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# Highly sensitive quantification of vancomycin in plasma samples using liquid chromatography–tandem mass spectrometry and oral bioavailability in rats

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**Abstract**

We developed a highly sensitive liquid chromatography–tandem mass spectrometry assay (LC–MS–MS) for a glycopeptide antibacterial drug, vancomycin (VCM), in rat plasma. After precipitating 100 μl of plasma with 300 μl of 10% trifluoroacetic acid–methanol (2:1, v/v), the supernatant was diluted with 300 μl of distilled water and was passed through a filter. LC–MS–MS equipped with electrospray ionization in the positive ion mode used a pair of ions at 725/144 *m/z* for VCM in the multiple reaction-monitoring mode with a sample injection volume of 20 μl. The calibration curve had a linear range from 0.01 to 20 μg/ml when linear least square regression was applied to the concentration versus peak area plot. The drug in the sample was detected within 5 min. Precision, accuracy and limit of quantitation indicated that this method was suitable for the quantitative determination of VCM in rat plasma. Using this method, we defined for the first time that the oral bioavailability of VCM in rats was 0.069%. This method can be applied to basic pharmacokinetic and pharmaceutical studies in rats.


*Keywords:* Vancomycin

## 1. Introduction

The glycopeptide antibacterial drug, vancomycin (VCM, Fig. 1) is widely used in hospitals for the therapy of infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) [1]. Moreover, as a prophylaxis, VCM is used in patients with bone marrow transplantation to sterilize the intestinal bacteria that cause fatal infectious disease under immunosuppression [2]. Since the dose-limiting factors of VCM therapy are side effects such as nephrotoxicity [3] or ototoxicity [4], prevention of these toxicities without decreasing its efficacy is strongly desired. On the other hand, in the field of pharmaceutical experiments, VCM is widely used as a model drug to improve intestinal permeability because of its extreme hydrophilic property [5]. To date, however, there has been no accurate information on the oral bioavailability of VCM. Therefore, the development of a highly-sensitive method for the determination of plasma concentrations of VCM is important to facilitate satisfactory clinical

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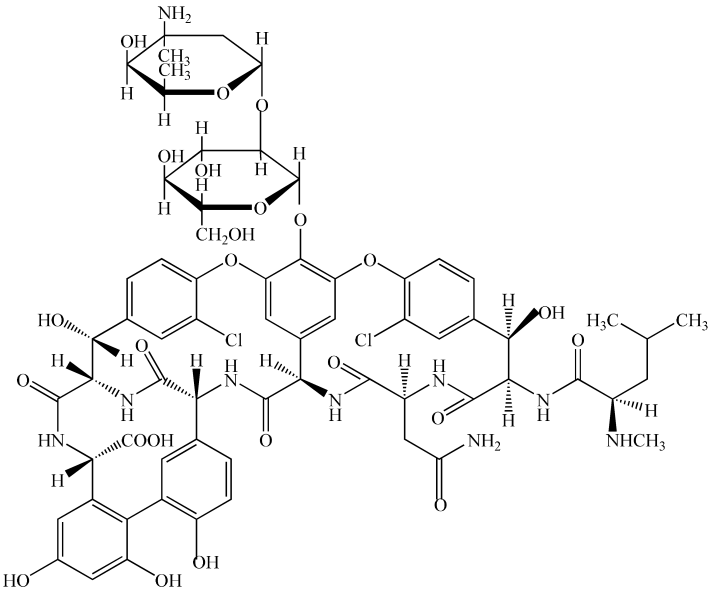

Fig. 1. Chemical structure of VCM.

outcomes, and to detect the VCM pharmacokinetic profiles to apply for the development of a new oral formulation of VCM.

This paper describes a new highly-sensitive and rapid method for the quantitation of VCM concentrations in rat plasma by LC–MS–MS and the application of this method to a pharmacokinetic study in rats.

## 2. Experimental

### *2.1. Materials and reagents*

Vancomycin hydrochloride and trifluoroacetic acid (TFA) were purchased from Wako (Osaka, Japan). The solvents for the mobile phase were of HPLC grade. All other reagents used were of analytical grade and were used without further purification.

### *2.2. Preparation of standards*

The standard stock solutions of VCM were prepared by dissolving in deionized water at various concentrations, and were stored at 4 °C in the dark. Using these standard stock solutions, rat plasma samples for the calibration curve were prepared by adding known amounts of VCM to drug-free plasma in a volume ratio of 1:100. The test solutions of VCM for intravenous and oral administration were prepared by dissolving 250 mg of VCM in 10 ml of 0.9% saline and 200 mg in 10 ml of deionized water, respectively. The doses of VCM for intravenous (i.v.) and oral (p.o.) study were 5.0 and 20.0 mg/kg, respectively.

### *2.3. Calibration curve, precision, accuracy and limit of quantitation*

The VCM concentration range for the calibration curve in plasma was 0.01–20 μg/ml. The inter- and intra-day precisions of this method were evaluated using plasma samples at eight concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5, 10 and 20 μg/ml prepared for the calibration samples. For inter-assay precision, eight samples of each concentration, a total of 56, were assayed on 7 different days. For intra-assay precision, a total of 56 samples of each concentration were assayed within a day. Means and standard deviations were obtained for the peak areas of each sample, and relative standard deviations (RSDs) for the eight different samples were determined. The

accuracy was evaluated using control plasma samples separately prepared at three concentrations of 0.01, 1.0 and 20.0 μg/ml using a set of calibration curves within a day ($n=5$), and was calculated by dividing the calculated concentration by the nominal concentration. The limit of quantitation was defined as the lowest concentration in rat plasma sample such that the deviation between the value of the concentration calculated by the calibration curve and the nominal concentration was less than 20% [6].

### 2.4. In vivo pharmacokinetic studies using rats

Under light anesthesia with ether, male Wistar rats, weighing 300±20 g, fasted overnight with free access to water for at least 12 h received an intravenous (i.v., 5.0 mg/kg) or oral (p.o., 20.0 mg/kg) dose of VCM without constraint. All experiments were performed in accordance with the Guidelines for Animal Experimentation in Kyoto Pharmaceutical University. At 30 min before the administration of drug, 0.25 ml of blank blood samples were withdrawn from the external left jugular vein. Thereafter, 0.25-ml blood sample were collected into heparinized centrifuging tubes at 30, 60, 90, 120, 180, 240, 300 and 360 min after p.o. administration, and 10, 20, 30, 45, 60, 90, 120, 150, 180, 240 and 360 min after i.v. administration. To maintain the blood circulation, rats were given an equivalent volume of 0.9% saline at every sampling time. The plasma samples were obtained by centrifuging the blood samples at 9000 *g* for 10 min and were immediately frozen at −80 °C until analysis.

### 2.5. Extraction procedure

Exactly 300 μl of 10% TFA–methanol (2:1, v/v) were added to an aliquot of 100 μl plasma sample in a 1.5-ml microcentrifuge tube, and the mixture was vortexed for 30 s and then centrifuged at 12 000 *g* for 10 min. Then the supernatant was decanted to a clean test tube, and diluted with 300 μl of distilled water. After the mixture was passed through a Millex-LG filter (0.2 μm, Millipore, MA, USA) to remove particulates, the filtrate was placed into a clean HPLC vial and 20 μl of the sample were injected into the LC–MS–MS system.

### 2.6. Instrumentation and chromatographic condition of LC–MS–MS

The LC–MS–MS system consisted of a PE-Sciex API 365 triple quadrupole mass spectrometer equipped with turbo ion spray sample inlet as an interface for electrospray ionization (ESI) and Analyst workstation (Perkin-Elmer-Sciex, Ontario, Canada), an LC-10AD micropump (Shimadzu, Kyoto Japan), an AS8020 automatic sample injector (Toso, Tokyo, Japan) and a six-way switching valve (Kyoto Chromato, Kyoto Japan). The mobile phase of distilled water–acetonitrile (9:1, v/v) containing 0.1% acetic acid was degassed and pumped through an Inertsil ODS-3 column (100 mm×2.1 mm I.D., GL Science Tokyo, Japan) at a flow-rate of 0.2 ml/min, and the column temperature was maintained at 40 °C. In order to remove the front impurities of the samples eluted from the outlet of the column after the injection, the waste drain was released for measurement start post-40 s using a six-way switching valve. The ionization was via the turbo ion spray inlet in the positive ion mode. The flow-rates of nebulizer gas, curtain gas and collision gas were set at 8, 8 and 2 l/min, respectively, and the ion spray voltage and temperature were set at 4500 V and 425 °C, respectively. The declustering potential, the focusing potential, the entrance potential, the collusion energy and the collision cell exit potential were set at 20, 200, −10, 30 and 6 V, respectively.

### 2.7. Data analysis

Pharmacokinetic parameters for the oral and intravenous concentration vs. time data of VCM were calculated by a noncompartmental pharmacokinetic analysis method using a computer program, WinHARMONY [7]. The area under the concentration vs. time curve (AUC) was calculated by a trapezoidal rule, and was extrapolated to infinity. The elimination half-life ($t_{1/2}$) at the terminal phase, the total body clearance ($Cl_{tot}$) and the oral clearance ($Cl_{p.o.}$) were determined by following equations $t_{1/2} = \ln 2/\beta$, $Cl_{tot} = Dose_{i.v.}/AUC_{i.v.}$, $Cl_{p.o.} = Dose_{p.o.}/AUC_{p.o.}$, respectively, where $\beta$ represents the elimination rate constant at the terminal phase. The absolute bioavailability ($F$) of VCM was calcu-

lated from the AUCs after p.o. and i.v. administrations using the following equation:

$$F\ (\%) = (\mathrm{AUC}_{\mathrm{p.o.}}/\mathrm{AUC}_{\mathrm{i.v.}}) \times (\mathrm{Dose}_{\mathrm{i.v.}}/\mathrm{Dose}_{\mathrm{p.o.}}) \times 100$$

## 3. Results and discussion

By introducing 10 μg/ml VCM diluted by the LC mobile phase via a syringe pump into the LC inlet, parent and product ions for VCM under ESI positive ion conditions were determined. Full scan mass spectra for the parent and product of VCM are shown in Fig. 2. The parent mass spectrum due to the molecular mass of VCM (1449.2) was not observed, instead, the parent mass spectrum of VCM was found at 725 *m*/*z* (Fig. 2A). This result suggested that VCM changed into diprotonated mass $[M+2H^+]$ as the parent spectrum (Q1). This phenomenon may be due to the fact that VCM has multiple acidic and basic ionizable groups [8]. As the product mass spectrum, we selected main product fragment (Q3) at 144 *m*/*z* (Fig. 2B). Other fragmentations for the product mass spectrum were not likely to improve the sensitivity when the selected reaction monitoring was performed. Therefore, the Q1/Q3 of VCM for the selected reaction-monitoring mode was set at 725/144 *m*/*z*.

Fig. 3 shows typical mass chromatograms of a standard VCM (Fig. 3A) and plasma samples after i.v. or p.o. administrations of VCM (Fig. 3B and C). The peaks of mass chromatograms corresponding to VCM at high and low levels were clearly detected by the selected reaction-monitoring mode. No interfering peaks resulting from the extraction procedure, which were associated with the peaks of VCM, could be detected on the chromatograms. Retention time for VCM was 4.0 min, and all separations were completed within 5 min. Ion suppression effects due to co-eluting matrix components of plasma was below 10%.

Calibration curves of VCM in the concentration range of 0.01–20 μg/ml passed through the origin with correlation coefficients of 0.999 or higher. However, at plasma concentration above 20 μg/ml, the regression line showed a nonlinear shape due to the saturation of ionization. Therefore, samples with a concentration >20 μg/ml should be diluted with drug-free rat plasma. Intra- and inter-day accuracies for VCM determination in plasma were evaluated at three different concentrations (Table 1). The intra-day RSD of peak area of VCM at known eight concentrations (0.01–20 μg/ml) ranged from 2.0 to 6.9%, while the inter-day RSD ranged from 2.9 to

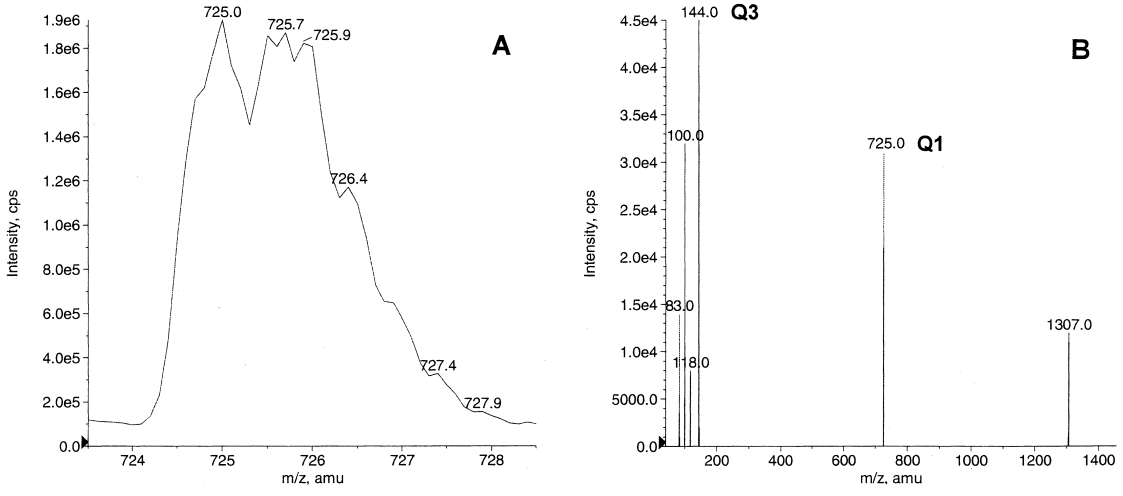


Fig. 2. LC–MS–MS spectra for the parent and product ions. (A) The optimization for parent spectrum by a multiple-channel acquisition scans. (B) Final parent (Q1) and product (Q3) spectra by a multiple-channel acquisition scans. The intensities of spectra are expressed in units of counts per second (cps).

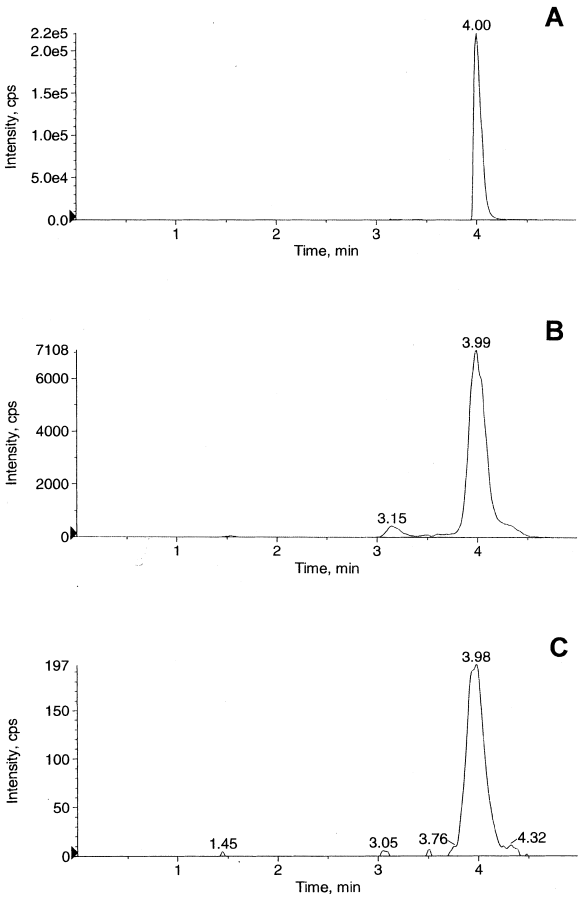


Fig. 3. Typical mass chromatograms of VCM for extracted rat plasma. (A) Standard VCM (400 ng). (B) Plasma sample at 4 h after i.v. administration (calculated concentration was 8.21 μg/ml). (C) Plasma sample at 4 h after p.o. administration (calculated concentration was 0.016 μg/ml). The intensities of spectra were expressed as a unit of counts per second (cps).

10.3%. Using the control plasma, the percentage of accuracy was obtained for the calculated drug concentrations over all 5 days at three different levels (0.01, 1.0 and 20.0 μg/ml, Table 2). The percentage accuracy in VCM control samples at 0.01, 1.0 and 20.0 μg/ml were 113.3±3.7, 108.3±2.7 and 99.0±0.7%, respectively. The limit of quantitation for VCM in plasma was 0.007 μg/ml, with a signal-to-noise ratio for VCM above 6. Although we did not use an internal standard in this study, periodic maintenance of the LC–MS–MS system was not necessary until more than 500 samples were analysed. Within this limit, marked signal suppression did not occur because of sample clean-up system

Table 2
Results of accuracy for VCM assay using control plasma

| VCM concentration (μg/ml) | Peak area (cps) | Calculated concentration (μg/ml) | Accuracy (%) |
|---|---|---|---|
| 0.01 | 145 | 0.011 | 107.980 |
| | 164 | 0.012 | 117.320 |
| | 151 | 0.012 | 115.860 |
| | 170 | 0.011 | 113.710 |
| | 158 | 0.011 | 111.490 |
| 1.0 | 9265 | 1.050 | 104.994 |
| | 9404 | 1.076 | 107.632 |
| | 9507 | 1.083 | 108.310 |
| | 10 443 | 1.080 | 107.954 |
| | 9893 | 1.126 | 112.585 |
| 20.0 | 155 402 | 19.741 | 98.707 |
| | 153 397 | 19.646 | 98.229 |
| | 154 630 | 19.687 | 98.437 |
| | 162 227 | 19.871 | 99.353 |
| | 175 908 | 20.012 | 100.060 |

Table 1
Results of intra- and inter-day precisions for VCM peak response data assay

| | | VCM concentration in plasma (μg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0.01 | 0.05 | 0.1 | 0.5 | 1 | 5 | 10 | 20 |
| Intra-day | Mean | 166 | 373 | 723 | 4395 | 9588 | 48 795 | 88 048 | 171 515 |
| $n=7$ | SD | 11 | 22 | 41 | 144 | 430 | 1609 | 1750 | 3481 |
| | RSD (%) | 6.9 | 5.9 | 5.7 | 3.3 | 4.5 | 3.3 | 2.0 | 2.0 |
| Inter-day | Mean | 171 | 478 | 946 | 4622 | 10 572 | 46 925 | 89 061 | 169 714 |
| $n=7$ | SD | 18 | 32 | 63 | 173 | 613 | 1337 | 3840 | 6219 |
| | RSD (%) | 10.3 | 6.6 | 6.7 | 3.8 | 5.8 | 2.9 | 4.3 | 3.7 |

Data represent the peak area (cps).

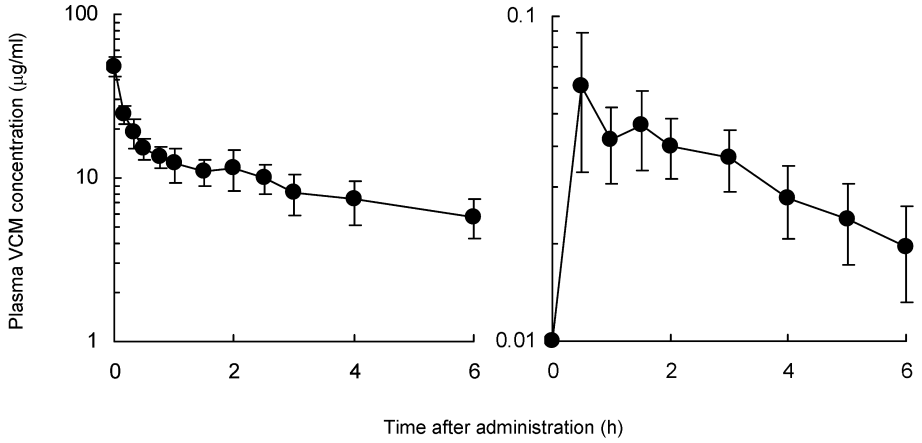


Fig. 4. Plasma concentration–time profiles of VCM after i.v. (A) and p.o. (B) administrations to rats. Doses of VCM for i.v. and p.o. administration were 5.0 and 20.0 mg/kg, respectively.

using a six-way switching valve. At the periodic maintenance, just by removing soot on the inlet of the interface components, such as the curtain plate, orifice plate, skimmer plate and skimmer, good intra- and inter-day precision could be obtained.

Fig. 4 shows application of the LC–MS–MS method developed here to in vivo pharmacokinetic studies in rats. The pharmacokinetic parameters of VCM after i.v. or p.o. administrations are listed in Table 3. After i.v. administration (5 mg/kg), VCM in plasma rapidly distributed to tissue compartments within 30 min. At 1 h after injection, mean plasma VCM levels decreased to about one fifth as compared to the initial plasma VCM levels (Fig. 4a). After p.o. administration (20 mg/kg), the time to reach the peak concentration and the maximal plasma concentration were found to be 0.5 h and 0.061 μg/ml, respectively (Fig. 4b). At the elimination phase, plasma VCM levels decreased in accordance with the first-order kinetics. The AUCs of VCM after i.v. and p.o. administrations were 105.62±69.54 and 0.29±0.18 μg h/ml, respectively. The total body clearance, $Cl_{tot}$, and oral clearance of VCM, $Cl_{po}$ were 61.42±30.07 ml/h/kg and 85.68±36.94 l/h/

Table 3
Pharmacokinetic parameters of VCM after p.o and i.v administration

| | | AUC μg h/ml | $t_{1/2}$ (h) | $Cl_{tot}$ (ml/h/kg) | $Cl_{po}$ (l/h/kg) | $F$ (%) |
|---|---|---|---|---|---|---|
| I.v. | Rat 1 | 61.84 | 2.6 | 80.85 | | |
| | Rat 2 | 55.38 | 3.7 | 90.28 | | |
| | Rat 3 | 99.49 | 3.9 | 50.26 | | |
| | Rat 4 | 205.77 | 7.0 | 24.30 | | |
| | Mean | 105.62 | 4.3 | 61.42 | | |
| | SD | 69.54 | 1.9 | 30.07 | | |
| P.o. | Rat 5 | 0.23 | 2.1 | | 87.26 | |
| | Rat 6 | 0.21 | 4.0 | | 96.26 | |
| | Rat 7 | 0.16 | 1.9 | | 123.69 | |
| | Rat 8 | 0.57 | 4.2 | | 35.37 | |
| | Mean | 0.29 | 3.0 | | 85.68 | |
| | S.D | 0.18 | 1.2 | | 36.94 | |
| | | | | | | 0.069 |

$F$, absolute bioavailability.

kg, respectively. The absolute bioavailability of VCM, *F*, was found to be 0.069% (Table 3). It has been reported that the value of *F* for VCM was below 4% [9] in rats. The *F* value we estimated using the LC–MS–MS system developed here is far below the previously-reported value.

In clinical practice, fluorescence polarization immunoassay (FPIA) is usually used for therapeutic drug monitoring of VCM [6,10]. However, it has been reported that FPIA results in overestimation of plasma VCM in patients with renal failure [6]. This overestimation is caused by crossreactivity of the degradation product, CDP-1, in the FPIA method [6]. Therefore, a more accurate measurement is required for patients with renal failure, because overestimation would lead to insufficient VCM therapy. On the other hand, a large amount of VCM is used in patients with bone marrow transplantation as an intestinal disinfectant [2]. Although it is believed that VCM is not entirely absorbed from the gastrointestinal tract, there is a possibility that VCM is transferred from the gastrointestinal tract into the blood circulation and may cause toxicities in patients who have an ulcerative injury in the gastrointestinal tract. Therefore, regular monitoring of VCM is advocated for these patients to avoid accumulative toxicities. Moreover, in the field of pharmaceutical experiments, VCM has often been used as a model drug to improve the intestinal permeability because of its extreme hydrophilic property [8,11]. Kajita et al. reported on use of a VCM formulation using water-in-oil-in-water multiple emulsion by unsaturated fatty acid in rats. However, the oral bioavailability of VCM in aqueous solution was not calculated because of the luck of sensitivity in measurements by FPIA [11].

Regarding analytical methods of VCM for basic and clinical studies, high-performance liquid chromatography (HPLC) equipped with ultraviolet detection [8,13], electrochemical detection [12] and FPIA [6,10,14] has been described. However, the lower limits of detection for these analytical methods were 0.25–2.0 μg/ml, and these detection limits were not adequate for determining the minimal inhibition concentration of VCM against methicillin-resistant *Staphylococcus aureus* (MRSA) and its oral bioavailability. More recently, an LC–MS–MS method for the quantitation of VCM has been developed [15]. The sample volume required and the detection limit of this method are 100 μl and 0.001 μg/ml, respectively [15]. Although the detection limit of this LC–MS–MS method is lower than that of our method and there is no extraction procedure, it requires a complicated procedure such as a column-switching technique to prepare sample specimens [15]. Our extraction procedure adopts a one-step extraction in a 1.5-ml microcentrifuge tube and saves time in preparing the plasma samples. Therefore, it is considered that the LC–MS–MS method for VCM we developed here will facilitate obtaining quality data for basic pharmacokinetic and pharmaceutical studies.

In conclusion, the present LC–MS–MS method provides a highly sensitive and reliable assay procedure for plasma VCM determination in rats. The sample volumes required are small, the extraction is rapid and simple, and the mass spectrometric detection system has the reliability and sensitivity to facilitate measurement of plasma VCM levels following oral or intravenous administration. This LC–MS–MS method will facilitate further basic pharmacokinetic or pharmaceutical studies of VCM.

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