Pharmacokinetics of Vancomycin in Porcine Cancellous and Cortical Bone Determined by Microdialysis

Research Year Thesis

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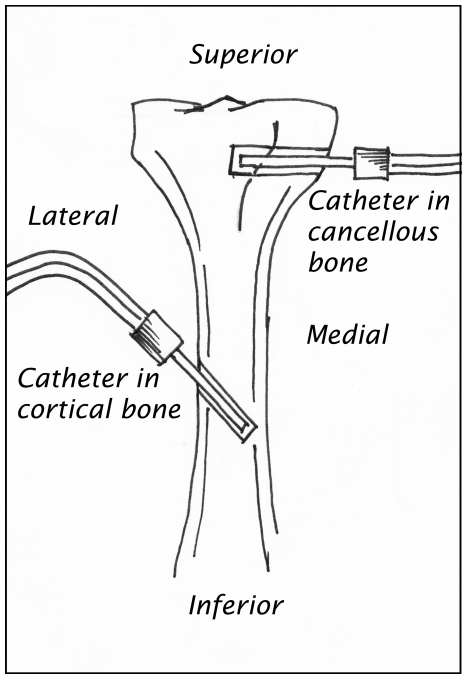

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Summary English

Bone and implant-associated bone infections remain a major health care problem. Treatment is difficult and it often fails. Insufficient bone penetration of antimicrobials may partly account for these therapeutic failures. Historically, assessment of bone pharmacokinetics of antimicrobials has been a challenge due to considerable limitations of the used methods. The well-known pharmacokinetic tool, microdialysis, is an appealing method, with potential, to generate more usable and valid pharmacokinetic data.

The main objective of this study was to use microdialysis to obtain basic pharmacokinetic parameters for vancomycin in subcutaneous tissue, cancellous and cortical bone in a porcine study. We found a heterogeneous and delayed distribution of vancomycin from plasma to bone. Both cancellous and cortical bone area under concentration-time curve (AUC) and peak drug concentration (C_{max}) were significantly lower than those of plasma ($P < 0.01$). Additionally, the same parameters were found to be significantly lower in cortical than in cancellous bone ($P < 0.025$), suggesting that cancellous and cortical bone may not be considered as one homogeneous compartment. An impaired and delayed penetration of vancomycin to bone indicates that timing and dosing of vancomycin in an orthopaedic setting is of importance.

Summary Danish

Infektioner i knogle, med eller uden proteser implanteret, udgør fortsat et stort problem i vores sundhedssystem. Behandlingen er vanskelig og svigter ofte. Insufficient penetration af antibiotika til knogle spiller muligvis en rolle ved de mislykkede behandlinger. Historisk set, har det været en udfordring at beskrive de farmakokinetiske parametre for antibiotika i knogle pga. begrænset brugbarhed at den anvendte metode. Det velkendte farmakologiske redskab, mikrodialyse, er en attraktiv metode med potentiale til at generere mere anvendelige farmakokinetiske data.

Hovedformålet med dette studie var at anvende mikrodialyse til at bestemme en række farmakokinetiske parametre for vancomycin i fedtvæv samt i spongiøs og cortikal knogle i et grisestudie. Vi fandt en heterogen og forsinket fordeling af vancomycin mellem plasma og knoglevæv. Der var signifikant lavere areal under koncentrationsskurven (AUC) og maksimum koncentration (C_{max}) i både spongiøs- og cortikal knogle ($P < 0.01$) sammenlignet med plasma. Ydermere fandtes disse farmakokinetiske parametre ($P < 0.025$) lavere i cortikal end i spongiøs knogle, hvilket indikerer at knogle muligvis ikke bør anses

som ét fast organ. Langsom og nedsat penetration af vancomycin til knogle indikerer at at timing og dosering af vancomycin er vigtig i ortopædkirurgisk sammenhæng.

Manuscript for article

Title

Pharmacokinetics of Vancomycin in Porcine Cancellous and Cortical Bone Determined by Microdialysis

Abstract

Background: Antimicrobial penetration to bone remains sparsely explored. Impaired tissue penetration may partly account for failures when treating orthopaedic infections. Valid knowledge regarding antimicrobial bone-pharmacokinetic is therefore warranted.

Methods: Eight female pigs were included in the study. The pigs received 1,000 mg vancomycin over a 100 min period. Within plasma, subcutaneous tissue, cancellous and cortical bone pharmacokinetics were investigated for 12 hours. Microdialysis was applied for measurements in bone and subcutaneous tissue. Free concentrations of vancomycin in plasma were determined with Cobas c501, and measurements in dialysates were conducted with Ultra High Performance Liquid Chromatography (UHPLC).

Results: An impaired and delayed distribution of vancomycin from plasma to bone was found. For both bone compartments a lower AUC and C_{max} were found when compared to plasma ($P < 0.01$). Moreover both AUC and C_{max} were significantly lower in cortical bone than in cancellous bone ($P < 0.025$). Finally, T_{max} revealed a delayed penetration from plasma to bone.

Conclusions: Microdialysis is a reliable method for sampling of vancomycin in bone tissue. Decreased and delayed penetration of vancomycin to bone was found in this porcine study. Furthermore, cancellous and cortical bone may not be considered as one compartment.

1 Introduction

The concentration of unbound antimicrobials in plasma has previously been considered to provide a valid surrogate marker for the concentration in the interstitial space of tissues [1, 2]. Over the last decades, however, this assumption has been challenged by repeated findings of heterogeneous and incomplete tissue penetration for a number of different antimicrobials[3-9]. It is now widely appreciated that sufficient antimicrobial exposure at target site is a prerequisite for a successful therapeutic outcome. Treatment failure and recurrence rates remain high for osteomyelitis and implant-associated infections (IAI) despite long-lasting antimicrobial therapy and surgical debridement, suggesting that insufficient antimicrobial bone penetration may be one explanation[10].

For bone tissue, antimicrobial penetration remains poorly elucidated. So far, the majority of studies addressing this issue have done so by measuring the concentration in homogenized bone specimens[11]. This approach does, however, suffer from a number of methodological limitations, which may reduce the value of the findings[11, 12].

During the last decade, microdialysis (MD) has emerged as a promising tool for assessing bone pharmacokinetics of antimicrobials[13-20]. MD allows for continuously sampling of the unbound and active fraction of drug in the interstitial space. Thus, MD seems to solve some of the inherent limitations of bone specimens.

Vancomycin is a glycopeptide antimicrobial, used prophylactically and in the treatment of infections caused by susceptible gram-positive microbes. In the daily clinical setting, the ratio of area under concentration-time curve (AUC) for free plasma concentration and the minimal inhibitory concentration (MIC) may be the best predictor of efficacy [21-24]. A target ratio of AUC/MIC > 400 have been associated with therapeutic success in a clinical setting[25]. Recently published *Vancomycin Therapeutic Guidelines* supports this finding by recommending the achievement of a ratio larger than 400 when treating Staphylococcus aureus infections[24].
Protein binding is generally considered to be in the range of 30-60 % and the terminal elimination half-life of vancomycin is 3-9 hours[26, 27].

Vancomycin tissue penetration has been investigated for a variety of tissues[28-33]. For bone, penetration was found to be poor and highly diverse[32, 33]. All studies assessing bone penetration have done so by means of bone specimens[11].
In bone tissue, the majority of infections are caused by gram-positive microbes[34, 35]. Consequently, vancomycin is an important drug prophylactically and in the treatment of osteomyelitits and IAI, either as first choice or in cases where Methicillin Resistant Staphylococcus Aureus (MRSA) has been documented [10, 36].

The objective of this study was to investigate plasma, subcutaneous tissue (SCT) and bone pharmacokinetics of vancomycin using MD in a large animal model. Based on previous differences regarding penetration to cortical and cancellous bone for cefuroxime, separate measurements were conducted for these two compartments[19, 20]. Additionally, an *in vitro* experiment was initially conducted in order to assess the suitability of the MD-method for vancomycin measurements.

2 Materials and methods

This experimental study was conducted at the Institute of Clinical Medicine, Aarhus University Hospital Denmark. The surgical procedures were performed under the approval from The Danish Animal Experiments Inspectorate and carried out according to existing laws. All chemical analyses were performed at Department of Biochemistry, Aarhus University Hospital.

2.1 Microdialysis

The principles of MD have been described in details elsewhere[37, 38]. In brief, the method is based on sampling of analytes by means of a semipermeable membrane at the tip of a MD probe[6]. The diffusion takes place along the concentration gradient. Due to continuous perfusion of the probe, completely equilibrium will never be obtained. Accordingly, the concentration in the dialysate will only represent a fraction of the true concentration in the tissue. This fraction is referred to as relative recovery (RR). The RR has to be assessed for correction of the values obtained in order to achieve information regarding the absolute tissue concentrations. A number of calibration procedures have been described to determine RR. As inter-probe variation of RR is common, and RR is dependent on local tissue factors individual *in vivo* probe calibration is imperative[39]. In the present study, CMA 63 probes (membrane length 10 mm, molecular cut-off 20 kilo Daltons) from μ-Dialysis AB

(Stockholm, Sweden) were used, and CMA 107 precision pumps produced the flow rates of 1 µL/min and 0.5 µL/min for the *in vitro* and the *in vivo* experiments, respectively

In the *in vitro* experiments, the following equations were used to calculate relative recovery by gain (RR_{gain}) and by loss (RR_{loss}):

$$RR_{gain}(\%) = 100 \times (C_{dialysate} \times C_m^{-1})$$

$$RR_{loss}(\%) = 100 \times (1 - C_{dialysate} \times C_{perfusate}^{-1})$$

where $C_{dialysate}$ is the concentration in the dialysate, C_m the concentration in the media surrounding the probe and $C_{perfusate}$ is the concentration in the perfusate.

In the porcine study, the same equations were used with C_{tissue} representing C_m. The absolute tissue concentrations were calculated by correcting for RR using the following equation:

$$C_{tissue} = C_{dialysate} \times RR^{-1}$$

where C_{tissue} is the concentration found in the tissue.

Individual probe calibration was performed for all probes at location.

In the data analysis, measured dialysate concentrations were ascribed to the center point of the sampling interval.

2.2 Preliminary in vitro experiments

Isotonic saline solutions containing vancomycin concentrations of 1 and 25 µg/mL were used to determine *in vitro* RR_{gain} and RR_{loss}. Blank isotonic saline were used as media for determination of RR_{loss} and were used as perfusion fluid for determination of RR_{gain}. In the same experiment, the effect of temperature was assessed. Using 40-min intervals, 3 samples of 40 µL were harvested for both RR_{gain} and RR_{loss} at each concentration. This study was conducted at both 22 and 37°C ± 1°C, respectively. An equilibration period of 15 min was allowed for whenever the experimental design was switched from RR_{gain} to RR_{loss}. The same probe was used for both RR_{gain} and RR_{loss} for the same concentration at different temperatures. Using two catheters the entire experiment was conducted over two days.

2.3 Porcine study

2.3.1 Animals, anaesthetic and surgical procedures

Eight female pigs were included in the study (Danish Landrace Breed 65-75 kg). A combination of fentanyl (0.4 - 0.6 mg/h, continuous infusion), propofol (200 - 400 mg/h, continuous infusion) and sevoflurane (minimal alveolar concentration: 1.1%±0.1) maintained the anaesthesia. Arterial pH was kept within the range of 7.36 – 7.46 throughout the study by regulating ventilation. Core temperature was kept within the range of 36.5°C - 39.5°C by using Bair Huggers. Before inclusion in the study, normal kidney function was confirmed by evaluating plasma creatinine.

MD probes were placed in drill holes in cancellous bone (depth 20±1 mm) in the tibia condyles by a medial approach and in the cortical bone (depth 15±0.5 mm) at the anterior margin of the tibia by an anteromedial approach. In both bone compartments, a 2 mm drill was used to make the holes. Drilling was paused every few seconds to prevent overheating of the tissue. All operations were made in the left tibia. Additionally a probe was placed in the SCT of the abdomen using an introducer according to the guidelines of the manufacturer. Correct location of the probes was evaluated visually by autopsy. After euthanasia, the entire tibia of each pig was harvested for post mortem CT-scans to evaluate correct intra-cortical location of the drill holes.

2.3.2 Sampling procedures

Immediately after placement, the probes were perfused with isotonic saline containing vancomycin at a concentration of 2.5 μg/mL, and a 30-min tissue equilibration was allowed for. The calibration procedure were then started using the retrodialysis method by collecting two 60-min samples[40]. The mean of these two samples was used for recovery calculation in the subsequent data analysis. Following calibration, the perfusate was changed to blank isotonic saline, and a 180-min washout period was allowed for. The system was flushed (15 μl/min) for 5 minutes at times 0, 15, 30 and 45 minutes in the washout period. Three dialysates were collected in the intervals 60-100 min, 100-140 min and 140-180 min of the washout period to evaluate the effectiveness of washout. 1,000 mg of vancomycin were then administered intravenously over a 100 min period. Due to the low flowrate and the ambition of not sampling dialysate, which already had passed the membrane, another 10 minutes was awaited before start of the sampling. Thus, the first dialysate was harvested between 10 - 50 min after initiation of vancomycin infusion. The first blood sample was drawn 25 min after

vancomycin administration. The first three dialysates were harvested with 40 min intervals, and thereafter with 60 min intervals for the following 10 hours, giving a total of 13 samples in a sampling period of 12 hours. Venous blood samples were drawn from a central venous catheter. Except for the first blood sample, all blood samples were drawn in the middle of a dialysate sampling interval.

Dialysates were instantly frozen and stored at -80 °C until analysis. Venous blood samples were stored at 5°C for a maximum of 6 hours before being centrifuged at 3,000 *g* for 10 minutes. Plasma aliquots were then frozen and stored at -80°C until analysis.

2.4 Chemical analysis of vancomycin

The free concentration of vancomycin in plasma was measured with a homogen enzyme immunoassay-technic on the Cobas c501 platform (Roche, Switzerland). Intrarun (total) impresicions [percent coeficient of variation (%CV)], for this assay were 2.5 (3.0) at 16.7 µg/mL and 3.7 (4.4) and 61.0 µg/mL.

Measurement of vancomycin in dialysates was performed with Ultra High Performance Liquid Chromatography (UHPLC). Standards for analysis consisted of vancomycin hydrochloride (Sigma-Aldrich, Denmark) diluted in 0.9 % isotonic saline, resulting in concentrations of 2.6, 5.2 and 10.3 µg/mL. Before analysis, 15 µL of dialysate or standard was mixed with 20 µL 10 mM phosphate buffer, pH 3 (NaH2PO4, H2O adjusted with HCl; Merck, Germany). The UHPLC system (Agilent 1290 Infinity; Agilent Technologies, USA) was equipped with a 1.7 µm 100 by 2.1 mm C18 column (Kinetex; Phenomenex, USA) which was pre-heated to 30 °C. Chromatography was performed with a gradient of acetonitrile (0 to 15% over 4 min) in 10 mM phosphate buffer, as the eluent. For analysis, 5 µL prepared sample was injected into to the UHPLC-system, and vancomycin was detected with a diode array detector at 280 nm. Calculation of the vancomycin concentrations was based of the peak areas of vancomycin, and was performed, with the ChemStation Software (Agilent Technologies, USA). Intra-run (total) imprecisions [percent coefficients of variation (%CV)] were 3.7% (5.7%) at 0.7 µg/mL, 3.0% (3.5%) at 3.7 µg/mL and 0.9% (2.2%) at 5.2 µg/mL. The limit of quantification was defined as the lowest concentration with intra run CV < 20%, and was found to be 0.05 µg/mL.

2.5 Pharmacokinetic analysis and statistics

Pharmacokinetic parameters were determined separately for each subject by non-compartmental analysis (NCA) using Stata (version 12.0; Statacorp, USA). The washout

concentrations were low, and therefore they were neglected in the analysis. The area under the concentration-time curve (AUC) for the sampling period was calculated using the trapezoidal rule. The terminal half life ($t_{1/2}$) was calculated as $\ln(2)/\lambda_{eq}$, where λ_{eq} is the terminal elimination rate constant estimated by linear regression of the log concentration on time. The appropriate number of points used for the calculation was determined by inspection of the individual concentration-time profiles.

Overall comparison of pharmacokinetic parameters was conducted using oneway ANOVA with a random animal effect. Post hoc pairwise comparisons were made for cancellous and cortical bone versus free plasma and for cancellous versus cortical bone. For the $T_{1/2}$ data, transformation to log scale improved normality. Consequently, values are given as medians with 95%-CIs, while the pairwise comparisons were conducted on the log scale. A P-value < 0.05 was considered significant. No correction for multiple comparisons were applied. Statistical analyses were also performed using Stata (version 12.0; Statacorp, USA).

3 Results

3.1 Preliminary in vitro experiment

Mean RR_{gain} and RR_{loss} for vancomycin were 38.3% and 32.4% at 1 µg/mL and 35.4% and 34.0% at 25 µg/mL, respectively. When assembling data for every probe ($RR_{gain+loss}$), mean $RR_{gain+loss}$ at 1 µg/mL were 34.1% at 22°C±1°C and 36.6% at 37°C±1°C. At 25 µg/mL $RR_{gain+loss}$ were 31.6% at 22°C±1°C and 37.8% at 37°C±1°C. See Figure 1a and 1b.

3.2 Pharmacokinetics of vancomycin in SCT, cancellous and cortical bone

All eight pigs completed the study. For the first two pigs, however, RR could not be reliably determined, and as such, they were excluded from analysis. Except for one cancellous bone probe, which was malfunctioning, data was obtained from all probes from the remaining 6 pigs. CT-scans confirmed pure intra-cortical location of all cortical drill holes.

Mean (±SD) RRs were 17.5±5.6%, 26.5±11.1% and 20.4±11.0% for SCT, cancellous and cortical bone, respectively. For the same positions, the mean (±SD) concentrations in the last washout samples were 1.28±0.98 µg/mL, 0.59±0.53 µg/mL and 0.93±1.1 µg/mL, respectively.

Tissue and plasma concentration-time profiles are shown in Figure 2. The corresponding pharmacokinetic parameters can be found in table 1. The mean (95%-CI) $AUC_{0\text{-}last}$ were 9375 (7445; 11304) for plasma, 9304 (7374; 11233) for SCT, 5998 (3955; 8040) for

cancellous bone and 3451 (1522; 5381) for cortical bone (ANOVA $P < 0.001$). Both cortical and cancellous bone AUC were significantly lower than that of free plasma ($P < 0.01$). Moreover, cortical bone AUC were lower than cancellous bone AUC ($P = 0.013$). For C_{max}, statistical significant differences among the means were also found, and again, both cortical and cancellous bone C_{max} were lower than that of free plasma. Further, C_{max} was lower in cortical compared to cancellous bone ($P = 0.025$).
T_{max} (95%-CI) were 90.0 (63.7; 116.3) for plasma, and thereby significantly lower than for cancellous borne 172.0 (143.2; 200.8) and for cortical bone 230.0 (203.7; 256.3).

4 Discussion

This is the first article to report vancomycin bone pharmacokinetics obtained by means of MD. The main finding is that vancomycin bone penetration is impaired and delayed, and that this is most pronounced for cortical bone. An AUC/MIC ratio of 400 or above has been shown to be a good predictor of therapeutic success. However, for bacterial MICs of ≥ 2 mg/L, a target of ≥ 400 is probably not achievable with standard dosing regimens of vancomycin. In this context, incomplete tissue penetration may have the same effect as increasing MICs, namely insufficient vancomycin exposure at the site of infection.

MD has previously been successfully applied for measurements of antimicrobials in cortical as well as cancellous bone[13-20, 41]. In the preliminary *in vitro* study we found that RR_{gain} equalled RR_{loss} for the two concentrations and was independent of the concentration. Additionally, vancomycin transport across the membrane was fast, and no adherence to the probes was observed (unpublished data). This is in agreement with previously published studies [30, 31]. Based on these findings, *in vivo* vancomycin experiments were considered feasible.

To date, studies of vancomycin bone penetration have only been performed using bone specimens. Bone specimens suffer from a number of methodological limitations, which can make it hard to evaluate[11, 12]. Landersdorfer et al. illustrated how widely the range of mean bone concentration may vary using this method; e.g. Kitzes-Cohen et al. found a 8-10 fold higher concentration in sternal bone than Vuorisalo et al., although they both administered vancomycin at a similar dose[11, 32, 33].
The making of drill holes, which is necessary for insertion of the MD probes in bone, creates a dead space around the probe. Nevertheless, for gentamycin, cefuroxime and metabolites, it

has been shown that measurements in drill holes in bone is reflective of the actual bone concentrations[20, 41, 42]. Altogether, MD seems to be an attractive alternative to bone specimens.

Several studies have demonstrated how tissue penetration for different antimicrobials and tissues, under both physiological and pathological conditions, may be incomplete and heterogeneous[3, 5-7, 9, 28, 31, 43, 44]. This underlines why pharmacokinetics of virtually all antimicrobials ideally should be characterized both in a specific tissue and for a specific condition before use.

In this porcine study, we found a delayed and impaired penetration of vancomycin from plasma to bone. When comparing the two bone compartments with plasma, significant differences in AUC and C_{max} revealed a decreased penetration to bone and differences in T_{max} demonstrated a slow penetration. This suggests that timing and dosing of antibiotics in an orthopaedic setting may be of importance.

Furthermore, AUC and C_{max} values differed significantly between cancellous and cortical bone which may indicate that cancellous and cortical bone might be considered as two distinct compartments. This is supported by our previous studies where cefuroxim also showed reduced penetration to bone[19, 20].

A major limitation to this study is the obviously fact, that this was an experimental study and not a clinical study. As the terminal elimination half-life time of vancomycin is 3-9 hours[26] and we only obtained measurements for 12 hours, we could not determine AUC at steady-state over 24 hours. As such, we could not determine AUC/MIC and cannot extrapolate our results directly to humans. Nevertheless, we found plasma concentration measurements in the range of 3.5-45.6 mg/L and in subcutis 3.0-46.6 mg/L. These ranges resemble concentrations found in plasma and subcutis for humans[23, 31].

Furthermore, the pigs were anaesthetized during the entire study. This is known to cause physiological changes that may alter pharmacokinetics.

Low RR-values are more exposed to standard deviations associated with pre-analytical handling and chemical analysis, why it's recommended that RR should exceed 20%[39]. With decreasing RR the resulting variations will increase exponentially. When interpreting results obtained by MD, this possible limitation should always be taken into account. In the present study, we found and average RR of 23.4% for MD measurements in bone. Due to

limitations by the depth of the drill holes and so membrane length, an average bone RR of approximately 23.4% seems acceptable.

In conclusion, we have demonstrated that MD is a reliable method for sampling of vancomycin and our study brings new knowledge about the penetration of vancomycin into bone.
Our findings of vancomycin bone concentrations are the first results that correspond to unbound concentrations found in other compartments.
The substantially impaired and delayed bone penetration of vancomycin demonstrated in this study is noteworthy and may partly explain the rather high rate of failures in treatment of osteomyelitis and IAI. Clinical trials are warranted to evaluate AUC/MIC in bone. However, pharmacokinetic studies alone cannot replace large clinical trials with clinical endpoints.
Further, it seems relevant to conduct experiments to assess bone penetration by means of other infusion settings for vancomycin, e.g. continuous infusion as well as studies evaluating the penetration of vancomycin into infected bone. More knowledge about vancomycin bone pharmacokinetics may enable evidence-based dosing regimens in the future. If the grade of bone penetration, time course of bone concentration and clinical outcome can be evaluated, the most suitable PK-target for treatment of a bone infection can be derived[11].

5 Funding

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Supplementary information

Background

Overview

Bone and implant-associated bone infections (IAI) are a major problem in the Danish health system. In Denmark, more than 85.000 orthopaedic operations are performed annually and approximately 2% of these are followed by a bacterial infection[45]. Appropriate management of e.g. osteomyelitis require long-lasting antimicrobial therapy, often in combination with surgical intervention[10]. Treatment is highly complicated and difficult, and is often associated with considerable sustained and/or permanent morbidity for the patients[10]. Consequently, treatment of orthopaedic infections can be very costly for the patient as well as the health system

Proper antimicrobial dosing is essential when treating orthopaedic infections. Therapeutic exposure at the site of infection (i.e. target site) must be achieved, to optimize bacterial killing and prevent development of resistance. However, it is difficult to make accurate and valid measurements of the concentration of antimicrobials in bone. Accordingly, dosing, when treating orthopaedic infections, is based on tradition and expert opinion rather than scientific evidence.

The available knowledge of antimicrobial penetration into bone is minimal and has limited usability due to methodological limitations of the used methods.

To overcome some of these limitations, our research group has applied the well-known pharmacokinetic tool microdialysis (MD) for measurement of antimicrobials in bone[19, 20]. In this research-year-project, we used MD to obtain pharmacokinetic parameters for vancomycin in subcutaneous tissue, cancellous and cortical bone and compared them to those found in plasma in a large animal model. Based on previous findings of different cancellous and cortical bone penetration of cefuroxime, measurements were conducted separately in these two locations [19, 20].

Measurement of antimicrobials in bone

Over the last decades, several studies have demonstrated how tissue penetration for different combinations of antimicrobials and tissue, under both physiological and pathological conditions, may be incomplete and heterogeneous[3-9]. The pharmacokinetic (PK) parameters for antimicrobials found in plasma do not necessarily equal the PK-parameters found in extravascular tissue. An ideal antimicrobial therapy is characterized by the

achievement of therapeutic concentration at target site, while concentrations in other tissues should be kept as low as possible to avoid side effects. Accordingly, US FDA recommends that manufacturers of antimicrobials assess target sites distribution of the drug[46].

This understanding has increased the need for development of new methods to determine concentrations of antimicrobials outside the bloodstream. When it comes to bone, the available knowledge of bone pharmacokinetics of antimicrobials is limited. So far, biopsies have been the predominant method to determine penetration of antimicrobials into bone. However, this method has considerable limitations, and seems unsuitable for the task[11, 12].

Bone biopsy

In the vast majority of bone penetration studies, PK-analysis have been done by comparing concentrations found in plasma with those found in bone samples. When taking a biopsy from bone, there is a list of steps from gathering the material to analysis that must be conducted before a result can be found. At first, the bone sample is resected. The sample often contains a lot of blood, as the sample is taken during surgery. Blood, bone marrow and soft tissue are often removed from the sample. An antimicrobial with poor tissue penetration can show artificial high bone concentrations, unless excess blood is removed. Next, the sample is homogenized and the drug is extracted. The homogenization-process can be done a variety of ways. As a consequence of method, bone homogenate will represent the total antimicrobial concentration (including both the intra- and extracellular fraction), and not the unbound pharmaceutical active fraction. Further, results from a bone homogenate cannot distinguish between concentrations in different bone compartments. Conclusively, the total antimicrobial concentrations found by means of bone biopsy represent the total antimicrobial concentration in bone homogenate[11].

After extraction the concentration is measured, and also the choice of method for measuring the antimicrobial concentrations may vary depending on the investigated drug. Moreover, the bone biopsy method often only allows for one bone sample per patient. This makes the timing of both the antimicrobial dosing and the biopsy harvesting very crucial to the method. If taking a biopsy after a bolus administration of an antimicrobial e.g. with a short terminal half life, it can be very difficult to know at which point of the concentration-time profile, the biopsy is taken. As such, harvesting of one (or few) sample(s), removes the course of time from the method. Thereby, it is difficult to evaluate the pharmacokinetics and thus

antimicrobial penetration to bone, as both time > minimal inhibitory concentration (MIC) and the ratio of area under concentration-time curve (AUC) and MIC cannot be determined.
At present no explicit guidance or standardization of when and how to perform these steps from harvesting a bone biopsy to analysis have been published. Landersdorfer et al. calls for new techniques to give more insight[11].

Alternative techniques

Radiolabelled drug molecules – Positron emission tomography, PET

An alternative or supplement to the bone biopsy method might be the method of monitoring radiolabelled drug molecules using PET[11]. In healthy volunteers, Fischman et al. investigated bone pharmacokinetics of [^{18}F]trovafloxacin and [^{18}F]leroxacin[47, 48]. Cremieux et al. investigated [^{14}C]sparofloxacin in an experimental infection model in rabbits[49].
This method does not have the potential to distinguish between intra- and extracellular concentrations of antimicrobials. Only total antimicrobial concentration per bone mass can be determined. Moreover, this method has some practical challenges and limitations, and ethical considerations due to involvement of a PET-scan for every measurement. As it allows for multiple measurements, time course is available, and thus, it may provide more valuable information of bone pharmacokinetics.

Microdialysis

Microdialysis (MD) has in a number of studies, in different tissues, been used to determine the unbound and thus active concentrations of antimicrobials [9, 30, 50-53]. MD allows for continuously sampling from the interstitial space and thus, can offer information about the pharmaceutical active fraction of antimicrobial as a function of time. Sampling is done within the same animal/human without causing any pain or practical issues to the animal/human. Thus, MD seems to be an appealing method for the determination of bone pharmacokinetics.
Briefly, the technique is performed by placing a small probe with a semipermeable membrane in the tissue of interest. Driven by a small precision pump, the catheter is then continuously perfused with a physiological fluid. The fluid that has passed through the catheter is called the dialysate and can be collected in small vials for subsequent analysis[37]. The diffusion across the MD-membrane takes place along the concentration gradient. A schematic overview of the basic principles of MD is depicted below in Figure 3.

Due to continuous perfusion of the probes, complete equilibrium across the semipermeable membrane will never occur. Thus, the concentration found in the dialysate will only represent a fraction of the actual unbound extracellular concentration. This can be accepted, if the interest is changes and ratios of the concentrations, but in pharmacokinetic studies where determination of absolute concentrations is the main objective, there is a need of correction. As correction, relative recovery (RR) is used. RR is the fraction of the true concentration in the tissue of interest to be found in the dialysate. RR depends on factors such as chemical conditions of the compound being analyzed, diffusion coefficient, perfusion rate and probe-membrane, but is independent of the concentration gradient across the membrane[37]. A number of methods to determine RR have been described. The MD-probes is manufactured for clinical use and are delivered in sterile packaging.

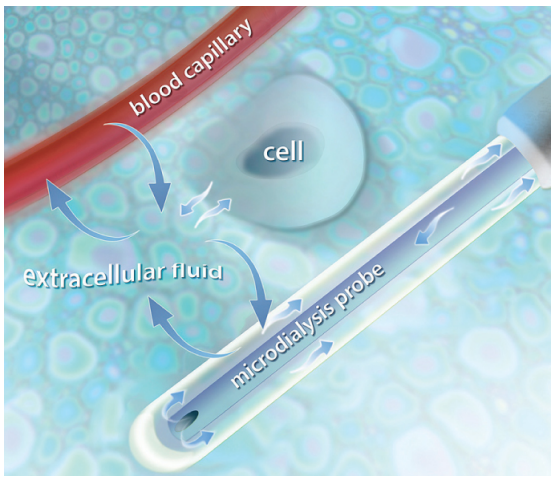

Figure 3. Schematic overview: Basic principle of microdialysis

Recently, MD has been applied and validated for measurement of various antimicrobials in healthy bone[13-17, 19, 20, 41].

Table 2 below illustrates a comparison of the three methods, bone biopsy, radiolabelled drug (PET) and MD, on some of the most important parameters.

Table 2	Invasive	Possibility of continuous measurements	Measurements of unbound and active antimicrobial	Methodological severity	Reproducibility
Bone biopsy	Substantial	No	No	High	Low
PET	No	Yes	No	Medium	High
Microdialysis	Minimal	Yes	Yes	Medium	High

At present, nine studies have been published, in which MD has been used to obtain knowledge regarding antimicrobial concentrations in bone[13-20, 41]. Six of these studies were conducted on pigs[14-16, 19, 20, 41], while three were clinical studies[13, 17, 18]. Stolle et al., found that MD was capable of generating dynamic *in vivo* concentration measurements in bone[15, 41]. There have been no reports of complications with the use of MD-catheters in clinical studies.

Vancomycin

The antimicrobial agent vancomycin has been on the Danish market for over 30 years. The drug is a glycopeptide antimicrobial, today used prophylactically and in the treatment of infections caused by susceptible gram-positive microbes. However, the pharmacokinetic profile of vancomycin in bone has not yet been evaluated.

The PK-parameter to predict efficacy best, is suggested to be the ratio of AUC for free plasma and MIC[21-24]. It has been found that a target ratio of AUC/MIC greater than 400 is correlated with a successful therapeutic outcome in a clinical setting[25]. Furthermore, the recently published *Vancomycin Therapeutic Guidelines*[24] underpins this finding by endorsing the achievement of a ratio > 400 when treating Staphylococcus aureus infections.

Vancomycin has a terminal elimination half-life of 3-9 hours[26] and the protein binding for vancomycin is generally considered to be in the range of 30-60 %[27].

Tissue penetration for vancomycin has been explored for a number of tissues [28-33]. In the particular case of bone, tissue penetration has solely been assessed by means of bone biopsy[11]. The results showed poor and highly diverse penetration [32, 33].

In bone, the gram-positive microbes are responsible for the majority of infections[34, 35]. Consequently, vancomycin is an important drug in both the prevention and treatment of bone infections. Thus, vancomycin may be used when treating osteomyelitits and IAI, either as first choice or in situations where Methicillin Resistant Staphylococcus Aureus (MRSA) has been documented [10, 36].

Studies using MD to assess the penetration of vancomycin into cancellous and cortical bone have not previously been published.

Chemical analysis of vancomycin

Owing to small volumes and low concentrations in the dialysates, the analysis of vancomycin concentration needs to be highly sensitive, accurate and precise. Thus,

assessment of the vancomycin in dialysates was performed with ultra high performance liquid chromatography (UHPLC). Intra-run (total) imprecisions [percent coefficients of variation (%CV)] were 3.7% (5.7%) at 0.7 µg/mL, 3.0% (3.5%) at 3.7 µg/mL and 0.9% (2.2 %) at 5.2 µg/mL. The limit of quantification was defined as the lowest concentration with intra run CV < 20 %, and was found to be 0.05 µg/mL.

Minimal Inhibitory Concentration (MIC) for e.g. staphylococcus aureus is generally between 0.5 and 2 µg/ml[54]. Thus, UHPLC gives the opportunity to measure considerably lower concentrations, than those clinical relevant.

The free concentration of vancomycin in plasma was determined with a homogen enzyme immunoassay-technic on the Cobas c501 platform (Roche, Switzerland). Intrarun (total) impresicions [percent coeficient of variation (%CV)], for this assay were 2.5 (3.0) at 16.7 µg/mL and 3.7 (4.4) and 61.0 µg/mL.

Vancomycin has earlier been found to show good stability over several days at -70°C and over more freeze and thaw cycles[55, 56].

Application in an animal model

In this project we applied MD for measurement of vancomycin in bone in a large animal model. We chose to use a porcine model, because of favorable local conditions for pig experiments. Further, a large pig has large bones, which makes surgery and implantation of MD-probes possible. Moreover, pigs correspond to humans in terms of physiology and anatomy[57].

Methodological considerations

Microdialysis in drill holes

In bone, due to its compact structure, MD probes must be implanted into drill holes in the bone[13-18, 41]. This approach has been questioned – do MD measurements of antimicrobials in drill holes actually reflect bone concentration or rather a mixed concentration originating from the bone and the surrounding tissue? For gentamycin, cefuroxime and metabolites, it has been found that measurements in drill holes in bone do reflect the actual bone concentrations[20, 41, 42]. In one of the studies, the concentration in unsealed drill holes in cortical bone were compared to that in cortical bone wax sealed drill holes. No significant differences between key PK parameters were found, and the concentration-time profile of unsealed drill holes resembled that sealed drill holes[20]. Sealing of drill holes with e.g. bone wax has been associated with an increased infection

risk[58]. For future clinical studies using MD in bone, this understanding is important, as it seems favorable to avoid the use of bone wax.

In our setup, we have measured vancomycin concentrations in both cancellous and cortical bone. To evaluate the intra-cortical placement of the cortical drill hole, a post-mortem CT-scan was conducted on every included pig. We found pure intra-cortical placements with no contact to the bone marrow. Examples of cross-sectional views from one of the CT-scans are depicted below in Figure 4.

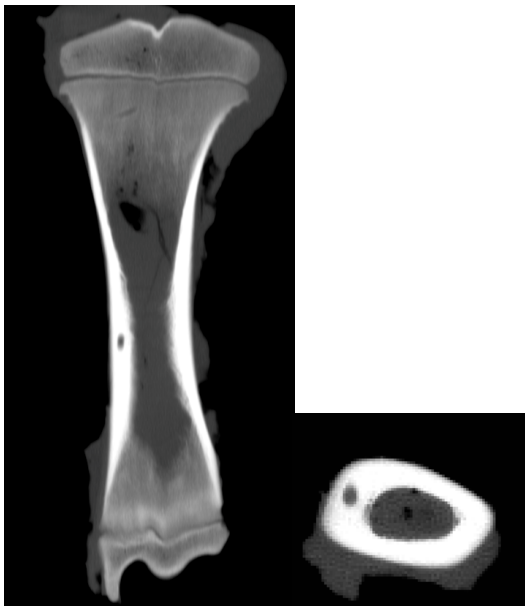

Figure 4. Examples of cross-sectional views from one CT-scan.

Limitations to MD

When performing MD-studies, the outcome should always be interpreted with the possible limitations of the method in mind. Limitations can e.g. be found in the analytical assay and its lower limit of quantification. The UHPLC-method in this study provided us with a very low limit of quantification (0.05 µg/mL). Moreover, the method has fulfilled the demands of stability for measuring vancomycin (unpublished data).

As said, relative recovery depends on a various number of factors such as chemical conditions of the compound being analyzed, diffusion coefficient, perfusion rate and probe-membrane. Thus, we tested two different membrane types (MD70-Polyamide and MD63-Polyarylethersulphone) to find the most suitable probe-membrane for sampling of vancomycin (unpublished data). We also explored the temporal resolutions effect on RR by testing different perfusion rates (unpublished data). We found that MD70 might be favorable

when sampling vancomycin, and that a reduction of flow rate from 1 µL/min to 0.5 µL/min, *in vivo,* increased RR with approximately 47 %. This illustrates how and why the use of the MD-method involves a series of *in vitro* studies to determine which experimental setup that is most likely to be suitable for the task.

Normally, it is recommended to achieve a RR above 20% when conducting MD-experiments[39]. Lower levels of RR are relatively more exposed to standard deviations regarding the chemical analysis and pre analytical handling and the resulting variations will increase exponentially with decreasing recovery[39].

We found a mean RR of 23.4% for MD measurements in bone. Due to limitations by the depth of the drill holes and so membrane length, an average bone RR of approximately 23.4% seems acceptable.

As in other MD-studies, our setup was a trade-off, which we found acceptable, between our experimental needs and the ideal setup.

Measurements in anesthetized pigs

We applied MD for measurement in bone in a pig model. As a consequence of this, we had to keep the pigs anesthetized during the entire study. A full anesthesia over several hours may cause physiological alterations that can affect pharmacokinetics. This must be taken into account when evaluating data.

The pigs were in full anesthesia for approximately 18-20 hours. This gave us no other reasonable ethical choice but to kill the pigs at the end of the study. As such, our setup only allowed us for measurements during one dosing interval. Further, we used young female pigs (65-75 kg). A young female pig at this weight is still growing, including the bones. This should also be born in mind when assessing pig bone pharmacokinetics.

Ethical and statistical considerations

In 1959 Russell and Burch published a concept known as the “three R’s” of laboratory animal science; **R**eplacement, **R**eduction and **R**efinement. Briefly, the meaning of this concept is that animals, if possible, should be substituted with insentient materiel, the number of sacrificed animals should be kept as low as possible, and animal welfare should always tried to be improved[59]. Today, these values constitute the foundation of laboratory animal research.

To ensure that ethical considerations are taken into account when using animal models, it is important to plan and execute the trial meticulously. The animal species should be selected

properly and procedure, sample size calculation, observation time etc. should all be considered fully. Furthermore, it is reasonable to test and apply the experiment to an animal model at first, before conducting a clinical trial. In the present project, these principles were pursued. The surgical procedures were performed under the approval from The Danish Animal Experiments Inspectorate and carried out according to existing laws.

We performed a sample size calculation to ensure the correct number of included pigs. A sample size calculation should demonstrate that the study is capable of answering the scientific questions posed and thereby justify the size of the study population[60]. It is in everyone's interest to not waste time and/or resources.

However, since antimicrobial penetration to bone is poorly elucidated, such a calculation can be difficult to perform. Given that a vancomycin AUC/MIC-ratio >400 is effective against most gram-positive bacteria, we found it reasonable to believe that this ratio also should be achieved in target site in order to be sufficient. In earlier experimental studies, we have found antimicrobial bone penetration-ratios from plasma to bone ranging from approximately 1/3 to 2/3, and thus our hypothesis was that this would also be the case for vancomycin.

Assuming that an AUC/MIC-ratio of 400 in plasma would be achieved with standard dosing regimens, and that this would lead to a mean (± SD) AUC/MIC-ratio in cancellous bone of 264 ± 115 (mean = 2/3 of successful treatment in plasma; the estimated SD is based on previous findings for cefuroxime), we had to include 8 pigs to demonstrate a significant difference[19]. Our assumptions on power and significance level can found below

Estimated sample size for one-sample comparison of mean to hypothesized value

Test Ho: m = 264, where m is the mean in the population

Assumptions:

alpha = 0.0500 (two-sided)
power = 0.9000
alternative m = 400
sd = 115

Estimated required sample sizes:
n = 8

Data were analysed using a Non-compartmental analysis (NCA). This approach was chosen because it is simple, requires few assumptions and because it allows for calculation of relevant PK parameters. As such, more advanced and assumption-demanding approaches like population PK modeling seemed unnecessary.

Discussion of results

This is the first study to evaluate vancomycin bone pharmacokinetics obtained by means of MD. The penetration of vancomycin to bone was shown to be incomplete and delayed, and this was most pronounced for cortical bone. It has been shown that an AUC/MIC ratio of 400 or greater is associated with therapeutic success. However, for bacteria MICs of ≥ 2 mg/L, it might not be possible to reach a target ratio of ≥ 400. In this perspective, an insufficient tissue penetration may have the same effect as increasing MIC-value: insufficient concentrations at target site.

MD has in a number of studies been successfully applied for measurements of antimicrobials in cortical as well as cancellous bone[13-20, 41]. In this study, our preliminary *in vitro* setup demonstrated that RR_{gain} equalled RR_{loss} over a relevant range of concentrations. RR was independent of the concentration and showed a fast movement across the membrane with no adherence to the probes (unpublished data). This is in agreement with earlier findings [30, 31]. Thus, *in vivo* sampling of vancomycin was considered feasible.

At present, studies investigating the penetration of vancomycin into bone have only been performed using bone biopsies. Landersdorfer et al. illustrate the variety in data by comparing two studies; Kitzes-Cohen et al. found an 8-10 fold higher concentration in sternal bone than Vuorisalo et al.[11, 32, 33]. In the two studies vancomycin was administered at analogous doses.

Tissue and plasma concentration-time profiles are illustrated in Figure 4. The corresponding pharmacokinetic parameters are depicted in Table 1.

Mean (95%-CI) $AUC_{0\text{-}last}$ were 9375 (7445; 11304) for plasma, 9304 (7374; 11233) for SCT, 5998 (3955; 8040) for cancellous bone and 3451 (1522; 5381) for cortical bone (ANOVA $P < 0.001$). In cancellous and cortical bone a significant lower AUC were found when comparing with plasma ($P < 0.01$ and $P < 0.001$, respectively). Additionally, AUC were significantly lower in cortical bone than in cancellous bone ($P = 0.013$).

For C_{max}, a statistical significant difference among the means was shown (ANOVA $P < 0.001$). Mean (95%-CI) C_{max} were 34.2 (28.6; 39.8), 27.2 (21.6; 32.8), 17.2 (11.2; 23.3) and 9.4 (3.8; 15.0) for plasma, SCT, cancellous and cortical bone respectively. C_{max} was found significantly lower in cancellous and cortical bone when compared to plasma (both $P < 0.001$) and C_{max} was found significantly lower in cortical bone than in cancellous bone ($P= 0.025$).

T_{max} (95%-CI) were 90.0 (63.7; 116.3) for plasma, 172.0 (143.2; 200.8) for cancellous bone and 230.0 (203.7; 256.3) for cortical bone. Thereby, T_{max} for plasma was significantly lower than T_{max} for the two bone compartments.

To summarize, AUC and C_{max} illustrated an impaired penetration and T_{max} a delayed penetration of vancomycin from plasma to bone. This suggests that timing and dosing of antibiotics in an orthopaedic setting may be of importance. Furthermore, p-values < 0.03 when comparing cancellous vs. cortical bone for both AUC and C_{max}, indicate that cancellous and cortical bone might be considered as two distinct compartments. These findings are in concordance with our earlier findings, with incomplete penetration of cefuroxim to bone tissue[19, 20].

With a terminal elimination half-life of vancomycin of 3-9 hours, our experimental setup didn't give us the opportunity to determine AUC at steady-state over 24 hours[26]. As such, we cannot compare our findings with human AUC/MIC-ratios. Nevertheless, our concentration measurements in plasma revealed a range of 3.5-45.6 mg/L and in subcutis 3.0-46.6 mg/L during the study. These ranges resemble concentrations found in plasma and subcutis for humans[23, 31]. Thus, a pig may be a feasible model to investigate tissue concentrations of vancomycin. In the particular case of bone, clinical trials for the measurement of vancomycin have not been conducted.

Perspectives

Studies evaluating the distribution of antimicrobials into bone by means of MD, will contribute with more pieces of knowledge to an area in science, where information currently is sparse. However, experimental studies alone cannot replace large clinical trials with clinical endpoints.

Today, dosing of antimicrobials is based on tradition rather than evidence. MD has the potential to bring more information about bone pharmacokinetics of antimicrobials. With greater knowledge, therapeutic concentrations of antimicrobials at target site can potentially be achieved. Thus, if accomplished, it is reasonable to expect that we in the future can provide the patients with a better antimicrobial treatment. As both prevention and treatment of infections are important, it is essential to get a better understanding of how, how often, when and in which doses antimicrobials should be administered to the patients. If the grade of bone penetration, time course of bone concentration and clinical outcome can be evaluated, the most suitable PK-target for treatment of a bone infection can be derived[11]. Larger knowledge and better antimicrobial treatment will eventually result in lower risk of orthopaedic-related infections and thus shorter hospitalization time and less morbidity for the patients. This is in great interest from a patient, a public health and socio-economic point of view.

Future studies

Determination of bone pharmacokinetics of antimicrobials with the use of MD is an area in research where many drugs, dosage-methods, timing of dosing, under different conditions etc. still are to be explored. It is research with the objective of potentially optimizing the antimicrobial treatment in an orthopaedic setting.

It is relevant to explore the bone pharmacokinetics of various antimicrobials in clinical studies, in infected bone, in bone with an inserted prosthesis, in the spinal bone and disc, in arthritic or calcified bone, in bone with low bone density, in bone cysts etc. Moreover it is important to evaluate treatment settings by e.g. comparing oral treatment with intravenous treatment and short-term infusion with continuous infusion. The site of pathogen in bone is also yet to be determined[11]. Thus, this also calls for future studies to complement this research field. The amount and relevance of future studies are massive.

At present, our MD-setup has already been applied on a randomized clinical trial for the measurement of cefuroxime in short-term infusion compared with continuous infusion, and we have also planned a clinical study on vancomycin bone penetration.

Conclusion

The obtainment of valid and reproducible pharmacokinetic measurements of antimicrobials in bone tissue is of significant relevance.

The distribution of vancomycin to bone is impaired and delayed, and may account for insufficient prophylaxis and treatment failures. These porcine-data cannot be applied directly to a clinical setting. However, our results represent an important step. Clinical trials are warranted.

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Tables and figures

Table 1

Key *in vivo* pharmacokinetic parameters for plasma, subcutaneous tissue and cancellous and cortical bone

Pharmacokinetic parameter	Plasma (unbound)	Subcutaneous tissue	Cancellous bone	Cortical bone	ANOVA[c]
$AUC_{0\text{-last}}$ (min µg/mL)	9375 (7445;11304)	9304 (7374;11233)	5998 (3955;8040)$^{\lambda}$	3451 (1522;5381)$^{\gamma x}$	P < 0.001
C_{max} (µg/mL)	34.2 (28.6;39.8)	27.2 (21.6;32.8)	17.2 (11.2;23.3)$^{\gamma}$	9.4 (3.8;15.0)$^{\gamma x}$	P < 0.001
T_{max} (min)	90.0 (63.7;116.3)	151.7 (125.4;177.9)	172.0 (143.2;200.8)	230.0 (203.7;256.3)	-
$T_{1/2}$ (min)[a]	248.1 (181.0;315.2)	241.5 (176.2;306.8)	223.5 (158.0;288.9)	404.7 (295.3;514.2)$^{\lambda}$	P < 0.01
$fAUC_{tissue}/fAUC_{plasma}$[b]		1.01±0.39	0.65±0.31	0.36±0.20	P = 0.014[d]

Values are given as mean (95%-CI) unless stated otherwise.

$AUC_{0–last}$, area under the concentration–time curve from 0 to the last measured value; C_{max}, peak drug concentration; T_{max}, time to C_{max}; $T_{1/2}$, half-life at β-phase; $fAUC_{tissue}/fAUC_{plasma}$, tissue penetration expressed as the ratio of free AUC_{tissue}/free AUC_{plasma}

[a] Values are given as medians (95%-CI)

[b] Values are givens as mean ± SD

[c] One way ANOVA for free plasma, subcutaneous tissue, cancellous and cortical bone

[d] T-test comparison of cancellous and cortical bone

$^{\lambda}$ P < 0.01, $^{\gamma}$ P < 0.001 for comparison with the corresponding free plasma value

^{x} P < 0.03 for comparison with cancellous bone

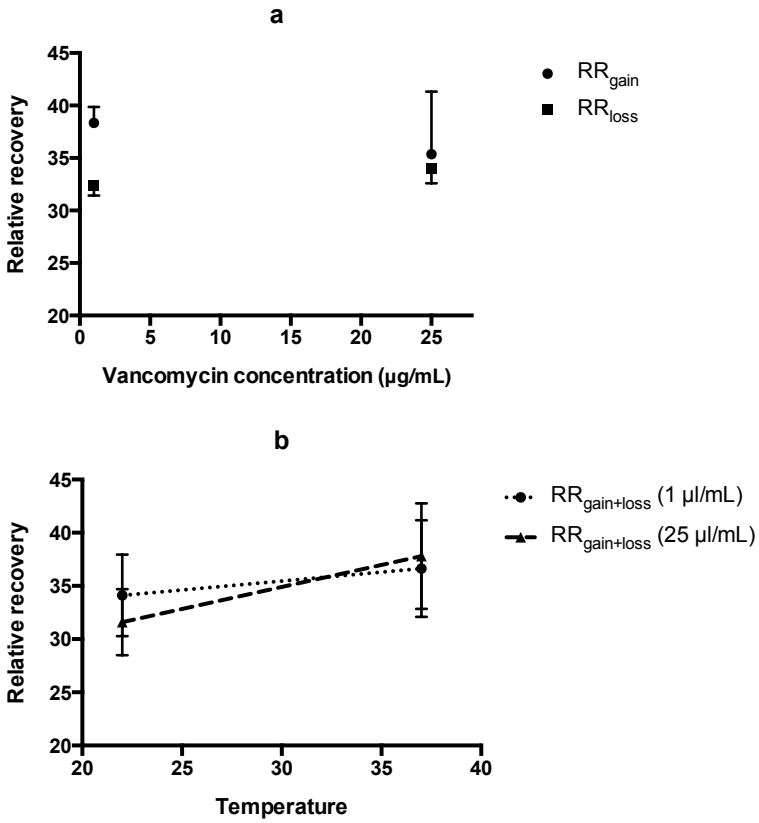

Figure 1a Mean RR_{gain} and RR_{loss} at different concentrations of vancomycin. **1b** Effect of temperature on recovery at different concentrations of vancomycin. Bars represent Standard Error of the Mean (SEM)

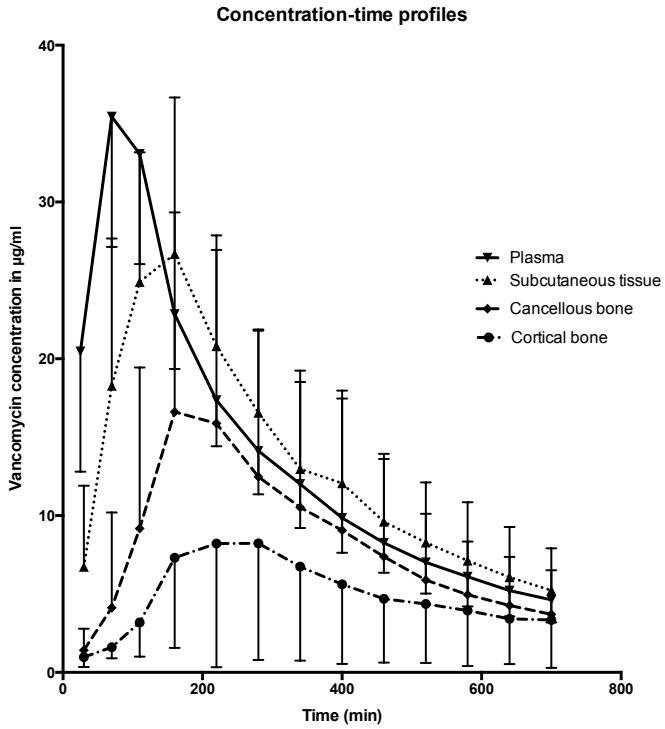

Figure 2. Mean concentration-time profiles for plasma, subcutaneous tissue, cancellous and cortical bone. Bars represent 95%-CI.