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Ready for TDM: Simultaneous quantification of amikacin, vancomycin and creatinine in human plasma employing ultra-performance liquid chromatography-tandem mass spectrometry

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ABSTRACT

Background: Amikacin (AMI) and vancomycin (VAN) are antibiotics largely used in intensive care in the empiric treatment of severe infections by multi-resistant gram-negative and gram-positive bacteria. AMI and VAN are eliminated untransformed by glomerular filtration, showing depuration ratio highly correlated with creatinine (CRE) clearance. AMI, VAN and CRE are highly polar structures, presenting poor retention in reversed-phase liquid chromatography when using conventional stationary phases.

Objective: This study aimed to develop and validate a simple UPLC-MS/MS method for simultaneous determination of AMI, VAN, and CRE in human plasma for therapeutic drug monitoring.

Results: Samples were prepared by protein precipitation, followed by dilution. Heptafluorobutyric acid (HFBA) was added to the mobile phase at low concentration (0.01%), and separation was performed in an ultra-performance reversed-phase column (particle diameter of 1.8 µm). These conditions allowed retention times of 0.92, 0.93, 2.12, 2.17 and 2.27 min for CRE, CRE-D3, AMI, KAN and VAN, respectively. The assay was linear from 0.5 to 100 mg L^{-1} for AMI and VAN and 5 to 100 mg L^{-1}. Precision, accuracy and stability assays were acceptable according to bioanalytical validation guidelines. Suitable results. Matrix effects were in the range of +10.5 to +11.6% for AMI, −4.3 to −4.5% for VAN, and − 1.7 to +0.7 for CRE.

Conclusion: The first assay for the simultaneous determination of AMI, VAN and CRE in plasma by liquid chromatography-tandem mass spectrometry was reported. This assay allows the obtention of the necessary analytical data for the clinical application of population pharmacokinetic methods for therapeutic drug monitoring of AMI and VAN.

1. Introduction

Amikacin (AMI) and vancomycin (VAN) are antibiotics largely used in the empiric treatment of sepsis [1–3]. AMI is active mainly against gram-negative bacteria and is frequently combined with VAN in the treatment of infections caused by gram-positive bacterial agents [4,5]. VAN is widely used for treating severe infections of multi-resistant gram-positive bacteria, like methicillin-resistant *Staphylococcus aureus* (MRSA) [6]. [6]. AMI and VAN are eliminated mainly untransformed by glomerular filtration, presenting body clearances highly correlated with creatinine (CRE) clearance, which is a common co-variate in population pharmacokinetic models used for dose individualization [5,7]. In this context, simultaneous measurement of AMI, VAN and CRE concentrations in the same assay can be advantageous for the therapeutic drug monitoring (TDM) of these antibiotics.

AMI presents concentration-dependent antibiotic activity, with target peak plasma concentrations (C_{max}) ten times higher than the minimal inhibitory concentration (MIC) of the pathogen for optimum effectivity [8]. AMI toxicity is reduced when trough concentrations are maintained below 7 mg L^{-1} [9]. Alternatively, another pharmacokinetic-pharmacodynamic (PK/PD) target for AMI treatment is a ratio of area under the curve to MIC ($AUC_{0\text{-}24h}$/MIC) of 75, which provides better detectability of interindividual and intraindividual alterations in clearance [10–13].

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VAN doses are usually individualized to achieved through concentrations in the range of 15–20 mg L^{-1} for severe infections [9]. More recently, a ratio of the $AUC_{0\text{-}24h}$ to the pathogen MIC ($AUC_{0\text{-}24h}$/MIC) higher than 400 have been proposed as more correlated to the therapeutic response, while avoiding excessive exposures to VAN [14,15]. VAN toxicity is increased when trough plasma levels are higher than 20 mg L^{-1} or $AUC_{0\text{-}24h}$/MIC is higher than 600 [16–18].

Routinely, immunoassays have been used to determinate AMI and VAN in biological matrices, even considering their limited sensitivity and precision [19–22]. Alternatively, liquid chromatography (LC) associated with different detectors can allow higher specificity and sensibility when compared to immunoassays [20,23]. Differently from VAN, which can be detected by optical LC detectors, AMI lacks a chromophore structure, requiring chemical derivatization when using UV or fluorescence detection [24–26]. Moreover, the high hydrosolubility of AMI prevents its retention in reversed-phase stationary phases, which limits the application of liquid chromatography for its measurement in the clinical setting. Liquid-chromatography-tandem mass spectrometry (LC-MS/MS) analysis of AMI has relied on the use of hydrophilic interaction chromatography (HILIC) stationary phases or in the presence of high concentrations of ion-pairing agents in the mobile phase, allowing retention in usual reversed-phase columns [24,27]. These approaches have limitations once HILIC stationary phases are known by its low robustness, and high concentrations of ion-pairing agents can be deleterious to chromatographic columns and mass detectors [22,26]. These unwanted effects can be reduced when the ion-pairing agent is present at very low concentrations [27,28]. Currently, there are few described assays for the determination of AMI in plasma by LC-MS/MS [29–33], and just two reports of the combined measurement of AMI and VAN [31,32]. Additionally, there is no described method for the simultaneous determination of AMI, VAN, and CRE in plasma using LC-MS/MS.

The aim of this study is to develop and validate a simple and sensitive ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) assay for the simultaneous determination of AMI, VAN and CRE vancomycin in human plasma for TDM, using a reversed-phase stationary phase associated to a low concentration of an ion-pairing agent.

2. Experimental

2.1. Reference standards, chemicals and blank plasma

Amikacin (AMI), kanamycin B (KAN) and vancomycin hydrochloride (VAN) were acquired from European Pharmacopeia (Strasbourg, France). Creatinine (CRE), formic acid and heptafluorobutyric acid (HFBA) were purchased from Sigma Aldrich (Saint Louis, USA). Deuterated creatinine (CRE-D3) was obtained from Toronto Research Chemicals (North York, Canada). Acetonitrile was supplied by Honeywell (New Jersey, USA). Blank plasma was from a healthy volunteer, with background CRE level of 8.1 mg L^{-1}. Ultra-pure deionized water was supplied by a Milli-Q RG water purifier from Millipore (Billerica, USA).

2.2. Reference standards solutions and mobile phases

AMI, VAN and CRE stock solutions were prepared by dissolution of respective powders in a mixture of water, methanol and formic acid (49.5:49.5:1, *v*/v/v) to obtain the concentration of 6.000 mg L^{-1}. Working standard solutions were prepared by dilution of the stock with the same solvent mixture. Working standard solutions had the concentrations of 10, 20, 50, 100, 200, 500, 1.000 and 2.000 mg L^{-1} for AMI and VAN, and 100, 150, 200, 300, 500, 1.000, 1.500 and 2.000 mg L^{-1} for CRE. Calibration and quality control plasma samples were prepared by diluting the working solutions with blank plasma (1:20, *v*/v). KAN and CRE-D3 stock solutions were prepared by dissolution of respective powders in the same solvent mixture used for AMI and VAN, producing solutions with the concentrations of 2.000 and 5.000 mg L^{-1}, respectively. Working internal standard solution contained both KAN and CRE-D3 at the concentration of 10 mg L^{-1}, in acetonitrile. Mobile phase A was purified water containing 0.1% formic acid and 0.01% HFBA, and mobile phase B was acetonitrile containing 0.1% formic acid and 0.01% HFBA.

2.3. Sample preparation

Fifty microliters of human plasma were transferred to polypropylene microtubes, added with 50 µL of the internal standard solution and vortex-mixed for 1 min. The resulting mixture was centrifuged at 14,500*g* for 5 min (4 °C). An aliquot of 70 µL from the supernatant was transferred to another polypropylene microtube and diluted with 210 µL of 0.1% formic acid in water, followed by another vortex mixing step of 1 min. After centrifugation for 5 min at 14,500*g* (4 °C), the supernatant layer was transferred to a vial and 1 µL was injected into the UPLC-MS/MS system.

2.4. Chromatographic and mass spectrometric conditions

Analyses were performed in an Acquity UPLC I-Class chromatography system coupled to a Xevo TQD triple quadrupole mass spectrometer, both supplied by Waters Technologies (Milford, USA). Separation was achieved on an Acquity HSS T3 (100 × 2.1 mm, p. d. 1.8 µm) column, also obtained from Waters, kept at 40 °C. Elution flow rate was 0.4 mL min^{-1}. The mobile phase gradient started at 90% mobile phase A, which was maintained for 0.5 min, followed by a linear gradient to 30% A in 3.5 min. Mobile phase composition returned to the initial conditions at 4.1 min, followed by 1.4 min of stabilization. Total run time was 5.5 min. Autosampler temperature was set to 10 °C. The Xevo TQD parameters were: ionization in ESI positive mode, capillary voltage of 1 kV, nitrogen was used as desolvation gas at flow of 1.100 L/h and temperature of 550 °C. The collision gas was argon. The optimized conditions of cone energy (CE), collision energy (ColE), MRM transitions, as well as retention times, are presented in Table 1.

2.5. Linearity

Calibration samples had concentrations at the range of 5 to 100 mg L^{-1} for CRE and 0.5 to 100 mg L^{-1} for AMI and VAN. Each batch of calibration and QC samples was processed using with the same blank plasma for the spikes, and resultant peak area ratios of CRE to CRE-D3 were subtracted from each calibrator and control ratios before CRE concentration calculations. Calibration curves were constructed relating the nominal concentrations of the calibrators with the peak area from VAN (external standardization) and peak area ratio of AMI to

Table 1
Mass-spectrometric acquisition parameters and chromatographic retention times of AMI, VAN and CRE in human plasma by UPLC-MS/MS.

Analyte	Cone energy (V)	Collision energy (V)	*MRM* transitions (*m*/*z*)[a]	Retention times (min)
AMI	40	33	586.3→163.1	2.12
		19	586.3→425.3	
VAN	30	14	725.2→144.2	2.27
		14	752.2→100.2	
KAN	30	25	484.2→163.1	2.17
		18	482.2→324.4	
CRE	35	14	144.1→44.1	0.92
		11	144.1→86.1	
CRE-D3	35	14	147.1→47.1	0.93
		11	147.1→89.1	

[a] Quantifier transitions were underlined. *MRM*: multiple reaction monitoring.

KAN and CRE to CRE-D3 (internal standardization). Heteroscedasticity analysis of data was performed with *F*-test at 95% confidence interval. Calibration models were tested at several weighting factors and evaluated using the cumulative percentage relative error (Σ%RE) and the coefficient of correlation (*r*) [34]. Additionally, linearity was considered as acceptable if back-calculated concentrations of the calibrators were within ± 15% of their nominal concentrations [35].

2.6. Selectivity

Selectivity was performed testing blank plasma samples collected from 6 different volunteers with the described procedure to evaluate the absence of interfering peaks at retention times and MRM transitions of AMI and VAN.

2.7. Precision and accuracy

Precision and accuracy were evaluated using QC samples at the concentrations of 1.5 mg L^{-1} (quality control at low concentration, QCL), 7.5 mg L^{-1} (quality control at medium concentration, QCM) and 75 mg L^{-1} (quality control at high concentration, QCH) for AMI and VAN. Concentrations of QC samples for CRE were 6 mg L^{-1} (QCL), 20 mg L^{-1} (QCM) and 80 mg L^{-1} (QCH). QC and blank samples were analyzed in triplicate in 5 different days, along with a calibration curve. The average CRE to CRE-D3 area ratio from the blanks was subtracted from the QC ratio, before calculation of the concentration. Intra-day and inter-day precision were evaluated based on ANOVA and expressed as coefficient of variation (CV%). Accuracy was calculated comparing the QC concentration calculated with the calibration curve to its nominal concentration and expressed as percentage. Accuracy of the assay was considered acceptable if values were in the range of 85 to 115%, and precision was acceptable if CV values were below 15% [36].

2.8. Lower limit of quantification

Precision and accuracy of an additional QC sample (quality control at the lowest limit of quantification, QCLLOQ), at the concentration of the lowest calibrator (0.5 mg L^{-1} for AMI and VCM and 5 mg L^{-1} for CRE), was also evaluated, as described above. This QC sample was tested in triplicate in 3 different analytical batches. The QCLLOQ was accepted as the limit of quantification if accuracy was in the range of 80 to 120% and precision was below 20% [36].

2.9. Autosampler stability

Stability of AMI, VAN, and CRE in extracts was assessed at the concentrations of QCH and QCL at autosampler conditions. These QC samples were extracted as described above and injected into the UPLC at time intervals of 1 h, over 12 h. Peak areas from VAN and peak area ratios of AMI and CRE obtained at the 1st injection was compared with those achieved at the end of series. Additionally, a linear regression between peak responses and injection time was obtained to evaluate trends to increases or decreases in detector signals. A decrease or an increase lower than 15% in peak areas of VAN and peak area ratios of AMI and CRE was considered as acceptable [36].

2.10. Freeze-thaw stability

QC samples at the concentrations of QCL and QCH were extracted in triplicate before and after each of 3 freeze-thaw cycles. For each freeze-thaw cycle, samples were kept frozen at −20 °C and thawed at room temperature before analysis. The concentration of QC samples was estimated with freshly prepared calibration curves, as described above. Deviations from the nominal concentration of the QC lower than 15% were indicative of analyte stability at the evaluated conditions [36].

2.11. Dilution integrity

In order to evaluate the possibility to quantify concentrations higher than the upper calibrator, dilution integrity was evaluated. Plasma control samples were prepared at the concentration of 375 mg L^{-1} for AMI, VAN, and CRE. These controls were analyzed in triplicate. and precipitate with acetonitrile containing internal standards at concentration 5 times higher than usual. Samples were extracted as previously described but using an internal standard solution 5 times more concentrated than usual. The extract was diluted 5 times with the initial mobile phase and quantified using a regular calibration curve, as previously described. The target concentration of the diluted extracts was 75 mg L^{-1}. Dilution integrity was considered acceptable if accuracy was in the range of 85 to 115% and CV% was below 15% [36].

2.12. Matrix effect and extraction yield

Matrix effect (ME) and extraction yield (EY) were determined after the analysis of three sets of samples (A, B, and C), with ME being evaluated by the post-extraction spike method [37]. The sample set A was composed of solutions of AMI, VCM, and CRE in water containing 0.1% formic acid at concentrations correspondent to an EY of 100%. The sample set B was composed of five independent blank plasma extracts. From these blank plasma samples, two were from medicated patients (using benzodiazepines, anticonvulsants, and antidepressants, but not AMI or VAN), two were from non-medicated volunteers and one was plasma from a non-medicated volunteer, filtrated with a 0.22 μm membrane [22]. These blank extracts were added post-extraction with AMI, VAN, and CRE in water containing 0.1% formic acid, at concentrations equivalent to an extraction yield of 100%. The sample set C was composed of QCL and QCH samples, prepared and analyzed as described above. Considering the endogenous presence of CRE, blank plasma samples were processed in triplicate and the resultant average peak area ratios of CRE to CRE-D3 were subtracted from those obtained in batches B and C. The evaluated response in each set of samples (A, B and C) was VAN peak area or AMI to KAN and CRE to CRE-D3 area ratios. ME was calculated as ME = [100-(B/A%)] and EY was obtained using the eq. EY = C/B%.

2.13. Assay application

The developed method was applied to 71 plasma samples obtained from 18 patients in antibiotic drug treatment from the intensive care unit of the Santa Maria University Hospital (Brazil). Plasma was separated by centrifugation after venous blood collection in EDTA-containing tubes. The study was approved by the institutional review board of Universidade Federal de Santa Maria and performed according to the Helsinki Declaration principles. All participants or their responsible persons provided informed consent for specimen collection and analysis.

3. Results and discussion

3.1. Method validation

AMI, CRE and VAN are highly polar compounds, which represents a challenge to obtain proper separation from the biological matrix, as well as adequate chromatographic separation from endogenous compounds. Additionally, the acidification of mobile phase, usually required to increase ionization of basic polar compounds before MS/MS analysis, also contributes to reduced retention of AMI, KAN and VAN in reversed-phase columns [24]. Considering clinical applications of CRE plasma levels as kidney function biomarker and a co-variate in several population pharmacokinetic models of AMI and VAN, this compound was also measured. LC-MS/MS assays for CRE quantification in different matrices were already reported [44–49]. Nevertheless, this assay

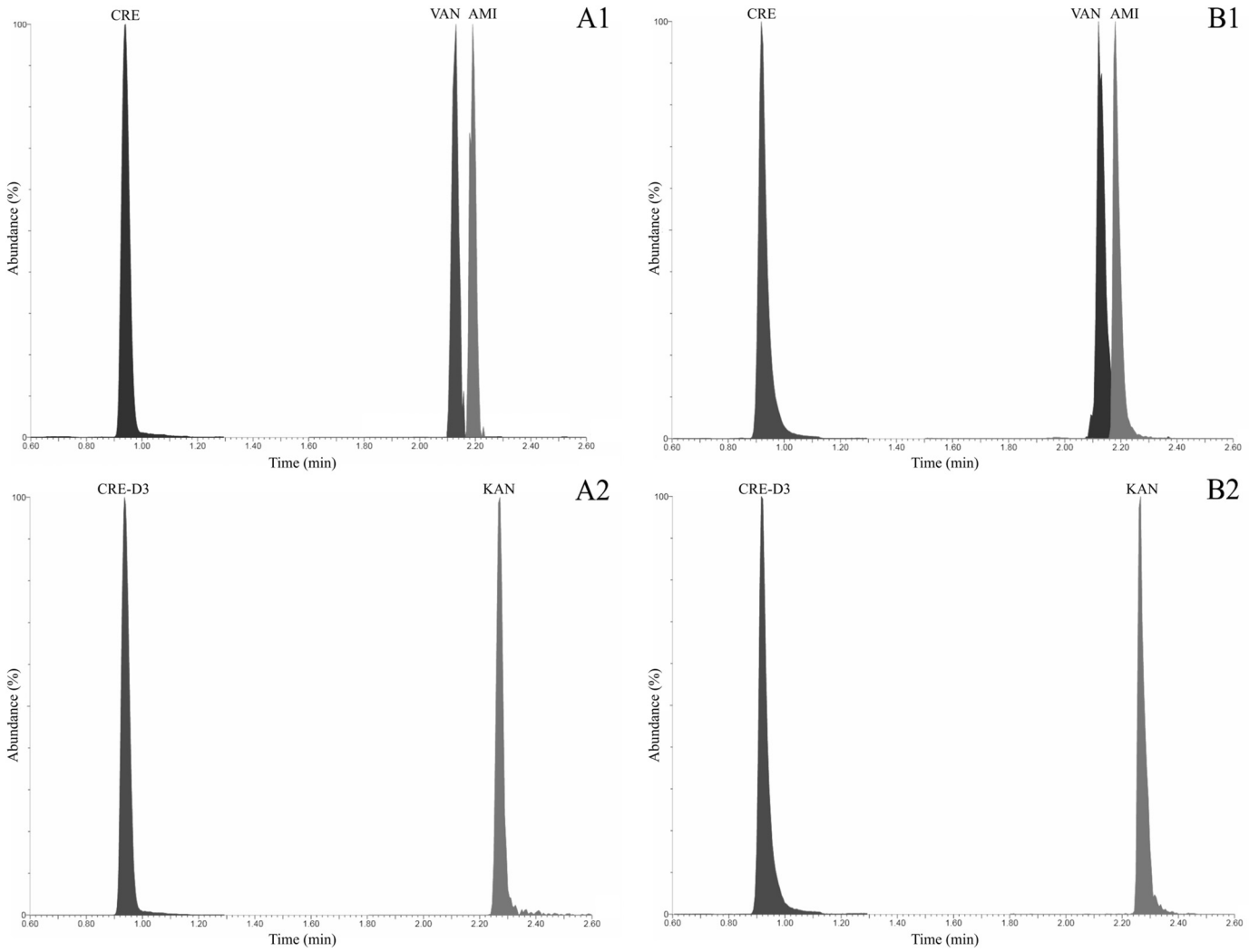

Fig. 1. Chromatograms obtained from plasma analyses (Quantification MRM transitions). A1 and A2: Quality control at low concentration (1.5 mg L^{-1} for AMI and VAN; 6 mg L^{-1} for CRE). B1 and B2: Patient sample containing AMI, VAN and CRE at the concentrations of 52.4, 4.4 and 11.3 mg L^{-1}, respectively.

is the first report of simultaneous determination of AMI, VAN and CRE, particularly fitted for TDM applications.

We used an ultra-performance (particle diameter of 1.8 µm) reversed-phase column. The chromatographic separation was achieved with a run time of 5.5 min, with retention times of 0.92, 0.93, 2.12, 2.17 and 2.27 min for CRE, CRE-D3, AMI, KAN and VAN, respectively (Fig. 1). The use of sub-2 µm particle chromatographic columns is considered a green technology once the combination of short analytical run time and low mobile phase flow rate leads to a small consumption of solvents [39,40]. In this particular case, the consumption of mobile phase was only 2.2 mL per injection. Previous LC-MS/MS methods for the determination of AMI showed retention times of 1.22, 2.53 and 3.62 min [29–31]. VAN measurement LC-MS/MS assays present largely variable retention times, varying between 2.02 and 9.87 min [30,31,41–43]. Previous CRE assays had retention times in the range of 0.9 to 1.9 min [44–48]. An alternative to reversed-phase stationary phases for highly polar compounds is HILIC separations. However, HILIC columns are known to be less robust than reversed-phase, once are crucially influenced by mobile phase pH and ionic strength [26,27]. HILIC assays were applied for determination of our target analytes presenting retention times of 3.99 min for AMI [33], 1.1 min for CRE [49] and 1.8 and 2.7 min for VAN [2,22].

To increase retention of AMI and its internal standard KAN in a reversed-phase column, the ion-pairing agent HFBA (heptafluorobutyric acid) was added to the mobile phase in a very low concentration (0.01%). The use of ion-pairing agents in the mobile phase to increase chromatographic retention of aminoglycosides was already reported, with the use of pentafluoropropionic acid (PFPA) at concentration of 7.8% [38] and HFBA at 0.013, 1.0 and 5.2% [25,28,29]. Although the use of ion-pairing agents is usually associated with ionization suppression in electrospray ionization, leading to reduced analytical sensitivity, there is no consensus of the ideal concentration to reach satisfactory retention time [27,31,33]. An alternative commonly applied to increase chromatographic retention of highly polar compounds is the use of ion-pairing agent as protein precipitants. However, this approach requires the use of high concentrations of ion-paring agents, resulting in very acidic extracts which can reduce considerably the lifespan of the chromatographic columns [22]. In the present study, HFBA at the concentration of 0.01% in the mobile phase allowed adequate retention for AMI and KAN. A previous study which used HFBA in the mobile phase for the analysis of AMI and KAN in serum employed much higher concentrations (1%), but obtained similar retention times of 2.53 min for AMI and 2.62 min for KAN, in an octadecilsilica column [29]. The presence of HFBA at 0.01%, as employed in our assay, did not affected the performance of the column, which maintained its efficiency even after 1.000 injections with this method.

The polar characteristics of AMI, VAN and CRE do not allow their

Table 2
Method validation parameters for the determination of AMI, VAN and CRE in plasma by UPLC-MS/MS: precision, accuracy, matrix effect, extraction yield and dilution integrity.

Analyte	QC sample	Nominal concentration (mg L^{-1})	Precision CV %)		Accuracy (%)	Matrix effect (%)	Extraction yield (%)	Dilution integrity	
			Intra-day	Inter-day				Precision (CV%)	Accuracy (%)
Amikacin	QCLOQ	0.5	5.9	7.3	105.8	–	–	–	–
	QCL	1.5	6.0	3.8	99.7	10.5	89.7	–	–
	QCM	7.5	4.8	4.6	94.2	–	–	–	–
	QCH	75	4.7	5.6	99.3	11.6	83.1	4.4	107.8
Vancomycin	QCLOQ	0.5	5.7	4.3	100.8	–	–	–	–
	QCL	1.5	5.1	3.7	104.2	−4.3	99.9	–	–
	QCM	7.5	5.7	2.4	100.6	–	–	–	–
	QCH	75	4.2	4.6	102.8	−4.5	97.3	3.7	99.3
Creatinine	QCLOQ	5	2.5	4.8	101.6	–	–	–	–
	QCL	6	3.0	4.1	107.7	0.7	94.3	–	–
	QCM	20	2.3	3.8	102.9	–	–	–	–
	QCH	80	2.5	4.7	101.4	−1.7	91.5	1.7	110.7

QCLLOQ: quality control at the lower limit of quantification, QCL: quality control low, QCM: quality control medium, QCH: quality control (precision and accuracy $n = 45$, matrix effect $n = 30$, extraction yield $n = 30$).

extraction from biofluids by liquid-liquid extraction (LLE) [25]. The use of solid-phase extraction (SPE) was reported for AMI and VAN, but this technique is usually avoided due to its long processing time [29,33,50]. Protein precipitation (PP) is the most commonly used sample preparation strategy for the analysis of AMI, VAN and CRE in plasma or serum by LC-MS/MS [2,20,22,29–32,43,44,47,51]. Nevertheless, PP produces extracts with high concentrations of endogenous compounds such as fatty acids and phospholipids, which negatively affects ionization in MS analysis [23,52]. In this study, we used a simple PP with acetonitrile, followed by dilution in 0.1% formic acid in water. This approach avoided the use of strong acids and provided a significant dilution of matrix compounds. The matrix dilution combined with the injection of small amount of the final extract (1 μL) contributed to low matrix effects, as presented below.

Matrix effects were in the range of +10.5 to +11.6% for AMI, −4.3 to −4.5% for VAN, and − 1.7 to +0.7 for CRE (Table 2). Small matrix effects were observed for VAN, even without the compensation of an internal standard. The combined sample preparation and chromatographic separation strategy were efficient to reduce matrix effects once these effects, calculated without the use of internal standards, were between 2.1 and 12.6% for CRE and − 12.9 and − 12.3% for AMI. Average extraction yield was 86.3% for AMI, 98.6% for VAN, and 92.9% for CRE.

Precision and accuracy of the developed assay were acceptable (Table 2). The intra-day precision was 4.7–6.0% for AMI, 5.1–5.7% for VAN, and 2.3–3.0% for CRE. Inter-day imprecision varied between 3.8 and 5.6% for AMI, 2.4–4.6% to VAN and 3.8–4.7% for CRE. Accuracy was in the range of 94.2–99.7%, 100.6–104.2% and 101.4–107.7% for AMI, VAN and CRE, respectively. The QCLOQ demonstrated an imprecision of 5.9–7.3% for AMI, 4.3–5.7% for VAN and 2.5–4.8% for CRE. At the LLOQ level, accuracy was 100.8% for VAN, 101.6% for CRE and 105.8% for AMI.

Freeze-thaw stability was satisfactory, with an observed deviation after the last freeze and thaw cycle between −4.2 and 14.6% for AMI, −0.3 and 14.1% for VAN, and − 0.6 and − 8.5% for CRE (Table 3). Analytes in extracts were stable at the autosampler, presenting signal variations of −9.6 to 2.7% for AMI, 2.4 to 7.7% for VAN and − 1.0 to −1.4% for CRE after 12 h. Linear regression of measurement signals *vs.* injection time showed no significant tendencies. Results support the possibility of re-analyzing samples several times after freezing, as well as the processing of long analytical batches. Dilution integrity evaluation showed that diluted samples can be adequately measured, once diluted extract presented accuracy of 107.8% for AMI, 99.3% for VAN and 110.7% for CRE, with CVs of 4.4, 3.7 and 1.7% for AMI, VAN and CRE, respectively. These results allow accurate reanalysis of samples with concentrations that exceed the calibration range of the assay.

3.2. Clinical application

Concentrations of AMI, VAN and CRE varied widely in clinical plasma samples. AMI concentrations of AMI were in the range of 0.5 to 113.6 mg L^{-1}, with one sample presenting concentration lower than the LLOQ. Among the 18 evaluated patients, 7 patients were receiving VAN simultaneously with AMI. In these patients, VAN concentrations ranged from 1.4 to 30.8 mg L^{-1}. CRE concentrations varied between 5.0 and 72.3 mg L^{-1}, with 14 samples presenting levels below the LLOQ. The LLOQ of CRE was set to 5.0 mg L^{-1} due to its estimation by the background subtraction method, a strategy already applied to this compound previously [45,53]. CRE plasma concentrations, in the context of TDM of AMI and VAN, are used for the calculation of CRE clearance, which is a co-variate in pharmacokinetic models for AMI and VAN [5,7]. However, extremely low CRE plasma levels could lead to an overestimation of CRE clearance and, as a consequence, of AMI and VAN clearance. This effect was described for gentamycin (GEN), with CRE serum levels lower than 6.8 mg L^{-1} being rounded to this value, which improved the correlation between CRE clearance and GEN clearance [54]. Considering the above, the CRE LLOQ of 5.0 mg L^{-1} is adequate for the clinical use of the assay. Combining the measurement of AMI and VAN with CRE, all required analytical information for the use of common pharmacokinetic dose individualization software is available.

4. Conclusion

AMI, VAN and CRE concentrations were measured with adequate analytical performance using a simple and fast UPLC-MS/MS assay. Adequate retention in a reversed-phase ultra-performance column was by the use of a low concentration of the ion-pairing agent HFBA. This study presents the first description of an assay for the simultaneous determination of AMI, VAN and CRE in plasma by UPLC-MS/MS. The developed assay provides all necessary analytical information for the clinical application of pharmacokinetic population models of AMI and VAN.

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Table 3
Autosampler (AS) and freeze-thaw stability of AMI, VAN and CRE.

Analyte	QC sample	Nominal concentration (μg mL^{-1})	Processed sample concentration change after 12 h in AS (%)	Stability after 3 freeze-thaw cycles (%)		
				Cycle 1	Cycle 2	Cycle 3
Amikacin	QCLOQ	0.5	–	–	–	–
	QCL	1.5	−9.6	6.8	14.6	10.5
	QCM	7.5	–	–	–	–
	QCH	75	2.7	1.3	−4.8	−4.2
Vancomycin	QCLOQ	0.5	–	–	–	–
	QCL	1.5	7.7	3.6	12.7	14.1
	QCM	7.5	–	–	–	–
	QCH	75	2.4	−0.3	8.9	−5.0
Creatinine	QCLOQ	5	–	–	–	–
	QCL	6	−1.4	−3.5	−7.4	−8.5
	QCM	20	–	–	–	–
	QCH	80	−1.0	−3.7	1.8	−0.6

QCLOQ: quality control at the lower limit of quantification, QCL: quality control low, QCM: quality control medium, QCH: quality control (freeze-thaw stability $n = 24$, processed sample stability $n = 24$).

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References

[1] C. Roger, B. Nucci, N. Molinari, S. Bastide, G. Saissi, G. Pradel, S. Barbar, C. Aubert, S. Lloret, L. Elotmani, A. Polge, J.Y. Lefrant, J.A. Roberts, L. Muller, Standard dosing of amikacin and gentamicin in critically ill patients results in variable and subtherapeutic concentrations, Int. J. Antimicrob. Agents 46 (2015) 21–27, https://doi.org/10.1016/j.ijantimicag.2015.02.009.

[2] S.L. Parker, Y.C.G. Valero, J.L.O. Mejia, C. Roger, J. Lipman, J.A. Roberts, S.C. Wallis, An LC-MS/MS method to determine vancomycin in plasma (total and unbound), urine and renal replacement therapy effluent, Bioanalysis. 9 (2017) 911–924, https://doi.org/10.4155/bio-2017-0019.

[3] J. Ruiz, P. Ramirez, M.J. Company, M. Gordon, E. Villarreal, P. Concha, M. Aroca, J. Frasquet, M. Remedios-Marqués, Á. Castellanos-Ortega, Impact of amikacin pharmacokinetic/pharmacodynamic index on treatment response in critically ill patients, J. Glob. Antimicrob. Resist. 12 (2018) 90–95, https://doi.org/10.1016/j.jgar.2017.09.019.

[4] S. Deresinski, Vancomycin in combination with other antibiotics for the treatment of serious methicillin-resistant staphylococcus aureus infections, Clin. Infect. Dis. 49 (2009) 1072–1079, https://doi.org/10.1086/605572.

[5] A. Marsot, R. Guilhaumou, C. Riff, O. Blin, Amikacin in critically ill patients: a review of population pharmacokinetic studies, Clin. Pharmacokinet. 56 (2017) 127–138, https://doi.org/10.1007/s40262-016-0428-x.

[6] G. Özcengiz Yılmaz, Antibiotics: pharmacokinetics, toxicity, resistance and multidrug efflux pumps, Biochem. Pharmacol. 133 (2017) 43–62, https://doi.org/10.1016/j.bcp.2016.10.005.

[7] A. Marsot, A. Boulamery, B. Bruguerolle, N. Simon, Vancomycin: a review of population pharmacokinetic analyses a review of population pharmacokinetic analyses, Clin. Pharmacokinet. 51 (2012) 1–13, https://doi.org/10.2165/11596390-000000000-00000.

[8] R.D. Moore, P.S. Lietman, C.R. Smith, Clinical response to aminoglycoside therapy: importance of the ratio of peak concentration to minimal inhibitory concentration, J. Infect. Dis. 155 (1987) 93–99, https://doi.org/10.1093/infdis/155.1.93.

[9] S. Devabhakthuni, Antibiotic pharmacokinetic monitoring, Am. J. Heal. Pharm. (2011) [Cited 2019 mar 5] Available from: https://cmapspublic.ihmc.us/rid=1M6LMRYWW-1MC64-2307/Antibiotic%20Pharmacokinetic%20Monitoring.pdf.

[10] J.A. Dijkstra, R. Van Altena, O.W. Akkerman, W.C.M. De Lange, J.H. Proost, T.S. Van Der Werf, J.G.W. Kosterink, J.W.C. Alffenaar, Limited sampling strategies for therapeutic drug monitoring of amikacin and kanamycin in patients with multidrug-resistant tuberculosis, Int. J. Antimicrob. Agents 46 (2015) 332–337, https://doi.org/10.1016/j.ijantimicag.2015.06.008.

[11] G.L. Drusano, P.G. Ambrose, S.M. Bhavnani, J.S. Bertino, A.N. Nafziger, A. Louie, Back to the future: using aminoglycosides again and how to dose them optimally, Clin. Infect. Dis. 45 (2007) 753–760, https://doi.org/10.1086/520991.

[12] B.P. White, B. Lomaestro, M.P. Pai, Optimizing the initial amikacin dosage in adults, Antimicrob. Agents Chemother. 59 (2015) 7094–7096, https://doi.org/10.1128/AAC.01032-15.

[13] E.J. Begg, M.L. Barclay, S. Duffull, A suggested approach to once-daily aminoglycoside dosing, Br. J. Clin. Pharmacol. 39 (1995) 605–609, https://doi.org/10.1057/9781137402240_6.

[14] A.E. Muller, B. Huttner, A. Huttner, Therapeutic drug monitoring of beta-lactams and other antibiotics in the intensive care unit: which agents, which patients and which infections? Drugs. 78 (2018) 439–451, https://doi.org/10.1007/s40265-018-0880-z.

[15] R.B. Turner, K. Kojiro, E.A. Shephard, R. Won, E. Chang, D. Chan, F. Elbarbry, Review and validation of Bayesian dose optimizing software and equations for calculation of the vancomycin area under the curve in critically ill patients, Pharmacotherapy. 38 (2018) 1174–1183, https://doi.org/10.1002/phar.2191.

[16] V. Peyko, M. Friedman-Jakubovics, Novel approach to vancomycin level monitoring: impact of a multidisciplinary monitoring system on timing of vancomycin levels, Am. J. Heal. Pharm. 75 (2018) 121–126, https://doi.org/10.2146/ajhp160760.

[17] J.M. Pogue, N.A. Finch, K.P. Murray, M.J. Rybak, R.P. Mynatt, E.J. Zasowski, T.D. Trinh, Identification of vancomycin exposure-toxicity thresholds in hospitalized patients receiving intravenous vancomycin, Antimicrob. Agents Chemother. 62 (2017) 1–9, https://doi.org/10.1128/aac.01684-17.

[18] E.L. Heil, K.C. Claeys, R.P. Mynatt, T.L. Hopkins, K. Brade, I. Watt, M.J. Rybak, J.M. Pogue, Making the change to area under the curve–based vancomycin dosing, Am. J. Heal. Pharm. 75 (2018) 1986–1995, https://doi.org/10.2146/ajhp180034.

[19] L. Mahmoudi, A.H. Mohammadpour, R. Niknam, A. Ahmadi, M. Mojtahedzadeh, Limited sampling strategy for estimation of amikacin optimal sampling time in critically ill adults, Anaesth. Intensive Care 42 (2014) 228–233.

[20] H. Brozmanová, I. Kacířová, R. Uřinovská, P. Šištík, M. Grundmann, New liquid chromatography-tandem mass spectrometry method for routine TDM of vancomycin in patients with both normal and impaired renal functions and comparison with results of polarization fluoroimmunoassay in light of varying creatinine concentrations, Clin. Chim. Acta 469 (2017) 136–143, https://doi.org/10.1016/j.cca.2017.04.003.

[21] C. Ezquer-Garin, L. Escuder-Gilabert, Y. Martín-Biosca, R.F. Lisart, S. Sagrado, M.J. Medina-Hernández, Fit-for-purpose chromatographic method for the determination of amikacin in human plasma for the dosage control of patients, Talanta. 150 (2016) 510–515, https://doi.org/10.1016/j.talanta.2015.12.057.

[22] M. Oyaert, N. Peersman, D. Kieffer, K. Deiteren, A. Smits, K. Allegaert, I. Spriet, J. van Eldere, J. Verhaegen, P. Vermeersch, S. Pauwels, Novel LC-MS/MS method for plasma vancomycin: comparison with immunoassays and clinical impact, Clin. Chim. Acta 441 (2015) 63–70, https://doi.org/10.1016/j.cca.2014.12.012.

[23] A. Veringa, M.G.G. Sturkenboom, B.G.J. Dekkers, R.A. Koster, J.A. Roberts, C.A. Peloquin, D.J. Touw, J.W.C. Alffenaar, LC-MS/MS for therapeutic drug monitoring of anti-infective drugs, TrAC - Trends Anal. Chem. 84 (2016) 34–40, https://doi.org/10.1016/j.trac.2015.11.026.

[24] C. Cheng, S. Liu, D. Xiao, S. Hansel, The application of trichloroacetic acid as an ion pairing reagent in LC – MS – MS method development for highly polar aminoglycoside compounds, Chromatographia. 72 (2010) 133–139, https://doi.org/10.1365/s10337-010-1614-x.

[25] C.Y. Lu, C.H. Feng, Micro-scale analysis of aminoglycoside antibiotics in human plasma by capillary liquid chromatography and nanospray tandem mass spectrometry with column switching, J. Chromatogr. A 1156 (2007) 249–253, https://doi.org/10.1016/j.chroma.2007.01.001.

[26] G. Kahsay, H. Song, A. Van Schepdael, D. Cabooter, E. Adams, Hydrophilic interaction chromatography (HILIC) in the analysis of antibiotics, J. Pharm. Biomed. Anal. 87 (2014) 142–154, https://doi.org/10.1016/j.jpba.2013.04.015.

[27] F. Farouk, H.M.E. Azzazy, W.M.A. Niessen, Challenges in the determination of aminoglycoside antibiotics, a review, Anal. Chim. Acta 890 (2015) 21–43, https://doi.org/10.1016/j.aca.2015.06.038.

[28] S. Bogialli, R. Curini, A. Di Corcia, A. Laganà, M. Mele, M. Nazzari, Simple confirmatory assay for analyzing residues of aminoglycoside antibiotics in bovine milk: hot water extraction followed by liquid chromatography-tandem mass spectrometry, J. Chromatogr. A 1067 (2005) 93–100, https://doi.org/10.1016/j.chroma.2004.10.033.

[29] J.A. Dijkstra, M.G.G. Sturkenboom, K. Van Hateren, Koster, quantification of amikacin and kanamycin in serum using a simple and validated LC – MS / MS method, Bioanalysis. 6 (2014) 2125–2133.

[30] A. Chahbouni, F.A.M. Dungen, R.M. Vos, J.C.G. Burger, A. Sinjewel, A.J. Wilhelm, A.I. Veldkamp, E.L. Swart, M.M. Weissenbruch, An UPLC – MS detection method for the quantification of five antibiotics in human plasma, Bioanalysis. 7 (2015) 2321–2329, https://doi.org/10.4155/bio.15.121.

[31] Y. Bijleveld, T. de Haan, J. Toersche, S. Jorjani, J. van der Lee, F. Groenendaal, P. Dijk, A. van Heijst, A.W.D. Gavilanes, R. de Jonge, K.P. Dijkman, H. van Straaten,

M. Rijken, I. Zonnenberg, F. Cools, D. Nuytemans, R. Mathôt, A simple quantitative method analysing amikacin, gentamicin, and vancomycin levels in human newborn plasma using ion-pair liquid chromatography/tandem mass spectrometry and its applicability to a clinical study, J. Chromatogr. B 951–952 (2014) 110–118, https://doi.org/10.1016/j.jchromb.2014.01.035.

[32] R. Cazorla-Reyes, R. Romero-González, A.G. Frenich, M.A. Rodríguez Maresca, J.L. Martínez Vidal, Simultaneous analysis of antibiotics in biological samples by ultra high performance liquid chromatography-tandem mass spectrometry, J. Pharm. Biomed. Anal. 89 (2014) 203–212, https://doi.org/10.1016/j.jpba.2013.11.004.

[33] R. Oertel, V. Neumeister, W. Kirch, Hydrophilic interaction chromatography combined with tandem-mass spectrometry to determine six aminoglycosides in serum, J. Chromatogr. A 1058 (2004) 197–201, https://doi.org/10.1016/j.chroma.2004.08.158.

[34] A.M. Almeida, M.M. Castel-Branco, A.C. Falcão, Linear regression for calibration lines revisited: weighting schemes for bioanalytical methods, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 774 (2002) 215–222, https://doi.org/10.1016/S1570-0232(02)00244-1.

[35] EMEA, Guideline on Bioanalytical Method Validation, [cited 2019 mar 10]; Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf.

[36] FDA, Guidance for Industry: Bioanalytical Method Validation, [cited 2019 mar 10]; Available from: http://www.fda.gov/downloads/drugs/guidance/ucm070107.pdf.

[37] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, Anal. Chem. 75 (2003) 3019–3030, https://doi.org/10.1021/ac020361s.

[38] Y. Liu, X. Zhang, S. An, Y. Wu, G. Hu, Y. Wu, Pharmacokinetics of neamine in rats and anti-cervical cancer activity in vitro and in vivo, Cancer Chemother. Pharmacol. 75 (2015) 465–474, https://doi.org/10.1007/s00280-014-2658-7.

[39] M.W. Dong, K. Zhang, Ultra-high-pressure liquid chromatography (UHPLC) in method development, Trends Anal. Chem. (2014), https://doi.org/10.1016/j.trac.2014.06.019.

[40] J.C.P. Zalewski, P. Garbacki, A. Jelin, UHPLC : The Greening Face of Liquid Chromatography, (2013), pp. 1429–1437, https://doi.org/10.1007/s10337-013-2434-6.

[41] K. König, U. Kobold, G. Fink, A. Leinenbach, T. Dülffer, Quantification of vancomycin in human serum by LC-MS / MS, Clin. Chem. Lab. Med. 51 (2013) 1761–1769, https://doi.org/10.1515/cclm-2013-0142.

[42] L. Javorska, L.K. Krcmova, P. Solich, M. Kaska, Simple and rapid quantification of vancomycin in serum, urine and peritoneal/pleural effusion via UHPLC–MS/MS applicable to personalized antibiotic dosing research, J. Pharm. Biomed. Anal. 142 (2017) 59–65, https://doi.org/10.1016/j.jpba.2017.04.029.

[43] F. Chen, Z.Y. Hu, S.C. Laizure, J.Q. Hudson, Simultaneous assay of multiple antibiotics in human plasma by LC-MS/MS: importance of optimizing formic acid concentration, Bioanalysis. 9 (2017) 469–483, https://doi.org/10.4155/bio-2016-0157.

[44] M. Ou, Y. Song, S. Li, G. Liu, J. Jia, LC-MS / MS method for serum creatinine : comparison with enzymatic method and Jaffe method, PLoS One 10 (2015), https://doi.org/10.1371/journal.pone.0133912.

[45] R.A. Koster, B. Greijdanus, Dried blood spot analysis of creatinine with LC-MS / MS in addition to immunosuppressants analysis, Anal. Chim. Acta 407 (2015) 1585–1594, https://doi.org/10.1007/s00216-014-8415-2.

[46] W. Kwon, J.Y. Kim, S. Suh, M.K. In, Simultaneous determination of creatinine and uric acid in urine by liquid chromatography – tandem mass spectrometry with polarity switching electrospray ionization, Forensic Sci. Int. 221 (2012) 57–64, https://doi.org/10.1016/j.forsciint.2012.03.025.

[47] Y. Zhao, G. Liu, A. Angeles, L.J. Christopher, Z. Wang, M.E. Arnold, J.X. Shen, A validated LC – MS / MS method for the quantitative measurement of creatinine as an endogenous biomarker in human plasma, Bioanalysis. 8 (2016) 1997–2005.

[48] X. Liu, Y. Luo, C. Zhou, A. Peng, J. Liu, A sensitive and accurate method to simultaneously measure uric acid and creatinine in human saliva by using LC – MS / MS, Bioanalysis. 9 (2017) 1751–1760.

[49] J. Dunn, G. Smith, A clinical biomarker assay for the quantification of d3-creatinine and creatinine using LC – MS / MS, Bioanalysis. 6 (2014) 745–759.

[50] T. Zhang, D.G. Watson, C. Azike, J.N.A. Tettey, A.T. Stearns, A.R. Binning, C.J. Payne, Determination of vancomycin in serum by liquid chromatography-high resolution full scan mass spectrometry, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 857 (2007) 352–356, https://doi.org/10.1016/j.jchromb.2007.07.041.

[51] L. Baietto, A. D'Avolio, F.G. De Rosa, S. Garazzino, M. Michelazzo, G. Ventimiglia, M. Siccardi, M. Simiele, M. Sciandra, G. Di Perri, Development and validation of a simultaneous extraction procedure for HPLC-MS quantification of daptomycin, amikacin, gentamicin, and rifampicin in human plasma, Anal. Bioanal. Chem. 396 (2010) 791–798, https://doi.org/10.1007/s00216-009-3263-1.

[52] A. Van Eeckhaut, K. Lanckmans, S. Sarre, I. Smolders, Y. Michotte, Validation of bioanalytical LC-MS/MS assays: evaluation of matrix effects, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 877 (2009) 2198–2207, https://doi.org/10.1016/j.jchromb.2009.01.003.

[53] N.B. Andriguetti, L.L. Lisboa, S.R. Hahn, L.R. Pagnussat, M.V. Antunes, R. Linden, Simultaneous determination of vancomycin and creatinine in plasma applied to volumetric absorptive microsampling devices using liquid chromatography-tandem mass spectrometry, J. Pharm. Biomed. Anal. 165 (2019) 315–324, https://doi.org/10.1016/j.jpba.2018.12.023.

[54] C.M.J. Kirkpatrick, S.B. Duffull, E.J. Begg, Pharmacokinetics of gentamicin in 957 patients with varying renal function dosed once daily, Br. J. Clin. Pharmacol. 47 (1999) 637–643, https://doi.org/10.1046/j.1365-2125.1999.00938.x.