5	0.4
14 days at -20 °C	110.8 ± 12.5	11.2	90.9 ± 3.5	3.8
30 days at -20 °C	100.8 ± 3.5	3.5	96.6 ± 2.1	2.2

Accuracy and precision

Accuracy and precision results are shown in Table 3. The results were within the accepted limits. It indicated that the method was accurate and precise.

Stability

The results of the stability are shown in Table 4. Data indicated that vancomycin in human plasma was stable after storage at -20°C for 30 days and two freeze-thaw cycles. It was also stable when left on a bench-top for 6 hours before processing and in an autosampler for 20 hours after being processed.

Methods comparison

Seventy-nine plasma samples obtained from patients receiving vancomycin for treatment were determined by LC-MS method and EMIT assay. Both methods can be used to measure the vancomycin concentration in plasma samples. The concentration of vancomycin obtained from validated LC-MS methods was in the range of 1.3 - 39.6 µg/mL, with a mean of 12.7 µg/mL, while the concentration of vancomycin obtained from EMIT assays was in the range of 2.2 - 47.4 µg/mL, with a mean of 15.6 µg/mL.
The results of Passing-Bablok analysis are shown in Figure 2. The coefficient of correlation was 0.957 with a 95% confidence interval ranging from 0.933 to 0.972, and the equation of linear regression model was as follows: $y_{LC\text{-}MS} = 0.78\ x_{EMIT} + 0.67$. In addition, a Bland-Altman plot of the differences between the two assays with respect to their mean concentration indicated that their results were similar, which is shown in Figure 3. The 95% confidence interval was -3.4 - 9.2 µg/mL, and most results (97.5%) were within the 95% CI. Only two results (2.5%) were outside of the 95% confidence interval, which showed clinically relevant differences in EMIT and LC-MS.
The mean bias amounted to 2.9 µg/mL (95% confidence interval of -3.4 - 9.2 µg/mL). Vancomycin concentrations measured with LC-MS were a bit lower than those obtained with the EMIT assay, but the results were within the acceptance range. It may be due to the lower specificity of EMIT, which cannot distinguish vancomycin from its degradation product.

DISCUSSION

A new LC-MS method with lower plasma volume, simple sample extraction and lower limit of quantification was developed and validated for routine TDM of vancomycin. Only 80 µL plasma was needed for analysis. This is very important to newborn and pediatric patients. A simple and rapid protein precipitation method

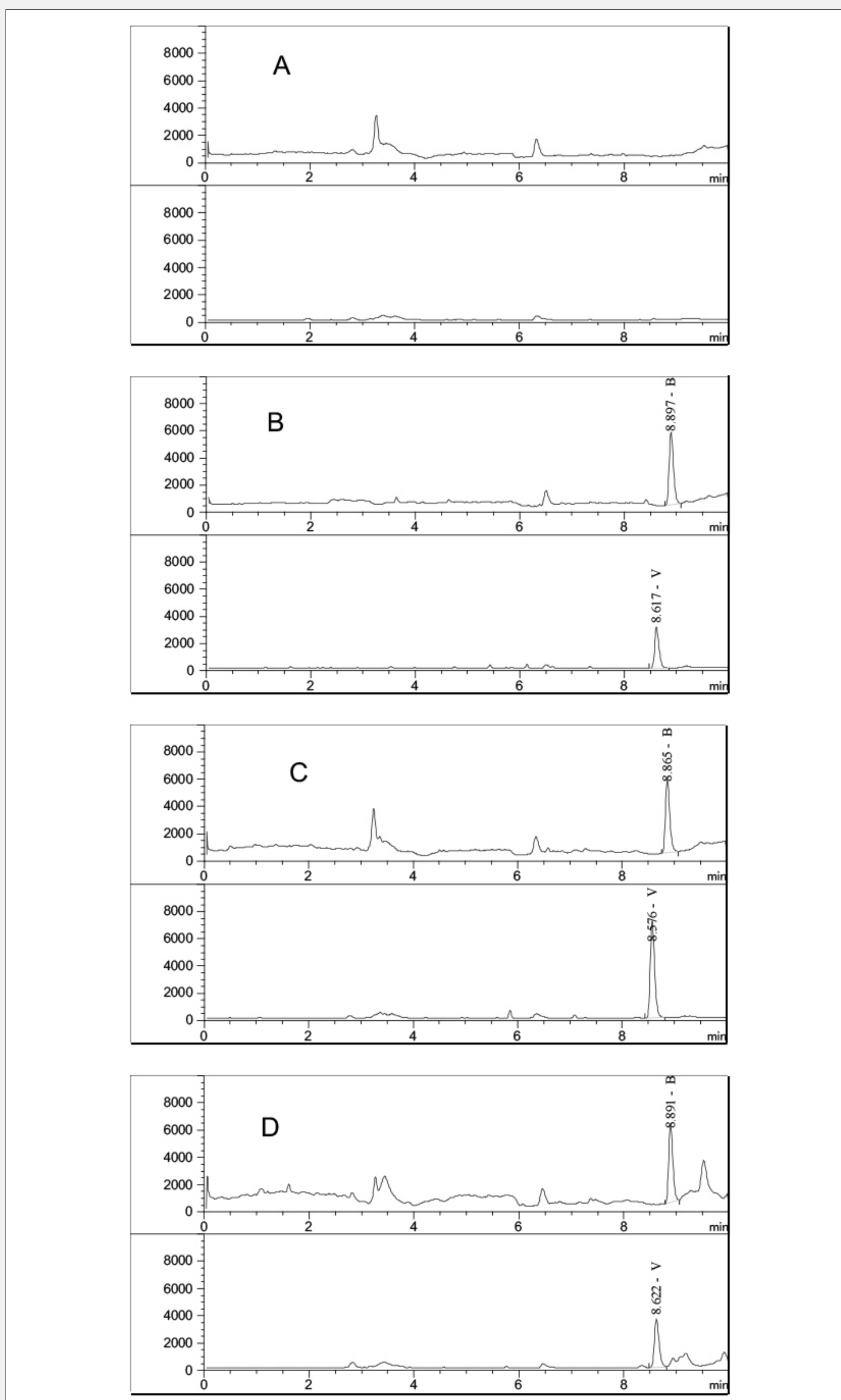

Figure 1. The typical chromatograms of vancomycin and IS.

A: blank plasma; B: vancomycin and IS standard; C: blank plasma spiked with vancomycin and IS; D: plasma of one patient spiked with IS. (V - vancomycin; B - bergenin, IS).

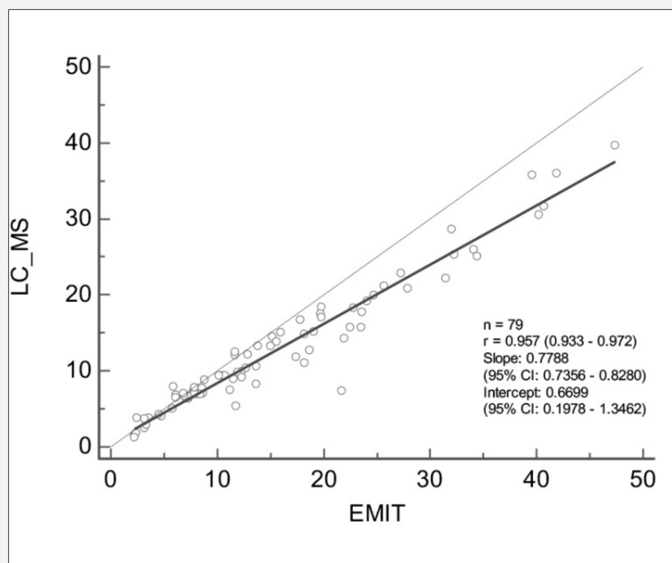

Figure 2. The correlation of LC-MS and EMIT using Passing-Bablok regression analysis.

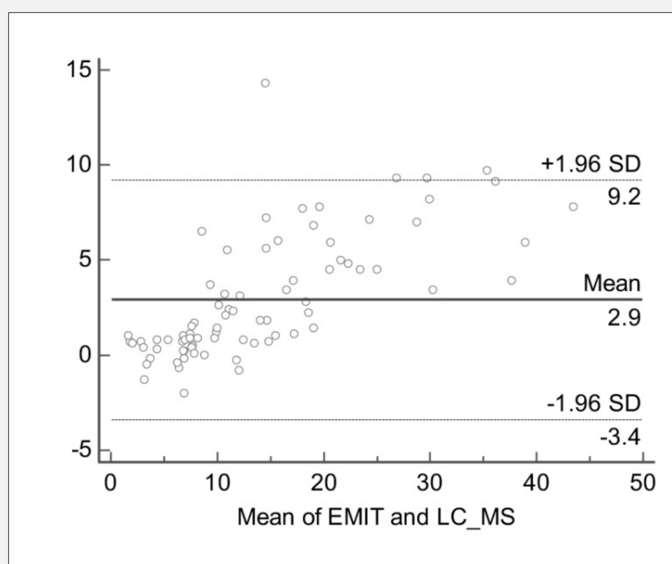

Figure 3. The Bland-Altman plot of the mean vancomycin concentration measured with LC-MS and EMIT.

was used for sample preparation with a mixture of TCA and acetonitrile (1:1, v/v) as precipitant. It has been reported that the best recovery was provided with 15 - 35% TCA [20]. In this study, 25% TCA was used. Moreover, in order to avoid the low pH of TCA shortening the lifespan of the chromatographic column and chromatographic system, the same volume of acetonitrile was added to the 25% TCA. This preparation meth-

od can provide high extraction recoveries for vancomycin, which were between 89.99% and 94.29% in all tested concentrations. The lower limit of quantification of this method was 0.25 µg/mL, more sensitive than that of the EMIT assay.
Because vancomycin is water soluble, a hydrophilic column is preferred. Therefore, in this study, an Inertsil ODS-SP (5 µm, 4.6 x 150 mm) column was used to provide good peak efficiencies. Internal standard (IS) is very important for the accuracy and precision of the method. According to the physico-chemical property of vancomycin, we tried ribovirin, pravastatin sodium and bergenin as internal standard. Finally, bergenin was selected because of the similar chromatographic behavior with vancomycin. Gradient elution with 0.1% formic acid and acetonitrile was used to elute the analytes because it could decrease the matrix effect and provide appropriate retention time for vancomycin and IS. Due to the polypeptide moiety of vancomycin, molecular ions with multiple charges were generated by the ESI source. In addition, 0.1% formic acid in the mobile phase also resulted in the formation of doubly charged molecular ions $[M+H]^{2+}$, which were 725.7 for vancomycin. Immunological methods, such as EMIT and FPIA, are often used to measure vancomycin in patient plasma or serum because of their speed and simplicity in the clinical routine laboratory practice. These methods are effective in the range of 5 - 20 µg/mL with a lower limit of quantification of 5 µg/mL for EMIT and 2 µg/mL for FPIA [21]. However, they also have some disadvantages, such as their susceptibility to interferences and cross-reactions. Some comparison studies among immunological methods or between immunoassays and HPLC methods both with ultraviolet or mass spectrumetric detection have been carried out. EMIT lost precision at concentrations above 30 µg/mL compared with FPIA [22]. When compared with HPLC, FPIA caused an overestimation in samples obtained from peritoneal dialysis patients, which probably was due to cross-reactions with CDP, the degradation product of vancomycin [23]. Recently, several studies on the comparison of immunoassay and LC-MS/MS for determination of vancomycin found only small differences in vancomycin concentrations between the two methods, with a slightly negative difference [13,14,17].
However, few studies have focused on the comparison of HPLC methods including LC-MS or LC-MS/MS and EMIT assay for determination of vancomycin. So, we developed and validated a new LC-MS method for determination of vancomycin and compared the results with those obtained from EMIT assays. A satisfactory agreement was found between the two methods. It indicated that both methods can be used to determine vancomycin in patient plasma as a part of TDM. Moreover, the increased sensitivity of the LC-MS method can allow an accurate determination of vancomycin in patients who are treated with lower doses.

CONCLUSION

A new LC-MS method for the determination of vancomycin has been validated and applied for routine TDM. This method used a simple and rapid protein precipitation, required only 80 µL plasma, which is significant particularly for newborn and pediatric patients, and provided high sensitivity for the determination of vancomycin. The results obtained from this method were correlated with those obtained from EMIT assay. It indicated that both methods can be used for TDM of vancomycin.

Declaration of Interest:
All the authors declare that they have no conflicts of interest.

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