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Ramadon, D., Courtenay, A. J., Permana, A. D., Tekko, I. A., McAlister, E., McCrudden, M. T. C., McCarthy, H. O., & Donnelly, R. (2020). A sensitive HPLC-UV method for quantifying vancomycin in biological matrices: Application to pharmacokinetic and biodistribution studies in rat plasma, skin and lymph nodes. *Journal of Pharmaceutical and Biomedical Analysis*, 189, [113429]. <https://doi.org/10.1016/j.jpba.2020.113429>

Published in:

Journal of Pharmaceutical and Biomedical Analysis

Document Version:

Peer reviewed version

Queen's University Belfast - Research Portal:

[Link to publication record in Queen's University Belfast Research Portal](#)

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**A sensitive HPLC-UV method for quantifying vancomycin in biological matrices:
Application to pharmacokinetic and biodistribution studies in rat plasma, skin and
lymph nodes**

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Abstract

Vancomycin (VCN) is an antibiotic used in the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA)-derived infections. As it has a relatively narrow therapeutic window, it is imperative to develop a sensitive and reliable analytical method for drug monitoring and pharmacokinetic purposes. In the present study, quick sample preparations and a sensitive high-performance liquid chromatography method using UV detection (HPLC-UV) have been developed and validated. The analytical method for detection and quantification of VCN in rat plasma, skin and lymph node samples was validated based on the Food and Drug Administration (FDA) and European Medicine Agency (EMA) bioanalytical method validation guidelines. The optimised plasma sample preparation involved a simple protein precipitation step, with extraction recovery of $100.3 \pm 0.92\%$. VCN in all biological matrices was analysed in a HPLC-UV system (215 nm) using a Cortecs® C18 column (4.6×150 mm, $2.7 \mu\text{m}$ particle size) fitted with a guard cartridge set at 20°C . Reverse phase HPLC under gradient conditions, with mobile phase consisting of 20 mM phosphate buffer containing 0.5% v/v of triethylamine and a mixture of methanol – acetonitrile (70:30, v/v), and runtime of 12 min/sample was employed. The calibration standards used for plasma ranged between 0.1-50 $\mu\text{g/ml}$, while in the skin and lymph node matrices, standards ranged between 0.05-50 $\mu\text{g/ml}$ with correlation coefficients (R^2) of ≥ 0.9995 for all matrices. The method was selective, sensitive, accurate and precise for detecting and quantifying VCN in the biological matrices used. The validated method was successfully utilised in the detection of VCN in a pharmacokinetic and organ biodistribution study carried out in rats following oral and IV bolus administration of the drug. This validated bioanalytical method offers a wide range of potential applications in clinical therapeutic drug monitoring, pharmacokinetics and toxicology.

Keywords

Vancomycin; HPLC-UV; skin; lymph node; pharmacokinetics

1. Introduction

One of the most effective antibiotics for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA)-derived infections is vancomycin (VCN) [1]. This glycopeptide antibiotic is produced by particular strains of *Streptomyces orientalis* and was first isolated by Dr. E. C. Kornfield, a chemist in the Eli Lilly company, in 1957 [1]. In both aerobic and anaerobic bacteria, VCN acts by blocking the transglycosylase reaction, binding the D-ala-D-ala section of the bacteria cell and hindering bonding between polymer of glycopeptide (N-acetylmuramic acid – N-acetylglucosamine) [1]. VCN may be used in the treatment of some illness such as endocarditis, osteomyelitis and acute bacterial prostatitis caused by Gram-positive cocci [1]. However, VCN must be administered intravenously for systemic infections because it is poorly absorbed in the gastrointestinal tract [1]. Filippone *et al.* [2] suggested the minimum level of VCN required to prevent resistance is 10 mg/L and associated nephrotoxicity may occur when the serum level is more than 15 mg/ml. It is, therefore, imperative to periodically measure VCN concentration in serum to ensure sufficient therapeutic concentration and to prevent toxicity.

Biodistribution studies have shown VCN to be widely distributed into most body organs after administration *via* intravenous injection or infusion [3], including cerebrospinal fluid (CSF) [4], heart valves [5], lungs [6] and interstitial fluid (ISF) [7]. As a consequence, and for therapeutic drug monitoring purposes, it is important to develop a sensitive and selective method for analysing and quantifying VCN in a range of different biological samples. A variety of different bioanalytical methods have already been employed, including immunologic, biological and chromatographic methods [8]. Chromatographic methods have the potential for greater sensitivity and reproducibility in routine analysis [8]. Thus, chromatographic methods are preferential for analysing VCN in various biological samples. To this end, recent studies have investigated a variety of chromatographic methods, using different detectors, to quantify VCN in biological tissues. Liquid chromatography (LC) coupled with mass spectrometric detection (MS/MS) has been utilised [9]. However, despite its high sensitivity, LC-MS/MS requires highly expensive, sophisticated instrumentation, suffers from limited sample throughput and may give rise to ion suppression [10]. Therefore, other types of LC detectors have also been employed in VCN analysis, such as electrochemical [11], fluorescence [12] and ultraviolet (UV) [13,14]. An exploration of the use of high-performance liquid chromatography (HPLC) with UV detection for the biological analysis of VCN would be advantageous due to

ease of instrumentation operation; good stability of response and the ability to utilise a wide range of wavelengths in analysis.

Analysis of VCN in the skin interstitial fluid (ISF) has previously performed by Ito *et al.* [7]. However, it was analysed by using LC-MS/MS, and the data obtained was limited to a 2 h-study. Moreover, the samples were limited to ISF and not the whole skin sample. It is necessary to quantify VCN in all skin compartments (interstitial and intracellular compartments) because the distribution of a drug may vary in these different compartments [15]. VCN has been quantified in a limited number of biological matrices, including plasma and ISF [7] but, to date, no HPLC-UV method for detecting and quantifying VCN in lymph node samples has been published. The intravenous administration of VCN is associated with a drug reaction with eosinophilia and systemic symptoms (DRESS), a rare-life threatening syndrome [16]. Recently, Zafar *et al.* [16] has reported that one of the DRESS symptoms is the lymphadenopathy, hence, it is necessary to investigate the biodistribution of VCN in the lymph node. Thus, this present work describes the development of a simple extraction method of VCN from rat plasma, skin and lymph node samples. A sensitive bioanalytical HPLC-UV method validation for quantification of VCN in a range of different biological samples was carried out, based on European Medicine Agency (EMA) 2011 [17] and Food and Drug Administration (FDA) 2018 [18] guidelines. The validated method was then utilised in an *in vivo* study to investigate the pharmacokinetic profile of VCN in rat plasma after oral and intravenous administration over a 48 h period.

2. Materials and Methods

2.1. Materials

Methanol, acetonitrile and triethylamine (TEA) were HPLC grade from Sigma-Aldrich (Dorset, UK). Vancomycin hydrochloride (VCN) was purchased from Alfa Aesar (Lancashire, UK). Vancocin[®] 500 mg powder (Vancomycin hydrochloride) solution for infusion and oral solution/gavage was obtained from Flynn Pharma Ltd. (Hertfordshire, UK). Sodium dihydrogen phosphate dihydrate was obtained from VWR International (Leuven, Belgium). Orthophosphoric acid was purchased from Amresco (Ohio, USA). Ultrapure water was obtained from a water purification system Elga PURELAB DV 25 (Veolia Water Systems, Ireland). HPLC column Cortecs[®] C18 (4.6 × 150 mm, 2.7 µm particle size) were purchased from Waters (Dublin, Ireland). All other chemical reagents were from Sigma-Aldrich (Dorset, UK). All reagents were of analytical grade.

2.2. Preparation of VCN calibration standards and quality control solutions in three different biological matrices

VCN stock solution was prepared by dissolving 10 mg of drug in ultrapure water and making up to 10 ml to obtain a concentration of 1 mg/ml. A series of VCN solutions of different concentrations were then obtained by serially diluting the stock solution in ultrapure water. A plasma calibration standard was prepared by adding 50 μ l blank rat plasma to 50 μ l of the relevant VCN standard to achieve serial VCN plasma concentrations in the range 0.1-50 μ g/ml. Quality control (QC) VCN plasma solutions at low (QCL) (0.3 μ g/ml), medium (QCM) (25 μ g/ml) and high (QCH) (40 μ g/ml) concentrations were prepared using the same method.

Blank skin and lymph node matrices were prepared by adding ultrapure water (0.45 ml) to 50 mg of skin or lymph node. The samples were then disrupted and homogenised using a tissue homogeniser, Tissue Lyser LT (Qiagen, Ltd, Manchester, UK) for 15 min at 50 Hz [19]. Following this, samples were centrifuged at 14,000 x g for 10 min and the supernatant was collected as the relevant blank matrix solution.

Calibration standards and QC solutions for skin and lymph node samples were obtained by spiking 80 μ l of the relevant concentration of VCN solution into 80 μ l of blank matrix (skin or lymph node). The concentration ranges used for skin and lymph node calibration standards were 0.05-50 μ g/ml, while QC solutions were 0.15, 25 and 40 μ g/ml, designated as low, medium and high concentrations, respectively.

2.3. Selection of plasma sample preparation

The extraction of VCN from rat plasma was tested using four previously published methods, with slight modifications, as listed in Table 1 below. A working solution of 50 μ g/ml VCN was used in these sample preparations and an aliquot (50 μ l) of each sample was injected into HPLC with the condition which will be explained in section 2.4. Parameter observed in this selection step was the area obtained from each sample after the extraction process with a specific method as listed below. Then, the area obtained was compared to the area of VCN standard solution (without extraction).

Table 1. Summary of methods tested for extracting VCN from plasma sample

Method	Process
A	Spiked plasma (volume) was placed in a centrifugal filtration unit, Amicon [®] Ultra (Sigma Aldrich (Dorset, UK)), with molecular weight cut off of 30 kD and centrifuged at 14,000 x g for 10 min, then the supernatant was collected [20].
B	Spiked plasma (100 µl) was added to 100 µl methanol in a 0.5 mL microcentrifuge tube, vortexed for 30 s, then centrifuged at 14,000 x g for 6 min at room temperature. The supernatant was then collected [21].
C	Spiked plasma (100 µl) was added to 100 µl of water in a 0.5 mL centrifuge tube and vortexed for 10 s. Methanol (100 µl) was then added to the sample and this was vortexed for 30 s, then centrifuged at 14,000 x g for 6 min at room temperature. The supernatant was then collected [22].
D	Spiked plasma (100 µl) was added to 100 µl methanol, as explained in method B, however the resulting supernatant was evaporated in a glass culture tube under nitrogen flow at 20°C for 20 min. The evaporation process was carried out using a Zymark TurboVap [®] LV Evaporator Workstation (Lab Equipment, UK). The residue was then reconstituted with water (100 µl) and collected in a microcentrifuge tube, vortexed for 30 s, and centrifuged at 14,000 x g for 6 min at room temperature. The supernatant was then collected [23].

2.4. Chromatographic and instrumentation conditions

The HPLC analysis of VCN was performed on an Agilent 1200 series system (Agilent Technologies UK Ltd, Stockport, UK) with UV detection at 215 nm. The chromatographic separation was achieved using a Cortecs[®] C18 column (4.6 × 150 mm, 2.7 µm particle size), fitted with a guard cartridge set at 20 °C. The mobile phase consisted of solvent A (20 mM phosphate buffer containing 0.5% v/v TEA, adjusted to pH 2.5) and solvent B (methanol – acetonitrile in the ratio, 70:30, v/v). The reverse phase (RP) HPLC gradient conditions and run times employed in this work are provided in Table 2. The total runtime of each sample was 12 min and the injection volume was 50 µl.

Table 2. Gradient condition utilised for VCN analysis

Time (min)	Solvent A (%)	Solvent B (%)	Flow rate (ml/min)
0	90	10	0.4
4	60	40	0.4
8	90	10	0.4
12	90	10	0.4

2.5. Bioanalytical method validation

A full validation of the bioanalytical method developed in this study was carried out, according to the EMEA 2011 and FDA 2018 bioanalytical method validation guidelines. Parameters validated in this work were: lower limit of quantification (LLOQ), linearity (calibration curve), accuracy, precision, selectivity, carry over, dilution integrity, extraction recovery and stability.

2.5.1. Selectivity

The selectivity of the bioanalytical method was investigated by analysing the chromatograms of blank plasma, skin and lymph node matrices from six different sources and the spiked matrices to assess possible interfering compounds in the chromatograms at the relevant VCN retention times.

2.5.2. Linearity of calibration curve

Calibration curves were prepared using plasma and working standard solutions. Each calibration curve consisted of one blank sample (plasma without analyte) and 7 non-zero samples (plasma spiked with VCN solutions, including lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ)). The concentration ranges used for plasma, skin and lymph samples were 0.1-50 µg/ml, 0.05-50 µg/ml and 0.05-50 µg/ml, respectively. According to EMEA (2011) [17], a new calibration curve must be prepared on each day of analysis and the percentage difference of each actual concentration, compared to the theoretical concentration (%diff), should not exceed $\pm 15\%$, except in the case of the LLOQ where it should not exceed $\pm 20\%$.

2.5.3. Lower limit of quantification

The lower limit of quantification (LLOQ) was determined by diluting the lowest concentration of VCN on the calibration curve two-fold. Each concentration was prepared in five replicates and injected onto HPLC. The %diff value of each sample and its coefficient of variation (%CV) was then determined. %CV was calculated by dividing the standard deviation (SD) with average concentration measured multiplied by 100%. The LLOQ was the lowest VCN concentration measured which showed the %diff not less than -20% and not more than +20%, compared to the theoretical value.

2.5.4. *Carry over*

Carry over is the possibility of a peak appearing in the blank/next injection after the injection of solution at upper limit of quantification (ULOQ) concentration. Carry over was evaluated by injecting standard solution at ULOQ concentration, equating to 50 µg/ml. Following the injection of ULOQ solution, a blank solution was then injected, and the area of analyte appeared in the blank plasma/matrix after the ULOQ injection was measured. At least five injections of ULOQ solution and blank plasma/matrix should be performed to determine the carry over parameters. The area of analyte that appeared in the blank plasma/matrix should not be more than 20% of LLOQ area.

2.5.5. *Accuracy and precision*

For accuracy and precision, the solutions used were QC sample solutions at low (QCL), medium (QCM) and high (QCH) concentration. In the present work, the concentration of QCL was three times of LLOQ, QC medium was 50% of the highest concentration, and QCH equates to 80% of upper limit of quantification (ULOQ) concentration. Moreover, the solution at LLOQ concentration was also used for this parameter. Five replicates of each concentration were prepared on each of three different days. The %diff and %CV of each sample injection, both within-run and between runs was determined. In terms of accuracy, the %diff of each replicate, at each concentration, should be $\pm 15\%$ and, to determine precision, the %CV should not exceed $\pm 15\%$. The %diff and %CV for the LLOQ concentration however should be $\pm 20\%$.

2.5.6. *Dilution integrity*

To determine the dilution integrity parameters, spiked plasma or matrix samples with VCN concentrations higher than the ULOQ were used. Accordingly, samples at a concentration of 60 µg/ml were diluted two-fold (30 µg/ml) and four-fold (15 µg/ml), then prepared and extracted using the appropriate method. Five replicates were prepared each day over three days. The acceptance criteria for the dilution integrity parameters were %diff (accuracy) and %CV (precision) of $\pm 15\%$ within and between runs.

2.5.7. *Extraction recovery*

In this study, a relative extraction recovery was obtained by comparing the measured value of extracted samples at QCL, QCM and QCH concentrations with the extracts of spiked blank matrix with the analyte post extraction at low, medium and high concentrations.

2.5.8. *Stability studies of VCN stock solution and VCN spiked matrices*

Stability studies were performed in plasma, skin and lymph node samples. The following stability studies were carried out: Short term stability of VCN stock solution and working solutions at room temperature (24 h); freeze and thaw stability (3 cycles) of the VCN in the various matrices stored at -20°C; long term stability of VCN stock solution and VCN in matrices following storage at -20°C for 30 days. The final stability study was carried out in the HPLC autosampler. The extracted VCN samples were injected at 0 h and stored in the HPLC sample chamber at room temperature for 24 h, and was injected at 24 h.

2.6. *Application of the validated HPLC method in an in vivo model*

Approval for animal experimentation was obtained from the Committee of the Biological Services Unit, Queen's University Belfast under Project Licence PPL 2794 and Personal Licences PIL 1466, 1583 and 1890. All animal experiments were carried out in accordance with the policy of the Federation of European Laboratory Animal Science Associations and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes, with implementation of the principles of the 3Rs (replacement, reduction and refinement).

Female *Sprague-Dawley* rats aged 8-10 weeks with average mass of 204 ± 11 g ($n = 20$), were acclimatised for 7 days before commencement of the study. The rats were divided into 2 groups, namely oral and intravenous (IV) groups. The animals were administered the same VCN dose of 30 mg/kg. Blood samples were taken at pre-defined intervals: 1, 2, 4, 6, 24 and 48 h *via* tail vein bleeds with a maximum of 200 µl collected at each sampling point into tubes (1.5ml Microtube Conical®, Sarstedt, United Kingdom) containing heparin (Wockhardt UK Ltd, Wrexham, UK). Blood sampling intervals adhered to the restrictions of the project licence and the rats were bled no more than two times per day. The plasma separation was carried out by centrifuging the whole blood at $3,000 \times g$ for 10 min, 4°C. Following this centrifugation step, the plasma supernatant was then collected and stored at -20°C prior to use.

Following the collection of the final blood samples, animals were sacrificed. Dorsal skin measuring approximately 2 cm x 2 cm was excised from the animals. Axillary and inguinal lymph nodes were removed. For skin and lymph node samples, the blood or other contamination was removed by rinsing the samples with phosphate buffered saline (PBS) pH 7.4 solution and then the samples were blotted using paper towels and stored in a -20°C freezer.

before being processed. Then, the plasma samples obtained were extracted by the selected method, as previously explained in section 2.3. With regards to skin and lymph node samples, VCN was extracted by adding each organ (skin or lymph node) (100 mg) to 900 µl of water. Then, it was processed using a tissue homogeniser, as previously outlined in section 2.2, and supernatants were collected. An aliquot (50 µl) of this supernatant was analysed *via* HPLC. To ensure that the drug was completely extracted from the tissue samples, a second extraction was carried out. Water (1 ml) was added to the residue obtained at the end of the first extraction and this was then processed as described. The supernatant from this second extraction was also collected and an aliquot (50 µl) was injected and analysed *via* HPLC.

2.7. Statistical analysis

All data presented was reported as mean ± standard deviation (SD). The mean, SD, linear regression analysis, %RSD and %CV of each sample in the validation method were processed using Microsoft® Excel® 2016 (Microsoft Corporation, Redmond, USA). In terms of pharmacokinetic profiles and parameters, a non-compartmental analysis of plasma samples was performed using PKSolver (add-in program of Microsoft Excel). Statistical analysis in this study was carried out using GraphPad Prism® version 8.3.0 (GraphPad Software Inc., San Diego, California), with the significance set at $p < 0.05$. In terms of VCN oral bioavailability, it was calculated using area under curve (AUC) for oral and IV administration using the following equation:

$$F = \frac{AUC_{oral}}{AUC_{IV}} \times 100\% \quad (1)$$

3. Results and Discussion

3.1. Chromatographic condition

The development of the bioanalytical method in this study was carried out using RP-HPLC with gradient method set at 215 nm. In terms of mobile phase, the pH of phosphate buffer used was 2.5. Based on its chemical structure, VCN is an amphoteric drug that has both carboxylic and amino groups. These functional groups are included into the ionisable functional groups. At pH values between 4 and 8, both carboxylic and amino groups are ionised, leading to shortened retention of this drug in the stationary phase. So, this range of pH was not appropriate to be selected as mobile-phase pH for such compounds. At higher pH ($pH > 8$), the amino

group becomes a neutral group but the carboxylic group is fully ionised. In contrast, at pH lower than 4, acidic group becomes neutral and the amino groups are fully protonated. Thus, pH of mobile phase that provides either the least or the highest ionisation of the samples is the most applicable for amphoteric compounds. In order to obtain a sharp peak, an amino modifier was added into the aqueous phase. One amine modifier which has been generally used for improving peak shape of analytes in RP-HPLC is TEA. Amine groups of VCN are positively charged at $\text{pH} < 8$ and the secondary interaction of amines with silanol groups may occur. The addition of TEA into the aqueous phase may, therefore, hinder the secondary interaction between these ionised amine groups and free silanol groups of stationary phases, thus maintaining the peak shape of amino groups-containing substances which are analysed using low pH mobile phase (acidic). For organic solvent, the mixture of methanol and acetonitrile was used. The type and content of organic modifier can interfere with the degree of ionisation of ionisable compounds. As mentioned previously, VCN has some ionisable functional groups, so the retention mechanism of this drug is dependent on both pH and organic modifier. LC analysis under gradient method for quantifying VCN in biological samples has been also reported in some previous studies [9,12,21,23].

3.2. Selection of sample preparation and drug extraction

Four methods were evaluated in the selection of plasma sample preparation. The extraction recovery for each method is illustrated in Figure 1 below. Results revealed that Method A showed recovery less than 10%. There may have been interactions between the VCN amino acid residues and plasma proteins resulting in a higher molecular weight product that could not pass through the filter membrane (3 kD cut-offs). Evaporation of the supernatant (Method D) was also shown to lead to a reduction in extraction efficiency. Evaporation may dehydrate the peptide constituent of VCN leading to drug loss. Additionally, dehydration of the liquid component, of plasma water and organic solvent, may destabilise the peptide structure and decrease the half-life of the peptide [24]. On the other hand, Method C (protein precipitation with the additional of water) which was previously reported by Shi *et al.* [22], gave the second highest extraction recovery when compared to other methods tested in this study. The highest extraction recovery was achieved using Method B, where VCN was extracted by using a simple protein precipitation method with methanol only. Therefore, Method B was selected for further studies because it was simple, quick and gave the highest recovery of drug.

Figure 1. Bar graphs showing the drug recovery percentage of each method when extracting VCN from plasma (means $\pm$ SD, n = 3).

3.3. Method validation

3.3.1. Selectivity

Based on the results presented in Figure 2, the extraction method was selective for analysing VCN in different biological matrices. No interferences were observed at the VCN retention time (approximately 8.20 min) in any of the blank matrices injected.

3.3.2. Linearity of calibration curve

In this study, calibration curve standards in different matrices were prepared using 7 drug concentrations and one blank per day for three consecutive days. Table 3 summarises the concentration ranges, slopes, y-intercept and correlation coefficient (R^2) values of each matrix. All concentrations in each matrix tested showed %CV < 8% and %diff not more than $\pm 15\%$, and %diff for LLOQ not more than $\pm 20\%$ for each replicate on three different days. Furthermore, the R^2 value obtained in all matrices was close to 1, which indicated the linearity of the concentration in the test system.

3.3.3. Lower limit of quantification (LLOQ)

The LLOQ values of VCN in plasma, skin and lymph node were 0.1 $\mu\text{g/ml}$, 0.05 $\mu\text{g/ml}$ and 0.05 $\mu\text{g/ml}$, respectively. The LLOQ determined for the plasma matrix in this study was lower than LLOQ from a range of previously published works, which reported LLOQs of 5 $\mu\text{g/ml}$ [13], 0.5 $\mu\text{g/ml}$ [25] and 0.25 $\mu\text{g/ml}$ [14], respectively. A method HPLC-UV method for quantifying VCN in the skin samples, was previously reported by Farin *et al.* [25], however, the LLOQ obtained (0.5 $\mu\text{g/ml}$) was also higher than LLOQs obtained in this study. Moreover, the LLOQ obtained for skin and lymph node samples were also low when compared to the

LLOQs mentioned above. These results revealed that the method was sensitive to analyse VCN in different biological matrices.

Figure 2. Representative HPLC chromatograms of blank: (1.a) plasma, (2.a) skin and (3.a) lymph node. Chromatograms of plasma, skin and lymph spiked with VCN standard solution (1.b) 50 $\mu\text{g/ml}$, (2.b) 1 $\mu\text{g/ml}$ and (3.b) 1 $\mu\text{g/ml}$, respectively.

Table 3. Linear regression of VCN standard solution in different matrices.

Matrix	Concentration range ($\mu\text{g/ml}$)	Slope	y-intercept	R ²
Plasma	0.1-50	51.85	-0.57	0.9998
Skin	0.05-50	109.45	-0.23	0.9999
Lymph	0.05-50	109.36	-0.23	0.9995

3.3.4. *Carry over*

For carry over assessment, no peaks appeared at the VCN retention time or any other peaks with values of more than 20% LLOQ area, in all matrices after the injection of ULOQ solutions. Thus, it was concluded that there was no carry over observed in this method.

3.3.5. *Accuracy and precision*

Accuracy and precision of VCN measurement using the optimised method in plasma, skin and lymph matrices are summarised in Table 4. These results showed that the method is accurate and precise based on EMEA 2011 and FDA 2018 guidelines, where the %diff and %CV within- and between-runs of the measurement should not exceed 15% for QCL, QCM and QCH and should not be more than 20% for LLOQ.

3.3.6. *Dilution integrity*

The dilution integrity accuracy of VCN measurements at a range of different concentrations in plasma, skin and lymph node matrices were 92-110%, 96-108% and 93-111%, respectively. All diluted samples exhibited acceptable accuracy which were not more than $\pm 15\%$ of the theoretical value in any case. The precision of the diluted samples (%CV) was in the ranges of 1.0-8.9%, all less than 15%. These results revealed that samples with concentrations higher than the ULOQ can be diluted and then accurately measured/quantified using this method.

3.3.7. *Extraction recovery*

The extraction recovery of VCN from all matrices is summarised in Table 5. The recovery obtained in this study was in the range of 85-115%. The FDA bioanalytical guidelines indicate the importance of efficiency, consistency and reproducibility of recovery, conceding that the recovery of analyte in bioanalytical method does not need to be 100%.

Table 4. Within-run (n=5) and between-run (n=15) accuracy and precision of VCN in plasma, skin and lymph node matrices.

Sample	Concentration (µg/ml)	Within-run						Between Run	
		Day 1		Day 2		Day 3		Accuracy (%diff)	Precision (%CV)
		Accuracy (%diff)	Precision (%CV)	Accuracy (%diff)	Precision (%CV)	Accuracy (%diff)	Precision (%CV)		
Plasma	0.1 (LLOQ)	-2.7	9.5	0.8	7.0	9.1	4.6	2.4	5.9
	0.3 (QCL)	-2.5	8.0	2.0	5.8	7.9	2.8	2.5	5.1
	25 (QCM)	7.5	2.4	-3.6	2.0	7.9	1.8	1.4	5.5
	40 (QCH)	7.8	0.9	-1.5	2.9	0.2	3.3	2.2	4.9
Skin	0.05 (LLOQ)	6.8	8.1	5.0	6.5	5.4	5.7	5.7	0.9
	0.15 (QCL)	0.2	5.0	-0.9	5.0	2.8	3.3	0.7	1.9
	25 (QCM)	2.2	1.2	3.0	2.7	2.8	2.0	2.9	0.6
	40 (QCH)	2.6	1.5	10.7	2.0	3.1	2.6	5.5	4.3
Lymph node	0.05 (LLOQ)	-3.5	8.4	8.3	5.4	10.4	4.4	5.1	7.1
	0.15 (QCL)	-2.1	3.0	1.5	2.9	1.7	3.9	0.4	2.1
	25 (QCM)	5.2	2.7	1.0	1.2	1.7	0.9	1.8	3.0
	40 (QCH)	7.4	2.9	8.9	2.4	-1.8	1.2	4.8	5.5

Table 5. Extraction recovery ranges of VCN from all matrices (n = 15)

Matrix	QC	Extraction Recovery Ranges (%)
Plasma	Low (0.3 µg/ml)	88 – 113
	Medium (25 µg/ml)	94 – 111
	High (40 µg/ml)	96 – 109
Skin	Low (0.15 µg/ml)	94 – 108
	Medium (25 µg/ml)	100 – 107
	High (40 µg/ml)	99 – 113
Lymph node	Low (0.15 µg/ml)	94 – 106
	Medium (25 µg/ml)	98 – 108
	High (40 µg/ml)	97 – 112

3.3.8. Stability study of VCN in different matrices

The stability study of VCN was carried out in ultrapure water (stock solution) and the three biological matrices at QCL and QCH concentrations. The data presented in Table 6 summarises the stability studies (short term, long term, freeze-thaw and autosampler) of VCN in all matrices. The short- and long-term stability results showed that VCN was stable for at least 30 days both in stock solution and in all biological matrices tested, based on the %diff of each measurement which did not exceed $\pm 15\%$ of the value obtained for day 0. In terms of freeze-thaw stability, VCN was still stable in plasma, skin and lymph node samples after the third cycle of freeze-thaw. Furthermore, VCN also remained stable based on the autosampler stability study over 24 h of storage in the HPLC sample chamber. The short- and long-term stability results showed that VCN was stable at least 30 days both in stock solution and all matrices tested based on the % drug recovered in each measurement (Table 7). They did not exceed $\pm 15\%$ of the values obtained on day-0.

Table 6. Stability of VCN at different QC concentrations in all matrices which showed by the ranges of drug recovered percentage obtained compared to the value at the initial conditions (n=3).

Matrix	Theoretical Concentration (µg/ml)	Drug Recovery Ranges (%)			
		Short term (24 h)	Long term (30 days)	Freeze-thaw (cycle-3)	Autosampler (24 h)
Plasma	0.3	107 – 110	97 – 101	104 – 107	98 – 105
	40	104 – 109	101 – 110	101 – 107	103 – 107
Skin	0.15	94 – 99	92 – 101	92 – 98	95 – 101
	40	94 – 102	94 – 104	98 – 103	98 – 105
Lymph node	0.15	91 – 98	90 – 98	91 – 104	92 – 98
	40	94 – 104	97 – 103	99 – 104	94 – 104

Table 7. Percentage of drug recovered from VCL stock solution in water after 24 h and 30 days storage (n = 3).

Theoretical Concentration ($\mu\text{g/ml}$)	Drug Recovery Ranges (%)	
	Short term (24 h)	Long term (30 days)
0.15	90 – 99	96 – 102
0.30	95 – 102	93 – 102
40	96 – 97	99 – 101

3.3.9. Animal studies

The validated bioanalytical method, with associated sample preparation parameters, was employed to determine the pharmacokinetics of VCN in rat plasma and its biodistribution in the skin and lymph nodes of treated animals. In terms of analyte extraction from skin and lymph node samples, VCN was only found in the first supernatant, equating to 100% recovery (98-101%). This demonstrated that VCN was completely extracted from the samples using only one extraction step. Thus, this one step extraction procedure was employed for extracting VCN from the skin and lymph node samples in the animal study.

The data in Figure 3 illustrates the mean plasma concentrations of VCN after administration of IV bolus injection or oral solution of drug. Following the construction of the mean plasma concentration and time profiles, the pharmacokinetics parameters were processed using the PKSolver, as previously explained, using a non-compartmental pharmacokinetic analysis. Then the following parameters were calculated: half-life ($t_{1/2}$), time to reach maximum concentration (t_{max}), maximum concentration (C_{max}), area under curve from 0 h to 48 h (AUC_{0-48}), area under curve from 0 h to infinity ($\text{AUC}_{0-\text{inf}}$) and mean residence time (MRT). The pharmacokinetic parameter summary is provided in Table 8.

Figure 3. Plasma concentration of VCN after administration of (a) IV injection and (b) oral solution quantified with the validated method. (Means $\pm$ SD, n=5).

Table 8. Summary of pharmacokinetic parameters after the administration of IV bolus injection or oral solution of VCN to rats (Mean $\pm$ SD, n=5).

Parameter	Intravenous Injection	Oral solution
$t_{1/2}$ (h)	3.12 ± 0.10	4.40 ± 0.28
$t_{\max}$ (h)	1.00 ± 0.00	2.00 ± 0.00
$C_{\max}$ ($\mu\text{g/ml}$)	50.34 ± 14.50	3.37 ± 1.84
AUC_{0-t} ($\mu\text{g.h/ml}$)	270.80 ± 97.18	30.50 ± 9.18
$\text{AUC}_{0-\text{inf}}$ ($\mu\text{g.h/ml}$)	270.80 ± 97.18	30.50 ± 9.18
MRT (h)	3.45 ± 0.41	9.43 ± 4.69

The dose of VCN administered in this study was 30 mg/kg for both IV injection or oral solution. This dose was the recommended dose for IV injection and infusion to reach rapid serum concentrations [26]. Following IV bolus injection, VCN was completely eliminated from rat plasma after 24 h (Figure 3). An administered dose of 30 mg/kg VCN led to $C_{\max}$ of $50.34 \pm 14.50 \mu\text{g/ml}$ at 1 h, with a decrease to approximately half that concentration at approximately 3 h (Table 8). In this study, AUC_{0-t} and $\text{AUC}_{0-\text{inf}}$ were the same because VCN was completely eliminated from the rat circulation at 48 h with MRT 3.45 ± 0.41 h. The results obtained in this present work are in line with that reported by Okochi and Nakano [27] who investigated the administration of VCN IV injection at a dose of 15 mg/kg to male Wistar rats. They reported that a VCN concentration equating to $25 \mu\text{g/ml}$ was reached 1 h post-administration and decreased to $\sim 13 \mu\text{g/ml}$ at approximately 3-4 h. Moreover, the $\text{AUC}_{0-\text{inf}}$ reported here was twice as high as the value obtained by Okochi and Nakano ($143.9 \pm 14.4 \mu\text{g.h/ml}$). There is a linear relationship between $C_{\max}$ and $\text{AUC}_{0-\text{inf}}$ of VCN with the dose administered. A faster $t_{1/2}$ was also reported by Blaskovich *et al.* who carried out a study with mice and IV injection of VCN at the dose of 2 mg/kg [28]. They reported that the $t_{1/2}$ was 0.64 h and VCN concentration at 1 h was $0.76 \pm 0.14 \mu\text{g/ml}$. Regardless of the dose administered, the drug clearance in smaller animals, such as mice, is often quicker than in rats or humans [15]. This faster clearance may be associated with the difference of drug metabolism behaviour in the smaller animals.

In terms of oral VCN administration, $C_{\max}$ was achieved at 2 h, with a concentration of $3.37 \pm 1.84 \mu\text{g/ml}$. Moreover, the VCN half-life in this study was approximately 4.5 h with MRT in the body circulation up to 9.43 ± 4.69 h. The $\text{AUC}_{0-\text{inf}}$ of VCN after 48 h-study was $30.50 \pm 9.18 \mu\text{g.h/ml}$. Based on these results, the absolute bioavailability of oral VCN can be calculated using equation 1. When compared to IV injection, oral bioavailability of VCN was 11.26%. Oral bioavailability data for VCN is quite limited, due to its low absorption in the

gastrointestinal tract. An investigations of VCN oral bioavailability were reported by Shibata *et al.* [29] and Yamazaki *et al.* [30]. The high oral bioavailability of VCN (54.5%) reported by Yamazaki *et al.*, was a result of the colitis condition in colitis model rats. Shibata *et al.*, however, who administered 20 mg/kg of VCN to male Wistar rats, found that the bioavailability of VCN was 0.069% over a 6 h study. Their results indicated that VCN was not completely eliminated from rat plasma at 6 h which might affect the AUCs, both of AUC_{0-t} and AUC_{0-inf}, resulting in the difference of F value obtained. Furthermore, the VCN dose and the gender of the rats used were different from the current study and, as previously mentioned, some factors relating to the *in vivo* model used can affect the reported pharmacokinetic parameters [15]. The biodistribution of VCN in the rat skin and lymph nodes after 24 h and 48 h of study are presented in Table 9.

Table 9. VCN concentrations in the skin and lymph nodes after administration of VCN *via* oral or IV routes. (mean $\pm$ SD, n=3)

Sample	Administration route	Concentration ($\mu\text{g/g}$)	
		24 h	48 h
Skin	Oral	0.27 ± 0.11	0.13 ± 0.06
	IV	0.07 ± 0.04	Not found
Lymph node	Oral	0.80 ± 0.24	0.11 ± 0.05
	IV	0.06 ± 0.01	0.05 ± 0.01

As can be seen in Table 9, the VCN was found in both skin and lymph node samples at 24 h, with concentrations of less than 1 $\mu\text{g/g}$. However, the IV route of administration resulted in lower VCN concentrations than the oral route. This may be due to the fast clearance of VCN in the plasma after the IV bolus administration and VCN excretion *via* glomerular filtration in the kidney. To elaborate on this, the MRT value in Table 8 suggests that VCN was retained for longer in the rat body when the drug was administered orally, specifically, approximately three times longer than when administered *via* the IV route. It is worth noting that supplementary to the organs investigated in the current work, VCN has also been reported to be distributed to a range of other organs including CSF [4], heart [5], lungs [6] and skin ISF [7]. VCN concentration in the skin has been previously reported by Farin *et al.* [25], but the samples (chest and leg skins) were obtained from patients undergoing cardiopulmonary bypass. Therefore, to conclude, the results of the present study help to complete the VCN biodistribution data set, with specific reference to skin and lymph node data sets.

4. Conclusions

The work presented here reports a simple and robust sample preparation and HPLC-UV method for quantification of VCN in a range of biological matrices. This study has provided a reliable, selective and sensitive bioanalytical method for VCN which was validated based on EMEA and FDA bioanalytical guidelines. The validated HPLC-UV method was successfully employed in a pharmacokinetic and biodistribution study of VCN in rats after oral and IV bolus administration. In addition, the method gave lower LLOQ in plasma (0.1 µg/ml) and skin/lymph node samples (0.05 µg/ml), when compared to previously reported HPLC-UV methods. The use of this new bioanalytical method, therefore, serves to enhance understanding of quantification and determination of the pharmacokinetics and biodistribution of VCN in future clinical and therapeutic drug monitoring applications.

Acknowledgements

This work was supported by The Indonesian Endowment Fund for Education (Lembaga Pengelola Dana Pendidikan / LPDP) scholarship. The authors also thank to Mr Gareth Geddis from the Biological Services Unit (BSU), Queen's University Belfast for his help with *in vivo* experimentation.

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