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Quantification of vancomycin and clindamycin in human plasma and synovial fluid applying ultra-performance liquid chromatography tandem mass spectrometry

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ABSTRACT

Periprosthetic joint infection is a challenging infection involving the joint prosthesis and adjacent tissue, such as synovial fluid, synovial tissue, and bone tissue. The current treatment consists of multiple surgical revisions and long-term antibiotic therapy. Treatment failure can cause poor functional outcome and reduced quality of life. Further research on the extent of antibiotic penetration into the infected tissues is of great importance. Our work aimed to develop and validate a novel ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method for the determination of the commonly administered antibiotics vancomycin and clindamycin in plasma and synovial fluid. An extraction procedure consisting of zinc sulfate precipitation and dilution with eluent was used for both analytes. Chromatographic separation was performed on a Waters Acquity UPLC HSS T3 C18 column (1.8 μm, 2.1 × 100 mm), and quantification was carried out by a Waters Xevo TQ-S micro mass spectrometer. Stable isotope-labeled vancomycin-d10 served as internal standard. The method validation was performed based on the guidelines of the EMA and FDA. The calibration curves were linear over the range of 0.5–50 mg/L, with a coefficient of determination above 0.990. The validation results for precision and accuracy, specificity, matrix effects and stability were all within the acceptance range. An accurate and rapid method for the simultaneous quantification of vancomycin and clindamycin in human plasma and synovial fluid on the UPLC-MS/MS was developed, optimized and validated. The analysis has a run time of 5.2 min and 50 μL sample volume is needed. This developed method was successfully applied in eight patients with PJI and is suitable to determine the exposure of antibiotics in plasma and synovial fluid in patients during current PK/PD studies.

1. Introduction

Each year, millions of people worldwide undergo hip and knee joint replacement surgery. It is a life-enhancing procedure that aims to reduce pain, increase mobility and improve the quality of life [1,2]. Although the procedure succeeds in the vast majority of patients, a small group (approximately 1–2 %) suffers from periprosthetic joint infection (PJI) [3]. PJI is a severe infection involving the joint prosthesis and adjacent tissue and is perhaps the most challenging complication associated with joint replacement [3]. Due to the ageing of the population, the number of implanted joint prostheses, and thus the absolute number of PJI, will increase [4].

The treatment of PJI is expensive and complex since multiple surgical revisions and long-term antimicrobial treatment are necessary [3].

Abbreviations: EMA, European Medicines Agency; ESI, electrospray ionization; FDA, U.S.A. Food and Drug Administration; HPLC, high-performance liquid chromatography; IS, internal standard; LLOQ, lower limit of quantification; MIC, minimal inhibitory concentration; MRM, multiple reaction monitoring; MS, mass spectrometry; PJI, periprosthetic joint infection; QC, quality control; QC H, quality control high; QC L, quality control low; QC M, quality control medium; RSD, relative standard deviation; ULOQ, upper limit of quantification; UPLC, ultra-performance liquid chromatography; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry.

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Insufficient treatment of PJI can lead to persistence of the infection and multiple surgical revisions, which can cause poor mobility, disability, and reduced quality of life [5]. To avoid undertreatment and therapy failure, adequate bone and joint penetration of the antimicrobial treatment is a requirement. Effective concentrations of the antibiotics at the target site are necessary to eradicate the infection [6].

For the treatment of PJI, the prosthesis is replaced in the same operating session (one-stage revision) or at a delayed interval (two-stage revision). The two-stage revision is the preferred method, as this method ensures the highest chance of eradication of the microorganisms that cause the infection [3,7]. Two-stage revision is a procedure where the treatment is divided into two parts. During the first stage, tissue cultures are taken, after which all the infected, necrotic tissue is debrided, prosthetic parts are removed and a temporary antibiotic-loaded spacer is placed [7,8]. The spacer is often loaded with gentamicin and/or vancomycin, due to its broad-spectrum [9]. Before the second stage, which involves re-implantation of the prosthesis, patients receive intravenous and oral antibiotics for a prolonged time [1,7]. The intravenous antibiotics (i.e., vancomycin, flucloxacillin, or cefuroxime) are administered for 2–4 weeks and are followed by oral antibiotics (i.e., flucloxacillin, clindamycin, or co-trimoxazole) for 4–6 weeks [7]. Antibiotic treatment supports the surgical treatment and targets residual microorganisms in the tissue surrounding the prosthesis (deep tissue infection) and in the affected part of the bone (osteomyelitis).

Adequate penetration of the antibiotics into the infection site remains the cornerstone of successfully eliminating the infection [1,7]. Suboptimal concentrations of antibiotics at the site of infection can lead to undertreatment of the infection, treatment failure, and development of more resistant bacteria populations, while excessive concentrations can lead to unnecessary systemic side effects [6]. In the case of PJI, the infection mainly affects the joint prosthesis and the surrounding tissues, such as the synovial fluid, synovial tissue, and bone tissue [7]. Although these sites are the main areas where PJI manifests, the antibiotic regimen and selection are based on the susceptibility of the microorganisms to the antibiotic and not on the degree of penetration into these tissues. The degree of antibiotic penetration in musculoskeletal tissues is in most cases unknown. Therefore, further research on the extent of antibiotic penetration into the synovial fluid, synovial tissue, and bone tissue is of great importance to optimally treat patients with PJI.

Two of the commonly administered antimicrobial agents during two-stage revision are vancomycin and clindamycin. By determining the extent of target site penetration at the infection site the dosage of these antibiotics can be optimized, resulting in a more efficient eradication of infection with fewer side effects. Up to now, various analytical methods have been reported for the quantification of vancomycin and clindamycin in human plasma [10–16]. However, these methods either involve time-consuming sample preparations [10–13], have long sequence running times [11,12], have a narrower linear range [13–15], or require larger sample volumes [15,16]. Although several methods are available for the measurement of vancomycin and clindamycin in plasma, it appears that no method of vancomycin and clindamycin in synovial fluid using UPLC-MS/MS has been reported. In addition, no methods have been reported in which vancomycin and clindamycin are simultaneously analyzed in plasma. Our aim was therefore to develop a sensitive, selective, and reliable ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) assay to measure the concentration of vancomycin and clindamycin in human plasma and synovial fluid.

2. Materials and methods

2.1. Subject recruitment and sample collection

The samples were obtained from patients with periprosthetic (hip or knee) joint infections undergoing a two-stage revision who were included in an ongoing clinical trial (ASTERICS). The study protocol (registration number MEC-2020–0279) has been approved by the Erasmus MC Medical Ethics Committee in Rotterdam, the Netherlands. Briefly, the ASTERICS study is a cross-sectional observational study, designed to analyze the target attainment of the administered antibiotics during the two-stage revision treatment, in order to determine the efficacy of the PJI treatment with respect to the dose and plasma concentrations.

All patients over the age of 18 years were eligible for inclusion if they received vancomycin or clindamycin. Patients were selected by the attending orthopedic surgeon. In case of a language barrier, drugs usage that interacts with the targeted antibiotics, or usage of the targeted antibiotics before the start of the study, the patient was excluded from participation.

On four occasions during the six-week treatment, patients' samples were collected. The first samples of venous blood and synovial fluid were collected 30 to 60 min after administration of the intravenous antibiotics. 90 to 120 min after the administration of the antibiotics venous blood was collected. For the third sample, at the end of the intravenous antibiotic administration (two weeks after treatment), venous blood and synovial fluid were drawn from the patient. The synovial fluid was collected through a joint puncture of the joint capsule. The final samples of venous blood and synovial fluid were collected during re-implantation, directly after continued use of oral antibiotic therapy. All blood samples were collected in EDTA blood tubes.

After accomplishing sample collection, the samples were delivered to the laboratory for cleaning procedures and storage. Within 24 h of collection, the blood and synovial fluid samples were centrifuged at 350 g for 6-min. The supernatant was pipetted into a clean cryo tube and stored at –80 °C until analysis.

2.2. Chemicals and reagents

Vancomycin hydrochloride (purity: 96.1 %) was purchased from Cayman Chemical (Uden, the Netherlands) and clindamycin hydrochloride (purity: 98.8 %) was purchased from Santa Cruz Biotechnology (Huissen, the Netherlands). The stable isotope-labeled internal standard (IS) vancomycin-d10 (purity: 97.2 %, isotopic purity: 97.6 %) was obtained from NucleoSyn (Olivet, France). Distilled, deionized water was produced by a MilliQ Water Advantage A10 Purification System (Merck Millipore, Darmstadt, Germany). Methanol LCMS-grade (99 %) was obtained from Biosolve B.V. (Valkenswaard, the Netherlands). The blank human EDTA plasma was obtained from the hemostasis laboratory of Erasmus MC (Rotterdam, The Netherlands). The blank synovial fluid was obtained with informed consent during surgical hip or knee interventions by the orthopedic surgeon. Directly after collection, the blank synovial fluid samples were stored at –20 °C until analysis.

2.3. Chromatographic and mass spectroscopy conditions

2.3.1. Instrumentation

Analysis was performed on a Waters Acquity UPLC-MS/MS system (Waters Corp., Milford, MA, USA) consisting of an Acquity binary solvent manager (chromatographic pump), a sample manager (auto sampler), and a column manager. The UPLC-system was coupled to a Waters TQ-S micro mass spectrometer with a triple quadrupole and electrospray ionization (ESI) probe. Data acquisition was performed using Masslynx™ V4.1 software and Targetlynx V4.1 (Waters Corp).

2.3.2. Chromatographic conditions

Chromatographic separation and optimization were performed on a reversed-phase Waters Acquity UPLC HSS T3 C_{18} column (1.8 µm, 2.1 × 100 mm) at a column temperature of 45 °C. The mobile phase consisted for 95 % of 0.1 % formic acid and 2 mM ammonium acetate in 1 L MilliQ water (eluent A) and for 5 % of 0.1 % formic acid and 2 mM ammonium acetate in 1 L LC-MS methanol (eluent B). A linear gradient elution with a constant flow rate of 0.35 mL/min was used. The initial condition of

95 % eluent A and 5 % eluent B was set for the first 0.8 min and then gradually changed to 10 % eluent A and 90 % eluent B. For 2 min, this condition was held and gradually returned to 95 % eluent A and 5 % eluent B, which lasted for the remaining 1.4 min. The total volume of injection was 1 µL, and the total analytical runtime was 5.2 min per sample. The auto sampler temperature was set at + 15 °C. Methanol was used as needle wash, whilst a mixture of water-methanol (9:1) functioned as a seal wash.

2.3.3. Mass-spectrometry conditions

Essentially, the optimal mass spectrometer (MS) conditions and MS/MS transitions for clindamycin, vancomycin, and the internal standard vancomycin-d10 were determined in electrospray ionization positive mode (ESI +). Solutions of each analyte were prepared with a concentration of 1 mg/L in methanol and directly infused into the MS/MS detector. For each analyte, the optimized cone voltages and collision energies were determined. The optimal MS conditions were as follows: capillary voltage of 3.0 kV, cone voltage of 20 V, desolvation temperature heated at 400 °C at a desolvation gas flow of 500 L/hour, and a cone gas flow of 10 L/hour. The optimal MS/MS transition results, collision energy, cone energy, and retention time of each analyte are summarized in Table 1.

2.4. Standards and quality control samples

2.4.1. Preparation of stock and working solutions

The stock solutions of vancomycin (2000 mg/L) and clindamycin (2000 mg/L) were individually prepared for the standards and the quality controls (QC) by dissolving 20 mg in 10 mL of MilliQ water. The stock solution of vancomycin-d10 (500 mg/L) was prepared by dissolving 5 mg in 10 mL of MilliQ water. The working solution of vancomycin-d10 (10 mg/L) was prepared by adding 400 µL of the stock solution in 20 mL 0.1 M zinc sulfate. The stock solutions of vancomycin and clindamycin were separately prepared for the calibration standards and QC. Between use, the stock and working solutions were stored at a temperature of + 5 °C for a maximum of three months.

2.4.2. Preparation of calibration standards

For the calibration curve, eight concentration levels were selected and prepared in both matrices, plasma and synovial fluid. Calibration standards 6–8 were prepared by diluting the stock solutions of vancomycin and clindamycin with vancomycin and clindamycin-free plasma or synovial fluid. Calibration standards 4 and 5 were prepared by diluting standard 8; calibration standard 3 was prepared by diluting standard 6, and standards 1 and 2 were prepared by diluting standard 3. The concentrations of the calibration standards, the lower limit of quantification (LLOQ), and the upper limit of quantification (ULOQ) are shown in Table 2. The standards were stored in aliquots of 50 µL in 1.5 mL safe lock Eppendorf tubes at –80 °C, until the day of analysis.

2.4.3. Preparation of quality controls

Three QC samples were prepared to consist of different concentrations than those of the calibration curve. In Table 3 the concentrations of the QCs are given. Quality control high (QC H) was prepared by diluting the stock standards with blank plasma or synovial fluid. Quality control medium and low (QC M and QC L) were prepared by further diluting QC H with blank plasma or synovial fluid. The QCs were also stored in aliquots of 50 µL in 1.5 mL safe lock microcentrifuge tubes at –80 °C, until analysis.

Table 1
Optimized MS/MS conditions of clindamycin, vancomycin and vancomycin-d10.

Analyte	Parent ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Retention time (min)
Clindamycin	425.0	125.9	2	38	1.20
Vancomycin	725.6	144.0	14	14	1.56
Vancomycin-d10	730.4	144.0	18	16	1.61

2.5. Sample preparation

The frozen standards and QCs were thawed, and 250 µL of the vancomycin-d10 working solution was added to the 50 µL samples. The working solution of vancomycin-d10 contained zinc sulfate for protein precipitation of the proteins in the plasma and synovial fluid. The aliquots were mixed for 10 s (Scientific Industries, Vortex Genie 2) and immediately centrifuged at 1811 g for 5-min (Eppendorf centrifuge). After centrifugation, 100 µL supernatant was pipetted into an auto sampler vial along with 900 µL of eluent A, followed by vortexing for 10 s. After all those steps were completed, 1 µL of each sample was injected into the LC-MS/MS system for analysis.

2.6. Method validation

The validation of the method was performed based on the guidelines of the European Medicines Agency (EMA) and the U.S.A. Food and Drug Administration (FDA) guidance on bioanalytical method validations [17,18]. Matrix effects and recoveries were validated according to the method of Matuszewski [11]. The following parameters were validated: linearity, limits of quantification, accuracy and precision, carry-over, auto sampler- and shelf-life stability, matrix effects, and recovery.

2.6.1. Linearity

For each analyte, a calibration curve was established and analyzed, consisting of eight non-zero calibration standards in duplicate. Aside from the eight calibration standards in duplicate, a blank sample without internal standard and a zero sample (blank with internal standard) were prepared. Non-zero calibration standards should be within 15 % of the nominal concentrations. Concentrations at LLOQ level should be within 20 % of the nominal concentrations [17,18]. The correlation coefficient (r) has to be above 0.995 and the coefficient of determination (r^2) has to be above 0.990 for both vancomycin and clindamycin.

2.6.2. Limits of quantification

The lower limit of quantification (LLOQ), and upper limit of quantification (ULOQ) were determined. The lowest standard of the calibration curve, reported in Table 1, was chosen to determine the lower limit of quantification (LLOQ). The LLOQ-standard was measured in duplicate on six different days. The accuracy and precision of the LLOQ should be between the limit of 80 and 120 %. The ULOQ corresponded to the highest concentration of the calibration curve, and the precision and accuracy should be < 20 % [17,18].

2.6.3. Precision and accuracy

The accuracy and intra-day precision were determined by measuring three different concentrations, QC L, M, and H, in sixfold on the same day. The inter-day precision was determined by measuring each QC level in duplicate on six different days. The RSD of the accuracy and precision should be between 85 and 115 % [17,18].

2.6.4. Carry-over

To determine the amount of carry-over of each analyte, a blank sample with internal standard was analyzed directly after the ULOQ was analyzed. Carry-over of the analytes in the blank sample should be lower than 20 % of the LLOQ-concentration [17,18].

2.6.5. Storage conditions and stability

The stability in the auto sampler was determined by comparing the QC L, M, and H in duplicate after 24, 48, and 72 h to a batch of freshly

Table 2
Calibration standards, LLOQ and ULOQ of vancomycin and clindamycin in plasma and synovial fluid.

Analyte	Calibration standards (mg/L)								LLOQ (mg/L)	ULOQ (mg/L)
	S1	S2	S3	S4	S5	S6	S7	S8		
Vancomycin	0.5	1.0	2.0	5.0	10.0	20.0	40.0	50.0	0.5	50.0
Clindamycin	0.5	1.0	2.0	5.0	10.0	20.0	40.0	50.0	0.5	50.0

S, standard; LLOQ, lower limit of quantification; ULOQ, upper limit of quantification.

Table 3
Quality control concentrations of vancomycin and clindamycin in plasma and synovial fluid.

Analyte	Quality controls (mg/L)		
	QC L	QC M	QC H
Vancomycin	6.3	12.5	25.0
Clindamycin	6.3	12.5	25.0

QC L, quality control low; QC M, quality control medium; QC H, quality control high.

prepared calibration standards in duplicate. The auto sampler vials were stored in the auto sampler at + 15 °C. The recovery of the stored QC samples and freshly prepared calibration standards should be between 85 and 115 %. The shelf life of the samples before sample preparation was also determined. Spiked plasma and synovial fluid QCs were stored under sample storage conditions for 72 and 168 h. The sample preparation was performed, and the concentrations were measured against freshly prepared calibration standards. The difference in signal between the stability sample and reference may not exceed 15 % [17,18].

2.6.6. Matrix effect and recovery

The effects of the matrix and the recovery were determined using the method of Matuszewski [19]. Three different sets were prepared to evaluate the recovery and absence or presence of matrix effects. The first set (set A) consisted of three known concentrations in MilliQ (QC L, QC H, and blank) in duplicate. The second set (set B) was prepared in five different batches of blank plasma and synovial fluid. The five batches of QC L, QC H, and blanks were spiked with vancomycin and clindamycin after sample preparation in duplicate. In the last set (set C), the same five batches were prepared, but the samples were spiked with vancomycin and clindamycin before sample preparation. The effect of the matrix was expressed as the ratio of set A and set B (B/A × 100 %). Recovery was calculated by comparing the results of set B and set C (C/B × 100 %). Process efficiency was expressed as the ratio of set A and set C (C/A × 100 %). The matrix effect, recovery, and process efficiency should be between the limit of 80 and 120 % and the RSD of these parameters should be < 15 %.

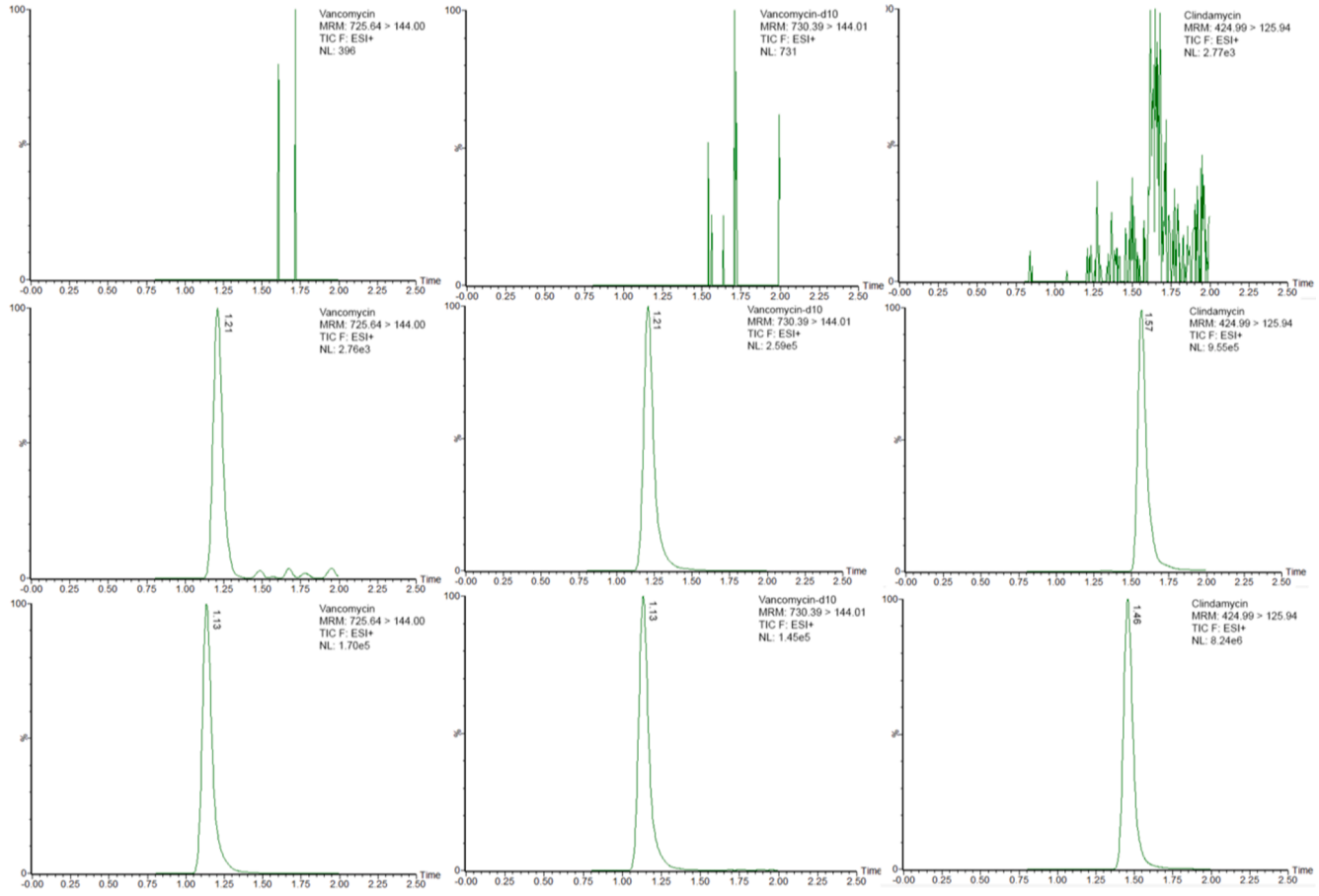

Fig. 1. Representative MRM chromatograms of a blank plasma sample: vancomycin (A1), vancomycin-d10 (A2) and clindamycin (A3). Representative MRM chromatograms of a plasma sample at the LLOQ level: vancomycin (B1), vancomycin-d10 (B2) and clindamycin (B3). Representative MRM chromatograms of a patient's plasma sample: vancomycin (C1), vancomycin-d10 (*C*2) and clindamycin (C3).

3. Results

3.1. Method validation

3.1.1. Linearity and selectivity

For the analysis of vancomycin and clindamycin, a minimum sample volume of 50 µL was required. The calibration range of 0.5–50 mg/L for plasma and synovial fluid covers the concentrations of vancomycin and clindamycin, which can be expected during the course of a regular treatment [20–22]. The concentration range was fitted by a 1/x weighting factor and the origin was included. The linearity was high enough to suggest that the calibration was sufficient. Linearity for vancomycin was demonstrated with $r^2 = 0.998$ for both plasma and synovial fluid. For clindamycin, linearity was demonstrated with $r^2 = 0.996$ and $r^2 = 0.993$ for plasma and synovial fluid, respectively. Measured concentrations were all within 15 % of their nominal values. As shown in the MRM (multiple reaction monitoring) chromatograms in Fig. 1 and Fig. 2, there was no significant endogenous interference in the retention time of each analyte and IS in plasma and synovial fluid, which proved that the selectivity of the method was acceptable.

3.1.2. Limits of quantification

The LLOQ was equal to the lowest calibration standard of 0.5 mg/L for both vancomycin and clindamycin in both matrices with an injection volume of 1 µL. The RSD for precision and accuracy of the LLOQ were within the acceptance value of < 20 %. This indicates that low concentrations of vancomycin and clindamycin in both matrices can reliably be measured. The exact values of the LLOQ are shown in Table 4. The ULOQ corresponded to the highest calibration standard.

3.1.3. Precision and accuracy

All precision and accuracy results met the acceptance criteria, suggesting the ability to achieve comparable analytical results using the same method on identical material under variable conditions. The results of the accuracy, intra- and inter-day precision are shown in Table 4 for plasma and synovial fluid, respectively. The RSD of the accuracy and precision values corresponded to the required value of ≤ 15 %.

3.1.4. Carry-over

The signal of the blank samples measured immediately after the ULOQ calibration standard (50 mg/L), in duplicate, revealed the extent of carryover of samples with higher concentrations to samples with lower concentrations. To reliably measure concentrations up to 50 mg/L of vancomycin and clindamycin in plasma and synovial fluid, the carryover should be < 20 % of the LLOQ. Fortunately, the carry-over for vancomycin and clindamycin was 0 % immediately after injection of the ULOQ standard in both matrices.

3.1.5. Stability

The stability of the analytes in the auto sampler was investigated by storing three QC levels in duplicate at + 15 °C after the first injection, for 24, 48, and 72 h. The recovery of vancomycin and clindamycin after 72 h was between the requirements of 85–115 % in both matrices. In unexpected situations where the analysis has to be postponed, the extracts can still be measured reliably up to 72 h after sample preparation when stored at + 15 °C. The mean recovery of vancomycin and clindamycin

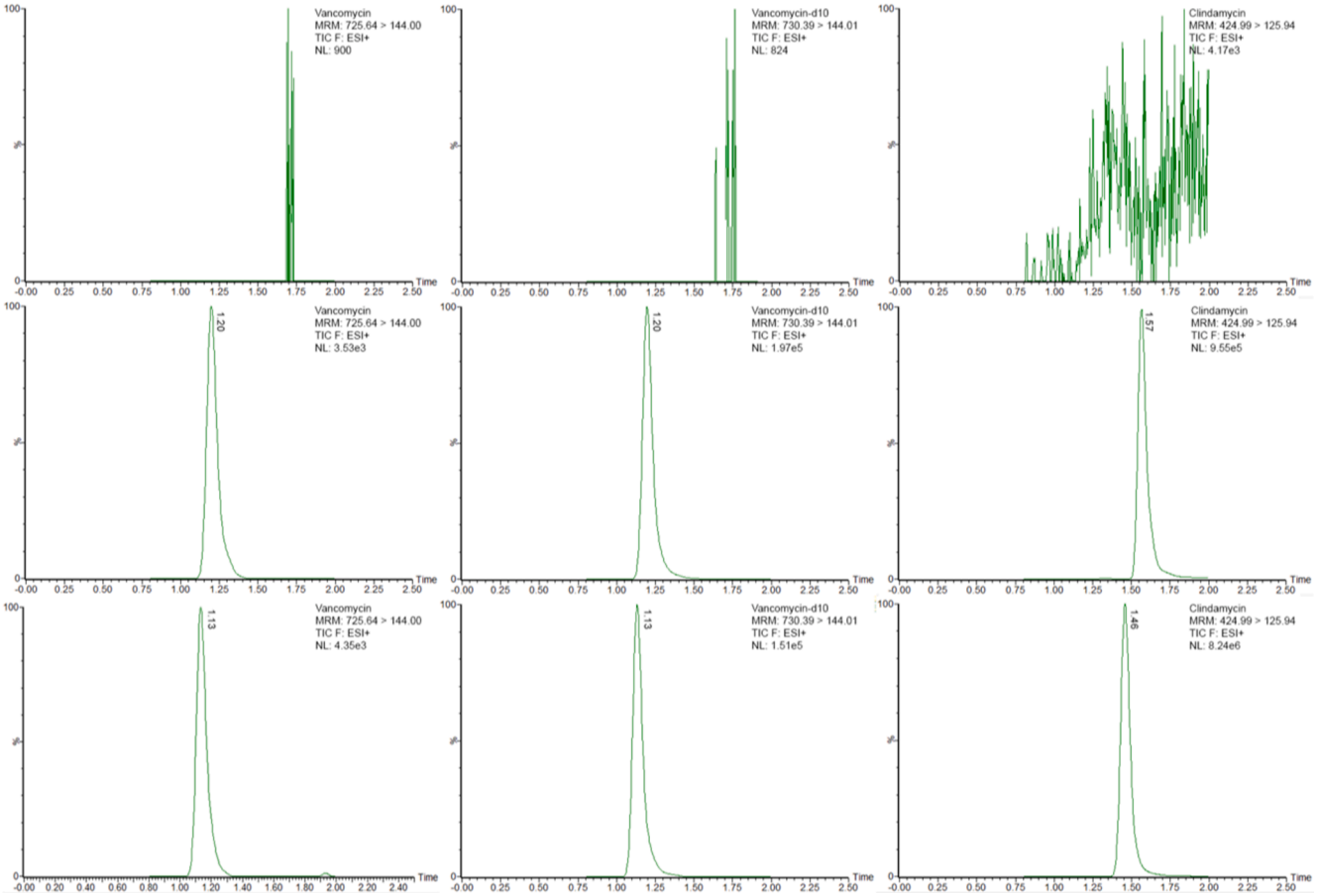

Fig. 2. Representative MRM chromatograms of a blank synovial fluid sample: vancomycin (A1), vancomycin-d10 (A2) and clindamycin (A3). Representative MRM chromatograms of a synovial fluid sample at the LLOQ level: vancomycin (B1), vancomycin-d10 (B2) and clindamycin (B3). Representative MRM chromatograms of a patient's synovial fluid sample: vancomycin (C1), vancomycin-d10 (*C*2) and clindamycin (C3).

Table 4
Accuracy, intra-day and inter-day precision values of vancomycin and clindamycin in plasma and synovial fluid in control materials.

Analyte	Matrix	Level	Concentration (mg/L)	Accuracy (%)	Intra-day precision (%)	Inter-day precision (%)
Vancomycin	Plasma	LLOQ	0.5	6	N/A	15
		QC L	6.3	−1.8	7.3	7.0
		QC M	12.5	1.3	9.1	4.7
		QC H	25.0	1.1	3.3	4.5
	Synovial fluid	LLOQ	0.5	10	N/A	12
		QC L	6.3	−2.8	2.5	4.6
		QC M	12.5	−2.2	4.6	5.2
		QC H	25.0	2.0	4.6	4.0
Clindamycin	Plasma	LLOQ	0.5	−8	N/A	12
		QC L	6.3	5.2	5.8	8.7
		QC M	12.5	0.0	7.1	5.2
		QC H	25.0	−2.1	4.2	7.7
	Synovial fluid	LLOQ	0.5	7	N/A	19
		QC L	6.3	−2.8	6.1	8.4
		QC M	12.5	−4.5	2.9	8.2
		QC H	25.0	−3.1	5.4	8.6

after 72 h is portrayed in Table 5.

3.1.6. Matrix effect and recovery

The matrix effects for clindamycin in plasma were between 80 and 120 %. For clindamycin a decrease in response was observed in synovial fluid and for vancomycin an increase in response was observed in plasma and synovial fluid compared to MilliQ water, indicating matrix influence. The recovery and process efficiency in plasma and synovial fluid were between 80 and 120 % for both analytes. The RSD of the parameters was < 15 %. The extent of matrix influence will be further discussed in the discussion. The data of the matrix effects of plasma and synovial fluid are presented in Table 6.

3.2. Application of the method in patient samples

The validated method was applied in eight participants of the *ASTERICS* study who were prescribed vancomycin or clindamycin and underwent two-stage revision treatment for PJI. Vancomycin and clindamycin in plasma and synovial fluid were successfully quantitated and found to be within the valid range for the assay.

Clindamycin concentrations were determined in plasma and synovial fluid in three patients diagnosed with PJI (Patient A, B, and C). Patient A (age 67, weight 87 kg) received a single bolus injection of 600 mg clindamycin. Plasma samples were obtained 30 min and 90 min after the injection. Synovial fluid was collected after 30 min, unfortunately there was insufficient synovial fluid for the sample collection after 90 min. The clindamycin concentration in plasma was 12.0 mg/L after 30 min and 8.7 mg/L after 90 min. In synovial fluid, the clindamycin concentration was 5.0 mg/L after 30 min, indicating high penetration into the fluid.

Patients B (age 69, weight 100 kg) and C (age 64, weight 92 kg) orally received 600 mg clindamycin three times a day, and plasma and synovial fluid samples were obtained after four weeks of treatment at a steady-state. The plasma samples for patients B and C were 2.1 mg/L and 1.1 mg/L, respectively. In the synovial fluid, 1.8 mg/L clindamycin was measured in patient B and 1.5 mg/L in patient C. At a steady-state, a virtual equal distribution of clindamycin between plasma and synovial fluid is observable. The clindamycin results of patients A, B and C are shown in Fig. 3.

Table 5
The mean recovery of vancomycin and clindamycin in plasma and synovial fluid after 72 h of storage in the auto sampler after sample preparation.

Analyte	Recovery in plasma (%)			Recovery in synovial fluid (%)		
	QC L	QC M	QC H	QC L	QC M	QC H
Vancomycin	106.8	100.3	103.5	98.0	105.8	102.6
Clindamycin	106.5	92.7	109.2	101.8	99.3	95.7

Table 6
Matrix effect, recovery and process efficiency of vancomycin and clindamycin in plasma and synovial fluid.

Analyte	Matrix	Parameter	Matrix effects (%) *B/A*	Recovery (%) *C/B*	Process efficiency (%) *C/A*
Vancomycin	Plasma	QC L	122	92	112
		QC H	118	93	110
		RSD	2.4	0.9	1.5
	Synovial fluid	QC L	119	83	99
		QC H	124	84	104
		RSD	3.2	0.4	3.5
Clindamycin	Plasma	QC L	105	95	100
		QC H	106	93	99
		RSD	0.3	1.5	1.3
	Synovial fluid	QC L	78	116	91
		QC H	88	105	92
		RSD	8.2	7.4	0.8

Patients B and C also had a vancomycin spacer in addition to oral administration of clindamycin. The extent of vancomycin release from the spacer after seven weeks was also determined during the analysis. The vancomycin concentration in synovial fluid due to the spacer release was similar for patients B and C, 0.48 mg/L and 0.47 mg/L, respectively. Two other patients (patients D and E) received different intravenous and oral antibiotics for their treatment of PJI, but they both had a vancomycin spacer. The vancomycin release from the spacer was measured in plasma and synovial fluid during re-implantation of the new prosthesis after approximately-seven weeks. In patients D (age 78, weight 120 kg) and E (age 68, weight 98 kg), respectively 0.7 mg/L and 1.0 mg/L vancomycin were measured in the synovial fluid. In plasma, no traces of vancomycin were detectable in all four patients.

In addition to the vancomycin concentration caused by the spacer, the concentration of intravenous vancomycin administration in plasma and synovial fluid was also determined in three patients with PJI without a vancomycin spacer (Patient F, G, and H). Patient F (age 84, weight 100 kg) and patient G (age 67, weight 109 kg) both received 2000 mg vancomycin as an intravenous bolus. Plasma samples were collected after 30 min and 90 min. Synovial fluid could only be collected after 30 min, due to the limited amount of fluid. For patient F, vancomycin concentration in plasma were 50.0 mg/L and 23.1 mg/L after 30 min and 90 min, respectively. The vancomycin concentration in synovial fluid at 30 min was found to be 1.61 mg/L. Similar concentrations in plasma and synovial fluid were measured in patient G. In plasma, the vancomycin concentrations were 45.8 mg/L after 30 min and 36.7 mg/L

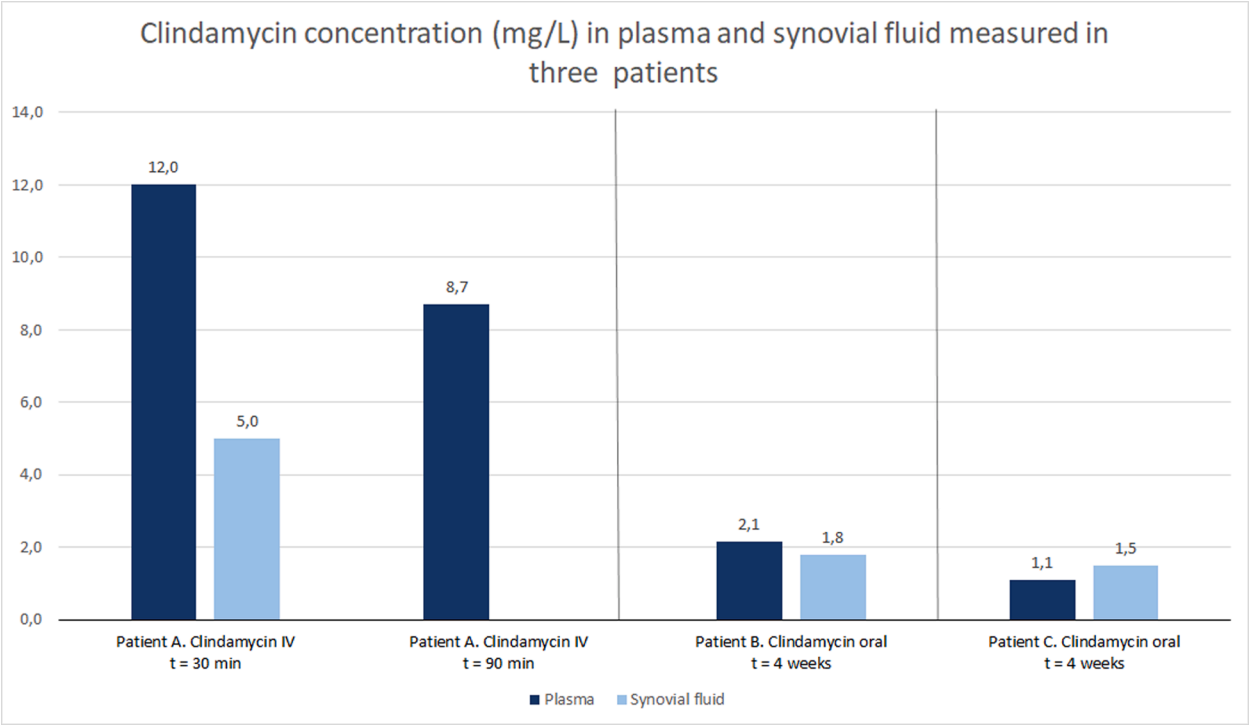

Fig. 3. The measured clindamycin concentration in plasma and synovial fluid in three patients presented in a graph.

after 90 min. In synovial fluid, 1.2 mg/L vancomycin was measured after 30 min.

Finally, the concentration of vancomycin was determined in a patient who received 3000 mg continuously per infusion for two weeks (Patient H, male: age 77, 107 kg). The concentration of vancomycin was observed at a steady-state and penetrated highly into the synovial fluid. The plasma concentration of vancomycin was 26.5 mg/L and the concentration in the synovial fluid was 20.2 mg/L. In Fig. 4, an overview of patients F, G, and H are structurally presented.

4. Discussion

For both antibiotics in both matrices, the parameters, linearity, limits of quantification, accuracy and precision, carry-over, auto sampler- and shelf-life stability, matrix effects, and recovery, measured with UPLC-MS/MS, met the requirements based on the guidelines of the EMA and

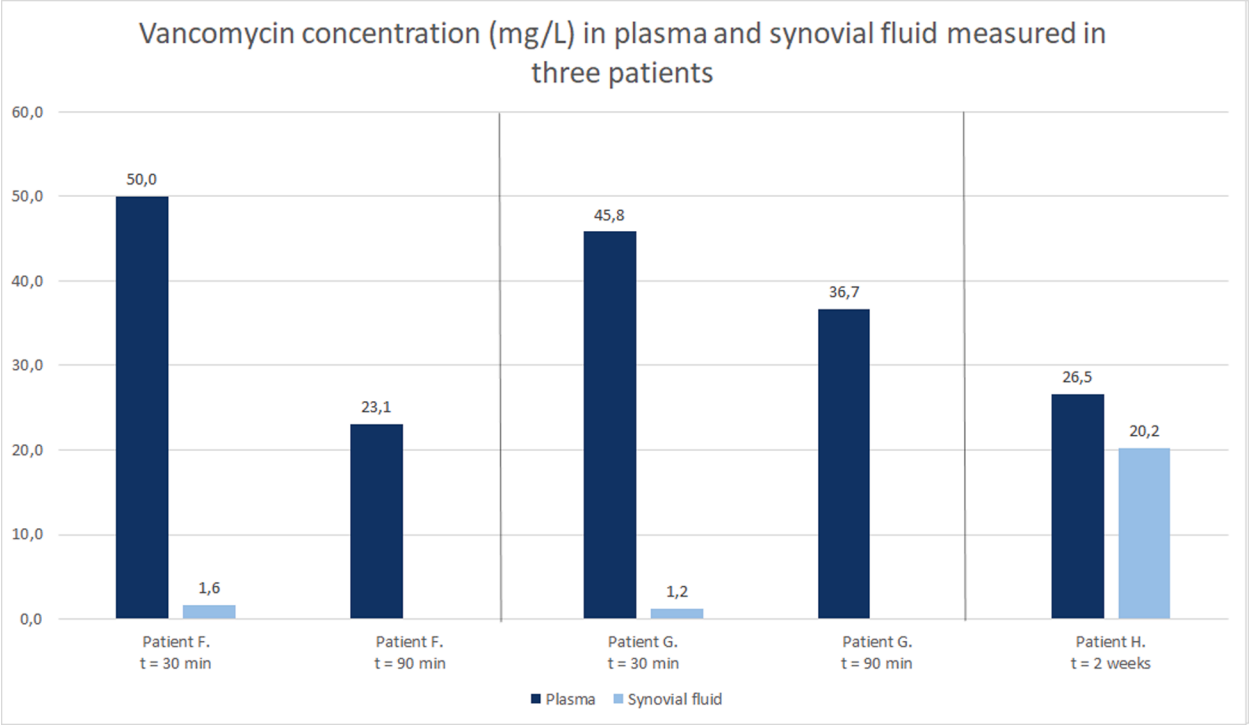

Fig. 4. The measured vancomycin concentration in plasma and synovial fluid in three patients presented in a graph.

FDA [17,18]. The thereby validated method for the quantification of vancomycin and clindamycin in human plasma and synovial fluid was subsequently used to successfully measure the concentrations of vancomycin and clindamycin in several patients with PJI.

The newly developed and validated method allows accurate and rapid determination of two frequently prescribed antibiotics in the treatment of PJI. Clindamycin is frequently prescribed during the four-week oral antibiotic treatment of the two-stage revision. Vancomycin is often prescribed during the two-week intravenous antibiotic treatment of the two-stage revision and is regularly present in the antibiotic-loaded spacer during the entire six weeks of antibiotic therapy. Previous studies have shown that vancomycin concentrations in the antibiotic-loaded spacer are sufficient enough to be measured six weeks after implantation in tissues surrounding the spacer [23,24]. The first results using this method have additionally shown that there are measurable concentrations of vancomycin in synovial fluid (0.5–1.0 mg/L) after a prolonged period due to spacer release. More patients are required to make a more reliable statement about the extent of release of the vancomycin-loaded spacer and its long-term clinical significance in patients with PJI. Nevertheless, at the end of the 6-week treatment, determining the vancomycin concentration at the same time as the clindamycin concentration in the case of vancomycin-loaded spacers has added value. Simultaneously, this method also allows assays to be performed to determine the concentrations of the two-week parenteral administration of vancomycin. Thus far, no other methods have been published in which vancomycin and clindamycin are simultaneously analyzed in plasma or synovial fluid.

The concentration range of 0.5 – 50 mg/L for both clindamycin and vancomycin was decided based upon the target levels of vancomycin and clindamycin in human plasma [20–22]. The range of the validated concentrations and the limits of quantification allow us to determine clinically relevant concentrations in both plasma and synovial fluid. Synovial fluid is an ultra-filtrate of plasma combined with hyaluronic acid [25]. The proteins present in the synovial fluid are mainly derived from the plasma and partly from the cartilage and the synovium. *Bennike et al.* conducted a study comparing the proteome of synovial fluid with the proteome of plasma. 113 of the 149 common proteins in synovial fluid are most certainly derived from the plasma [26]. Due to the high degree of similarity between the synovial fluid proteome and the plasma proteome, in combination with the plasma-derived nature of the synovial fluid, the validated calibration range of the study is also suitable for synovial fluid. However, during joint aspiration, there is a possibility that blood may contaminate the synovial fluid and alter the perceived concentration of the antibiotics in the synovial fluid [27]. In the future quantification of clindamycin and vancomycin in the synovial fluid, the number of erythrocytes could be determined before centrifugation. Determining the number of erythrocytes will provide relevant information about the possible presence of blood and its influence on the concentration of antibiotics in the synovial fluid.

The first results in which vancomycin and clindamycin have been measured in the synovial fluid have already shown that relatively high concentrations are achieved at the infected joint site. Vancomycin is mainly prescribed for positive cultures of (Methicillin-resistant) Staphylococcus aureus in case of PJI, which has a minimal inhibitory concentration (MIC) of 1 mg/L [28]. Initially, the vancomycin concentration is much higher in plasma than in the synovial fluid (i.e., Patient F: 50 mg/L in plasma and 1.6 mg/L in synovial fluid after 30 min). Long-term use of vancomycin shows that an equilibrium is established between plasma and synovial fluid (i.e. Patient H: 26.1 mg/L in plasma and 20.2 mg/L in synovial fluid after two weeks). The increase of vancomycin in the synovial fluid occurring between t = 30 min and t = 2 weeks predicts good penetration into the infected synovial fluid and exceeds the abovementioned MIC value. In the study of *Eshkenazi et al.* average peak synovial fluid levels of 19.0 mg/L were reported [29]. In other studies, synovial fluid levels between 6.0 and 7.0 mg/L were found [30,31]. However, in these studies, vancomycin was not determined with use of HPLC-MS (high-performance liquid chromatography mass spectrometry), but with less specific assays (i.e. immunoassay).

With clindamycin, on the other hand, a high concentration can be measured in the synovial fluid immediately after the first dose (i.e. Patient A: 5.0 mg/L in synovial fluid after 30 min) and an almost equal distribution between the plasma and synovial fluid is observable after 4 weeks. (i.e., Patient B: 2.1 mg/L in plasma and 1.8 mg/L in synovial fluid). The findings from the first results are consistent with previous studies, in which an equal distribution between the plasma and synovial fluid was also observed [32,33]. The MIC of clindamycin for both S. aureus and S. epidermidis is 0.125 mg/L. The first results already show that this value has been exceeded. More research on the penetration into the synovial fluid is necessary, as the requirement for the use of an antibiotic in PJI is the knowledge of its ability to penetrate the synovial joint at bacteriologically active concentrations.

A common problem when using ESI as an ionization technique is the signal suppression or enhancement of the analytes due to matrix effects [34]. Prevention of potential matrix effects was performed by using an optimal gradient program, a suitable chromatographic column, and an isotopic analog as IS. The matrix effect was determined by comparing three known concentrations of vancomycin and clindamycin in MilliQ (set A) with five batches of spiked blank plasma and synovial fluid (set B). Ion enhancement for vancomycin in plasma and synovial fluid (matrix effect $\geq$ 120 %) and ion suppression for clindamycin in at QC low in synovial fluid (matrix effect $<$ 80 %) was observed. The presence of other components in the matrix and the difference in pH between MilliQ and biological matrices are possible factors that may contribute to this phenomenon [35]. In addition, differences between the five batches were observed, especially in synovial fluid, indicating inter-patient differences regarding the sample composition of the biological matrices. Even though the matrix effects for vancomycin shows ion enhancement and for clindamycin ion suppression, the recovery and process efficiency for both analytes in both matrices meet the requirements, indicating adequate accuracy and precision for the overall method.

Moreover, we quantified the total fraction of clindamycin and vancomycin in plasma and synovial fluid, consequently, no statements can be made about the free fraction. The efficacy of an antibiotic is influenced by protein binding since only the unbound fraction is active. For vancomycin, protein binding can variate from$<$10 % to nearly 100 %. The variation in protein binding may be due to factors that affect serum proteins, such as obesity and critical illness [36–38]. The protein binding of clindamycin varies between 40 and 94 %, depending on the concentration [39]. Given the wide range of protein binding, developing a reliable method to determine the free fraction of vancomycin and clindamycin is challenging [40].

Over the years, several studies have examined the total fraction of vancomycin and clindamycin in different matrices (e.g., plasma, synovial fluid, and bone tissue) [32,33,41,42]. Therefore, comparing the results of the total fraction of vancomycin and clindamycin in the treatment of PJI to the results of the other studies is of great value. Further optimization of the method must be performed, leading to more reliable results of the free fraction of vancomycin and clindamycin in the future. To gain more insight into the exposure of vancomycin and clindamycin at the infection site in patients with PJI, a method will also be developed to measure vancomycin and clindamycin in synovial tissue and bone tissue.

5. Conclusions

An accurate method for the determination of both vancomycin and clindamycin in human plasma and synovial fluid on the UPLC-MS/MS was developed, optimized, and validated. Results of the validation in plasma and synovial fluid affirm the analysis to be sensitive, accurate, and precise over a concentration range of 0.5–50 mg/L with a sample volume of 50 μL. This method provides an efficient way to measure and

compare antibiotic concentrations in plasma and antibiotic concentrations in synovial fluid in patients with bone and joint infections, such as PJI.

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CRediT authorship contribution statement

L.T. Ringeling: Methodology, Investigation, Writing – original draft, Writing – review & editing. **S. Bahmany:** Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **J. van Oldenrijk:** Conceptualization, Writing – original draft. **P.K. Bos:** Conceptualization, Supervision. **E.S. Veltman:** Conceptualization, Writing – original draft. **B.C.P. Koch:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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