doi.org/10.36721/PJPS.2022.35.6.SP.1683-1690.1

HPLC method development and validation for *in vitro* and *in vivo* quantification of vancomycin in rabbit plasma

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Abstract: Vancomycin (VAN) is an effective antibiotic due to its broad-spectrum bactericidal action. High performance liquid chromatography (HPLC), a powerful analytical technique is used for the *in vitro/ in vivo* quantification of VAN. The current study was aimed to detect the VAN from *in vitro* as well as the plasma after the extraction from blood of rabbits. The method was developed and validated according to International Council on Harmonization (ICH) Q2 R1 guidelines. Results showed that the peak of VAN was recorded at 2.96 and 2.57 min, respectively *in vitro* and serum. The coefficient of VAN turned out to be >0.9994 each for *in vitro* and *in vivo* samples. VAN was found linear in the range of 6.2-25000ng/mL. The values of accuracy and precision in terms of coefficient of variation (CV) were less than 2%, indicating the validity of the method. The values for LOD and LOQ were estimated to be 1.5 and 4.5ng/mL, correspondingly, which were lower than the values calculated from *in vitro* media. Furthermore, the score of the greenness found out to be 0.81, depicting good score using AGREE tool. It was concluded that the developed method was found accurate, precise, robust, rugged, linear, detectable and quantifiable at prepared analytical concentrations and could be used for *in vitro* and *in vivo* VAN determination.

Keywords: Vancomycin, HPLC method validation, serum sampling, Greenness evaluation, AGREE analysis, ICH guidelines.

INTRODUCTION

Vancomycin, a bactericidal antibiotic which was discovered more than 50 years ago, has always been a drug of choice among all other antibacterial drugs, mainly because of its consistent efficacy against most of the gram-positive bacteria and methicillin resistant *S. aureus* (MRSA) (Akhtar *et al.*, 2021). Vancomycin is a glycopeptide antibiotic which shows its bactericidal effect by inhibiting the biosynthesis of cell wall and is therefore effective in treatment of severe infections caused by MRSA (Verma *et al.*, 2021). Severe staphylococcal and streptococcal endocarditis infections in patient's resistant to penicillin, penicillin G and cephalosporins can be treated by VAN therapy. Controlled serum levels of VAN results in non-toxic and effective therapeutic outcome (Wei *et al.*, 2021). The development and validation are ongoing processes in the analytical research and development. Methods are available in the literature for the determination of VAN from *in vitro* and serum sampling of the subjects. While some of the methods have issues of containing higher values of retention times as well as a bit mixed mobile phase concentration, which might also include methanol (Ramadon *et al.*, 2020). The use of methanol may be toxic to aquatic life as listed as the penalty point according to ecological safety (Pena-Pereira *et al.*, 2020a).

Therefore, aim of the current study to develop and validate a high performance liquid chromatography technique for the quantitative determination of VAN from dissolution media as well as serum from the subjects. For this, the conditions were optimized for the peak stability of the drug by adjusting the concentration of acetonitrile in the mobile phase. Then the method was validated according to ICH (Q2-R1) guidelines. Afterwards, the method was applied for the estimation of VAN dissolution media and then it was extracted from the rabbit. Lastly, the validated method was then evaluated for extent of greenness. In the industrial era, due to certain limitation and lack of technology advancement, it is straight away not possible to reuse the eluted solvents for which it is of prime value to estimate the impact of developed method to the nature as well as society (Gałuszka *et al.*, 2012, James *et al.*, 2015). The developed analytical method should be estimated in terms of it safety to the environment.

MATERIALS AND METHODS

Materials

Vancomycin was gifted from Szeged® (Hungary) while acetonitrile was purchased from Avonchem® (UK).

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Phosphoric acid and sodium hydroxide were obtained from Merck®, Germany and sodium dihydrogen phosphate as well as potassium dihydrogen phosphate were procured from Scharlau®, (Germany).

Methods

HPLC instrumental conditions

Instrumentation and chromatographic conditions for in vitro/ex vivo quantification of VAN. HPLC analysis was performed using Shimadzu® instrument, Series 200, Japan, having a C_{18} column (4.6 mm × 150 mm, 5 µm). The UV detector was operated at 230 nm on a C_{18} column. The mobile phase was composed of filtered and degassed mixture of 2% phosphoric acid aqueous solution and acetonitrile (84:16, v/v). The flow rate of the mobile phase was set at a rate of 1.0mL/min with injection volume at 20 µL. Instrumentation and chromatographic conditions for *in vivo* quantification in plasma samples was adopted similar to *in vitro* conditions.

Preparations of standard solutions

The standard solution was prepared by dissolving 10mg of VAN in 2-3mL of the phosphate buffer. The prepared solution was then diluted up to 10mL in a volumetric flask to produce a final concentration of the stock solution *i.e.*, 1mg/mL. The extraction and centrifugation from the plasma was performed at 10000rcf for 5 min and the top aqueous layer was injected into the HPLC column for subsequent detection (Vossen *et al.*, 2021).

System suitability

The specific retention time of the peak of drug in dissolution study and serum concentration was studied. The symmetry of the drug peak was evaluated using tailing factor as well as the theoretical count of plates according to USP method was found in accordance with the procedure as reported (SYED *et al.*, 2021, Phani Sekhar Reddy *et al.*, 2018).

Validation parameters

Accuracy

To measure accuracy, a minimum of three independent replicates were prepared at 80, 100 and 120% concentration of the analyte. It was accomplished by spiking the known concentration of the analyte to the prepared analytical concentration. The values were expressed as a mean of three peaks (Salam *et al.*, 2015).

Precision

Precision was estimated (inter and intra-day) by taking three independent repeats designated for low, medium, and high concentration (John *et al.*, 2015).

Linearity range

Different dilutions were prepared using the standard solution. Linearity was calculated by preparing different analytical concentrations and a plot was constructed in which area was plotted against concentrations of standard solution. The linearity of the drug was analyzed which included linear function, coefficient of linear regression and linearity range.

Limit of detection (LOD) and limit of quantitation LOQ)

LOD can be described as the minimum amount of the analyte that can be identified in a given sample but cannot be quantified exactly. On the other hand, the LOQ refers to the minimum amount of the analyte that can be quantified exactly with adequate accuracy and precision. The Equations 2 and 3 were used to determine LOD and LOQ respectively.

$$LOD = 3.3\ \sigma / S \quad \text{(Eq. 2)}$$

$$LOD = 10\ \sigma / S \quad \text{(Eq. 3)}$$

Where, σ= standard deviation of the output signal (area under the curve of the analyte peak in the chromatogram and S= slope of a linear regression.

Robustness

Robustness of the proposed method was performed by minor deliberate changes in the pH, flow rate and temperature of the column to evaluate changes. The results were expressed as a mean of standard deviation.

Specificity

Analytical procedures and validation (Q2 R1) describes specificity as the capability to evaluate the analyte unequivocally in the existence of components which might be present in, impurities, matrix, degradants or *ex vivo* (SYED *et al.*, 2021). To identify drug from expected degradants like the components of nanoparticles and hydrogel, at least three replicates were taken to identify VAN in various samples.

Synthesis of thiolated nanomicelles formulation

Vancomycin loaded polymeric thiolated nano-micelles were prepared by simple film hydration method. Initially, vancomycin (5mg) and thiolated pluronic (100mg) were solubilized in dichloromethane (DCM) in the round bottom flask, followed by slow evaporation at 60°C with rotation of 120 rpm to develop thin dried film of vancomycin loaded thiolated pluronic inside the flask walls. Remnants of the solvent were evaporated overnight under vacuum. Moreover, obtained dried film was hydrated and stirred in rotary evaporator for 1h at 120 rpm to obtain polymeric micelles followed by probe sonication for 1min (2 s pulses of on and off) [23].

In vitro dissolution studies

The *in vitro* dissolution of VAN based-nanomicelles was conducted in paddle over disk dissolution apparatus (Erweka, Germany). Dissolution vessels of paddle over disk were filled with 500mL of phosphate buffer (pH 7.4) equilibrated at 37±0.5°C (Razzaq *et al.*, 2018). Furthermore, VAN loaded thiolated nanomicelles formulation was filled in dialysis bags and placed in buffer solution.

Table 1: Investigation of accuracy of VAN in *in vitro* samples at various time points

Theoretical concentration (µg/mL)	Percentage recovery (%)		Mean recovery ± SD
1.56	Day 1	99.52	99.75 ± 0.48
	Day 2	99.74	
	Day 3	99.99	
0.19	Day 1	100.01	100.02 ± 0.36
	Day 2	99.97	
	Day 3	100.09	
0.024	Day 1	100.2	100.06 ± 0.27
	Day 2	99.93	
	Day 3	100.05	

Table 2: Investigation of inter and intra-day precision of the reported HPLC method for VAN quantification *in vitro* samples

	Intra-day precision	
Theoretical concentration (µg/mL)	Observed concentration ± SD (µg/mL)	Coefficient of variation (% CV)
1.56	1.54 ± 0.02	0.20
0.19	0.19 ± 0.01	1.01
0.024	0.023 ± 0.03	0.89
	Inter-day precision	
1.56	1.55 ± 0.03	0.25
0.19	0.18 ± 0.01	0.98
0.024	0.023 ± 0.01	1.21

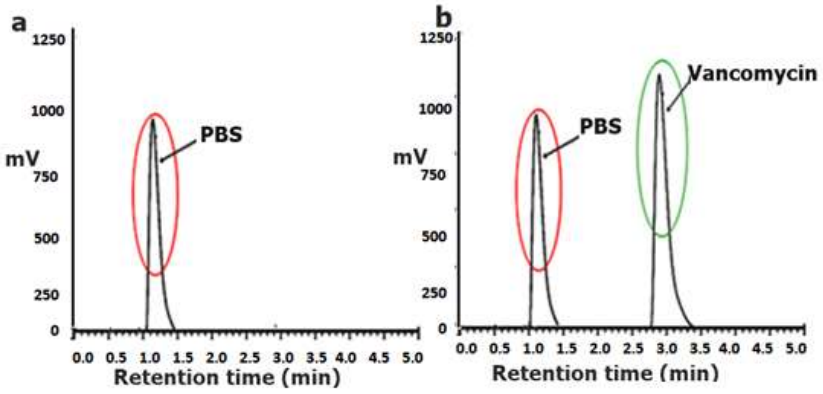

Fig. 1: Typical chromatogram of developed method showing, (a) the representation of chromatograms of PBS alone, and (b) VAN at 0.78µg/mL in PBS, the PBS peak is encircled by blue and VAN peak encircled by red color.

Commercially available drug powder was also used to pack in the dialysis bag to check the release for the comparison of results. The stirring speed was maintained at 150rpm and samples of 5mL were collected at specified time intervals for 48h. The withdrawn sample was replaced with the same amount of fresh media and evaluated for HPLC analysis (Zhang *et al.*, 2018).

Greenness evaluation

To evaluate the extent that the developed method will be green to the environment, AGREE analysis was performed. Briefly, developed instrumental conditions were evaluated partially closed questionnaire which quantified the safety of the developed method to the environment. Evaluation was performed using the AGREE® software v0.5 beta (Universida de Vigo). The score gained after the evaluation and penalty deduction were represented as sum were written on a circular pictogram with response of 12 parameters around the circle (Pena-Pereira *et al.*, 2020b). The parameter or step which was more ecologically safe was contoured green and vice versa (red).

STATISTICAL ANALYSIS

All the statistical tests applied in different section of validity of the results were performed in IBM SPSS statistical software package v. 21. The greenness was evaluated using AGREE software.

RESULTS

In the present study, different HPLC based reported methods with modifications were validated for quantification and detection of VAN in various samples. Instrumentation and chromatographic conditions for *in vitro/ex vivo* quantification of drug.

Table 3: System instrumental conditions, linearity and range for VAN *in vitro* samples

Parameters	Observations
Slope	48704
Intercept	12608
Linearity Equation	y = 48704x – 12608
r^2	0.9995
linearity (ng/mL)	3.1-25000
Area (IU)	247309
Tailing factor	1.14
Theoretical plate count (USP)	4667
LOD (ng/mL)	2.91
LOQ (ng/mL)	8.73

Table 4: Robustness of the developed method using *in vitro analysis*

Factors	CV of VAN at 0.78 µg/mL
	%
Optimized conditions = 1 mL/min, ambient conditions	
Flow rate	
0.85 mL	1.12
1.15 mL	0.87
Temperature change	
+5 ºC	0.66
-5 ºC	1.41
pH change	
0.5	1.96
-0.5	1.78

System suitability parameters

After the mobile phase, flow rate, pH and wavelength were selected, the calculations for retention time, peak area of the standard concentration, tailing factor and theoretical plate count (USP) found out to be 1.14 and 4667 (table 3), respectively.

HPLC method validation for vitro analysis

Accuracy and precision

Accuracy was evaluated at three concentrations of low, medium, and high (values taken from the calibration curve). The actual concentrations were measured from AUC and compared with theoretical concentrations and percentage recovery was estimated (Keyvan *et al.*, 2021). The percentage recovery was found to be 99-101% indicating a high extent of accuracy. The values of percentage recovery are shown in table 1.

Precision was evaluated in terms of intra-day repeatability (within 24h) and inter-day replication (*i.e.* three consecutive readings, each reading after 24h). In table 2, both inter-day and intra-day precision is illustrated with respect to CV (coefficient of variation) for VAN at low, medium, and high concentrations. In all the cases, the value of CV was measured less than 2%.

Linearity and range

The VAN was found linear in the range of concentrations 3.1-25000ng/mL (table 1). The correlation and least square linear regression analysis were carried out on the calibration curve. The correlation of coefficient was found to be 0.9995 suggesting a high degree of linearity within the selected range. The properties of the calibration curve, including slope, linearity equation, and y-intercept were evaluated and illustrated in table 3.

LOD and LOQ

The calculated LOD and LOQ values of VAN were 2.91 and 8.73 (ng/mL), respectively which showed that very minute amount of the drug could be detected from the dosage form.

Robustness

It was found that minor manipulations in the temperature, flow rate and pH depicted the value of CV less than 2% (table 4). These minor changes suggested that the developed method has robustness and ruggedness.

Specificity

To check the specificity of the reported method, a sample of phosphate buffer was injected into the HPLC as a blank, while the VAN solution in PBS was injected as a test solution. fig. 1a and 1b illustrate the chromatograms of PBS in the mobile phase at a concentration of 0.78 µg/mL. The chromatographic conditions exhibited a well-separated and sharp peak of vancomycin, without interfering peaks of the blank.

HPLC conditions for in vivo analysis

To quantify VAN *in vivo* samples, the same instrument and column were used, as described for the *in vitro* sample analysis, as follows.

Accuracy and precision

Accuracy was evaluated at three concentrations of low, medium and high (values taken from the standard calibration curve). The actual concentrations were measured from AUC and compared with theoretical concentrations and percentage recovery was estimated. The percentage recovery was found to 99-101% indicating a high extent of accuracy (table 5).

Precision was evaluated in terms of intra-day repeatability (within 24 h) and inter-day repeatability (*i.e.* three consecutive readings, each reading after 24 h).

Table 5: Investigation of accuracy and precision of the reported HPLC method for VAN quantification *in vivo* samples.

Theoretical concentration (µg/mL)	Percentage recovery (%)		Mean recovery ± SD
3.12	Day 1	99.91	99.97 ± 0.36
	Day 2	100.01	
	Day 3	99.99	
0.78	Day 1	100.05	99.95 ± 0.73
	Day 2	99.89	
	Day 3	99.92	
0.09	Day 1	100.2	100.06 ± 0.69
	Day 2	99.93	
	Day 3	100.05	

Table 6: Investigation of inter and intra-day precision of the reported HPLC method for VAN quantification *in vivo* samples.

Theoretical concentration (µg/mL)	Observed concentration ± SD (µg/mL)	CV (%)
Intra-day precision		
3.12	3.14 ± 0.09	0.15
0.78	0.76± 0.05	0.79
0.09	0.08 ± 0.02	0.92
Inter-day precision		
3.12	3.10 ± 0.03	1.05
0.78	0.79 ± 0.02	0.71
0.09	0.10 ± 0.01	0.29

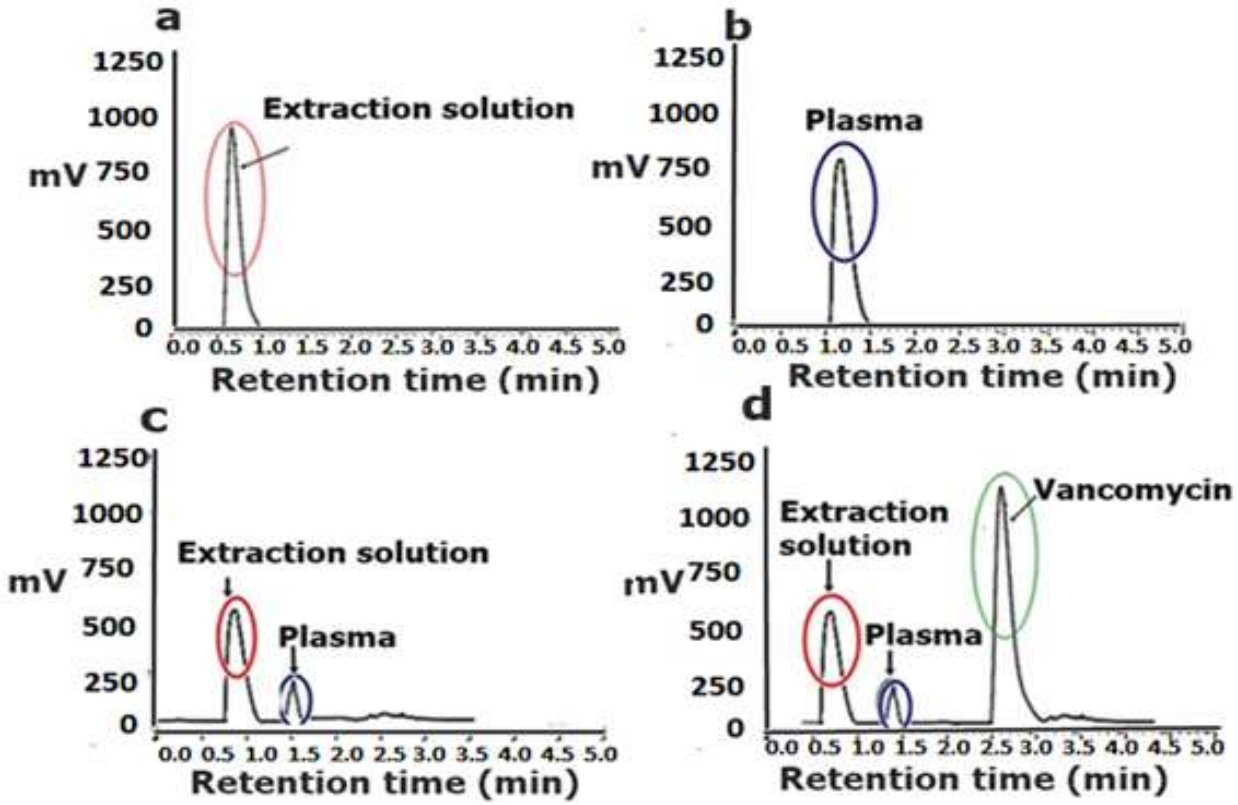

Fig. 2: Investigation of VAN specificity in *in vivo* samples, (a) chromatograms of extraction solution, (b) blank plasma, (c) plasma spiked with extraction solution, and (d) plasma spiked with 0.78µg/mL of VAN in the extraction solution.

In table 6, both inter-day and intra-day precision is illustrated with respect to coefficient of variation (CV) for VAN at low, medium, and high concentrations. In all the cases, the values of CV was measured less than 2%.

Linearity

A series of VAN concentrations in plasma samples were evaluated to check the linearity of the reported method. The correlation and least square regression line analysis were performed on three successive days in a triplicate manner. The linearity of the correlation coefficient was found to be 0.9996, depicting a high extent of linearity over a selected range of VAN concentrations. The properties of the standard calibration curve, including slope, linearity equation and y-intercept were evaluated and illustrated in table 7.

Table 7: Linearity and range of the reported HPLC method for VAN quantification in *in vivo* samples.

Parameters	Results
Slope	42572
Intercept	724.94
Linearity equation	y = 42572x - 724.94
r^2	0.9996
Area (IU)	273632
Linearity range (ng/mL)	6.2-25000
LOD (ng/mL)	1.50
LOQ (ng/mL)	4.50

LOD and LOQ

For the *in vivo* analysis, the values for LOD and LOQ were estimated to be 1.50 and 4.50ng/mL, correspondingly (table 7).

Robustness

Similar to the findings of *in vitro* data of the developed method, the robustness and ruggedness of the developed method was also achieved using serum analysis. The deliberate intentional changes produced a replicate values of CV less than 2% (table 8).

Specificity

To check the specificity of the reported method for *in vivo* samples, a sample of extraction solution (fig. 2a) and plasma spiked with an extraction solution (fig. 2b) were run on the HPLC as a blank. Likewise, the same plasma solution spiked with VAN and extraction solution (fig. 2d) was run as a test solution. The chromatographic conditions exhibited a well separated and sharp peak of drug in plasma, without interfering peaks of the blank samples. The fig. 1 and 2 exhibited that VAN was fully separated, and the validated range of VAN produced a linear response. The samples with VAN concentration higher than the validated range were diluted with the mobile phase before analysis to ensure that all the analyses were carried out within the validated linear range. The values for LOQ and LOD were comparable to the reported methods, which ensured that the analytical method is appropriate for VAN analyses in various *in vitro* and *in vivo* samples (Khatwani *et al.*, 2021). Both inter- and intra-day accuracy (99-101%) and precision (<5%) were found within the acceptable range as per previous literature (% CV and % recovery must not exceed 15% for QC samples).

Table 8: Robustness of the developed method using *serum analysis*

Factors	CV of VAN at 0.78 µg/mL
	%
Optimized conditions =	
Flow rate	
0.85mL	0.54
1.15mL	1.04
Temperature change	
+5ºC	1.10
-5ºC	1.24
pH change	
0.5	1.88
-0.5	1.14

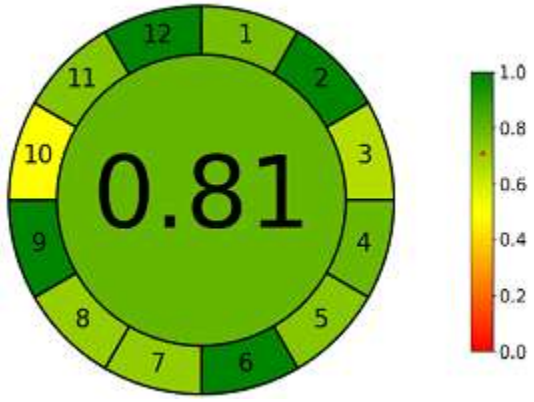

Fig. 3: AGREE score of the validated HPLC method indicating the greenness score.

Extraction of plasma

Animal handling was conducted according to the guidelines of Bio-Ethical Committee (BEC) of Riphah International University, Pakistan (Ethical code: Ref. No. REC/RIPS/2019/30). Male rabbits weighing 3.5-4.5kg were kept in free excess to food and water. Moreover, after giving oral administration of 1.0mg of vancomycin, 2.5mL of blood samples were withdrawn from the marginal ear vein at various time-points in the EDTA tubes. Afterwards, the blood samples were centrifuged for 10min at 4000 rpm and the extracted plasma was stored at -20°C until further use.

Greenness evaluation

Outcome of the AGREE analysis on the greenness of the developed method produced a aggregated response of

0.81 which fairly falls in the light green to darker contouring of the score. Since, some reagents were bio based due to which the penalty of tenth response was yellowish. Otherwise, the shorter sampling time allowed better score and overall more contribution towards greenness of the environment (Aqeela Raza *et al.*, 2022).

DISCUSSION

Pharmaceutical investigation is an integral process of the drug development process especially for the identification and quantification of active pharmaceutical ingredients (API). However high-performance liquid chromatography due to more sensitivity, selectivity, cost-effectiveness, and accessibility is most commonly used. According to ICH Q2 (R1), accuracy is defined as the extent to *which* current value matches the predictable true value or standard reference value. The accuracy was investigated for the mean percentage recovery along with the standard deviation (Quarterman *et al.*, 2021). The ICH recommends that percentage recovery must be within 15% of the conventional true value or standard reference value (Chaudhary *et al.*, 2021). Precision can be defined as the variation between the series of measurements acquired from various sampling days by different analyst on dissimilar machines. Linearity can be defined as the capability of a procedure to find the results that exhibit a positive relationship with the concentration of an analyte in a given sample. The method was found linear with a detectable value of less than 5ng/mL. In current study, the specificity was evaluated by investigating the samples having phosphate buffer alone and phosphate buffer with dissolved VAN. The phosphate buffer was chosen as a blank because it was utilized as a diluent for making different dilutions of VAN in various samples. Moreover, it was also employed as a medium for the dissolution of VAN in various analytical samples. The VAN was specifically extracted from the plasma samples and a characteristic peak was observed at 2.96 and 2.57 min for *in vitro* and *in vivo* serum respectively. In all cases, the percentage extraction efficiency was found to be >98% and CV<2%. These values were consistent with the reference methods and were considered acceptable for accurate quantification of VAN in plasma/tissue samples (Usman and Hempel, 2016). As compared with *in vitro* linearity range, the linearity range for *in vivo* samples was reduced slightly, however, this difference was statistically non-significant. On the other hand, LOD and LOQ of VAN in *in vivo* samples were slightly higher than *in vitro* samples, but again the difference was statistically non-significant (p<0.01). The results were comparable with previously reported methods that ensured the potential of the analytical method to be used efficiently for VAN quantification. Both inter- and intra-day recovery and precision were estimated to be 99-100% with a CV of less than 2%. Vancomycin loaded polymeric thiolated nano-micelles were prepared by simple film hydration method.

The *in vitro* dissolution of VAN based-nanomicelles was conducted in paddle over disk dissolution apparatus (Erweka, Germany). Dissolution vessels of paddle over disk were filled with 500mL of phosphate buffer (pH 7.4) equilibrated at 37±0.5°C.

Animal handling for extraction of rabbit plasma was conducted according to the guidelines of Bio-Ethical Committee (BEC) of Riphah International University, Pakistan (Ethical code: Ref. No. REC/RIPS/2019/30). Male rabbits weighing 3.5-4.5 kg were kept in free excess to food and water for extraction of plasma and the extracted plasma was stored at -20°C until further use.

The safety of the developed method or in other words, the extent of toxicity posed by the developed analytical method is evaluated using the greenness score. It is important since the eluted mobile phase is usually wasted after the analysis and therefore produces toxic pollutant that is hazardous to the ecosystem (Kannaiah *et al.*, 2021). Although various methods are available to evaluate the greenness score, out of which the AGREE tool provide a more detailed as well as quantifiable aspect of the analytical procedure (Pena-Pereira *et al.*, 2020a). The greenness of the developed method was also found to be in green contour with a penalty point of 0.19 which was probably due to the reason that some ingredients were bio-based. Another important greenness parameter was that the contribution of organic solvent was quite low which could possibly contribute the preservation of environment.

CONCLUSION

The current method was found out to be linear, specific, accurate, precise, robust and rugged. The extraction of VAN from the plasma/tissue sample was also evaluated successfully and did not show any interfering peak of serum. Same was the case observed for *in vitro* studies. It was concluded that detectable levels of <5 ng/mL were found from the plasma using the reported method, and validated according to the guidelines of ICH and successfully applied to the determination of dosage form. The greenness score above 0.8 using AGREE depicted a more green-shade in contour scale.

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