Plasma, Subcutaneous Adipose Tissue, Vertebral Cancellous Bone and Intervertebral Disc Pharmacokinetics of Cefuroxime

Research Year Thesis

Aarhus University Orthopaedic Research Unit, Aarhus University Hospital

Pelle Hanberg







Supervisors

Main Supervisor

Kjeld Søballe, Professor, MD, DMSc Department of Orthopaedic Surgery Aarhus University Hospital

Supervisors

Mikkel Tøttrup, MD, Ph.d. Departments of Orthopaedic Surgery Regional Hospital Randers And, Orthopaedic Research Unit Aarhus University Hospital

Hanne Birke Sørensen, MD, Ph.d. Orthopaedic Research Unit Aarhus University Hospital

Contact

Pelle Hanberg, MD-student Orthopaedic Research Unit, Aarhus University Hospital, Denmark Nørrebrogade 44, Bygning 1A, 8000 Aarhus C Tel: +4528744852 E-mail: pellehanberg@hotmail.com

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Preface

This research year thesis consists of one manuscript and a comprehensive review (supplementary information) dealing with plasma, subcutaneous adipose tissue, vertebral cancellous bone and intervertebral disc pharmacokinetics of cefuroxime. The manuscript is structured as a standard scientific paper with a presentation and discussion of the results, while the review is a thorough discussion of the applied pharmacokinetic parameters and methods. In the methodological consideration section, special attention has been put on the weaknesses and limitations of the applied methods.

The experimental studies were conducted at the Institute of Clinical Medicine, Aarhus University Hospital, Denmark, while Chemical analyses were performed at the Department of Clinical Biochemistry, Aarhus University Hospital.

Summary English

Pyogenic spondylodiscitis is associated with prolonged antimicrobial therapy and high relapse rates, and remains a significant therapeutic challenge. Assessment of antimicrobial bone and intervertebral disc (IVD) pharmacokinetics is difficult, and traditional methodological approaches like bone biopsy and discectomy exhibit important methodological limitations. The well-known catheter-based pharmacokinetic sampling tool, microdialysis (MD), offers significant advantages compared to traditional methods, and it may prove to generate more usable and valid pharmacokinetic data.

In the present study, MD was used to assess IVD, vertebral cancellous bone and subcutaneous adipose tissue pharmacokinetics of cefuroxime in a porcine model following a standard single dose of 1,500 mg. The main finding is that IVD cefuroxime time with concentrations above the minimal inhibitory concentration (T_{>MIC}) was significantly longer than those of the other compartments for a range of relevant MICs despite incomplete overall tissue penetration. Though tissue targets for prevention of infection is largely unknown, it is generally recommended that plasma as well as tissue concentrations of time dependent antimicrobials, like cefuroxime, exceed MIC values of relevant pathogens throughout the surgical procedure and until a few hours after the incision is closed. Based on this recommendation, our data suggest that superior tissue exposure of cefuroxime is obtained in the IVD compared to the other compartments. Combined with a delayed IVD penetration and using relevant *staphylococcus aureus* MIC values, our data indicates that cefuroxime should be administered in due time and repeatedly if effective concentrations are to be sustained in all tissues but the IVD throughout spine procedures lasting more than 2-3 hours.

Summary Danish

Bakteriel spondylodiscitis er associeret med både lang antibiotikabehandling og mange tilbagefald og er således en stor terapeutisk udfordring. Bestemmelse af antibiotikakoncentrationer i knoglevæv og discus intervertebralis (IVD) er vanskeligt. De tidligere anvendte metoder, knoglebiopsi og discectomi, har begrænsede anvendeligheder grundet metodologiske begrænsninger. Det velkendte farmakologiske redskab, mikrodialyse (MD), tilbyder signifikante fordele i forhold til de traditionelt anvendte metoder, og har derfor potentiale til at generere mere anvendelige farmakokinetiske data.

I dette studie blev MD brugt til at undersøge farmakokinetikken af cefuroxim i IVD, spongiøs vertebral knogle og subcutis i en grisemodel efter administration af en enkelt standarddosis cefuroxim på 1.500 mg. Det primære fund var, at tiden med koncentration over den minimale inhibitoriske koncentration (T_{>MIC}) for cefuroxim i IVD var signifikant længere end i de resterende kompartementer for en række klinisk relevante MIC værdier, på trods af signifikant nedsat samlet vævspenetration. Selvom det effektive behandlingsmål for profylaktisk antibiotikabehandling endnu ikke er bestemt, er det generelt anbefalet, at plasma- og vævskoncentrationer for et tidsafhængigt antibiotikum, som cefuroxim, overstiger MIC værdier for relevante bakterier fra start af kirurgi til ca. to timer efter kirurgi. På baggrund af denne generelle anbefaling, viser vores data, at den bedste vævseksponering af cefuroxim opnås i IVD sammenlignet med de øvrige kompartementer. Kombineret med en forsinket IVD penetration og ved brug af *staphylococcus aureus'* MIC værdier, viser vores data, at cefuroxim bør administreres tidsnok og over gentagende gange, hvis effektiv cefuroxim koncentrationer skal opnås i alle kompartementerne gennem rygoperationer varende længere end 2-3 timer.

Manuscript

Pharmacokinetics of Single Dose Cefuroxime in Porcine Intervertebral disc and Vertebral Cancellous Bone Determined by Microdialysis

Submitted to The Spine Journal

Pharmacokinetics of Single Dose Cefuroxime in Porcine Intervertebral Disc and Vertebral Cancellous Bone Determined by Microdialysis

Abstract

Background: Pyogenic spondylodiscitis is associated with prolonged antimicrobial therapy and high relapse rates. Nevertheless, tissue pharmacokinetic studies of relevant antimicrobials in both prophylactic and therapeutic situations are still sparse. Previous approaches based on bone biopsy and discectomy exhibit important methodological limitations.

Purpose: The objective of this study was to assess the C3/C4 intervertebral disc (IVD), C3 vertebral body cancellous bone and subcutaneous adipose tissue (SCT) pharmacokinetics of cefuroxime by use of microdialysis (MD) in a large animal model.

Study Design: This was a single-dose, dense sampling large animal study of cefuroxime spine penetration.

Methods: Ten female pigs were assigned to receive 1,500 mg of cefuroxime intravenously over 15 min. Measurements of cefuroxime were obtained from plasma, SCT, vertebral cancellous bone and IVD for 8 hours thereafter. MD was applied for sampling in solid tissues.

Results: For both IVD and vertebral cancellous bone, the area under the concentration-curve from zero to the last measured value (AUC_{0-last}) was significantly lower than that of free plasma. As estimated by the ratio of tissue AUC_{0-last} to plasma AUC_{0-last} , tissue penetration (95%-confidence interval) of cefuroxime was significantly incomplete for the IVD 0.78 (0.57; 0.99), while for vertebral cancellous bone 0.78 (0.51; 1.04) and SCT 0.94 (0.73; 1.15) it was not. The penetration of cefuroxime from plasma to the IVD was delayed, and the maximal concentration and the elimination of cefuroxime were also reduced compared to both SCT and vertebral cancellous bone. Due to this delay in elimination of cefuroxime, the time with

concentrations above the minimal inhibitory concentration ($T_{>MIC}$) was significantly longer in the IVD compared to the remaining compartments up to MICs of 6 µg/ml.

Conclusions: MD was successfully applied for serial assessment of the concentration of cefuroxime in the IVD and the vertebral cancellous bone. Penetration of cefuroxime from plasma to IVD was found to be incomplete and delayed, but due to a prolonged elimination, superior $T_{\text{>MIC}}$ was found in IVD up to MICs of 6 µg/ml.

Keywords

Cefuroxime; microdialysis; pharmacokinetics; intervertebral disc; vertebra; tissue penetration

Introduction

Pyogenic spondylodiscitis is a relatively rare disease but the incidence is increasing(1-3). The estimated incidence varies between 0.4-2.4/100,000 persons per year in developed countries(4, 5). Postoperative pyogenic spondylodiscitis accounts for approximately 30% of all cases of pyogenic spondylodiscitis, and, depending on the surgical procedure, the incidence varies between 0.24-3.6%(6, 7). Despite prolonged antimicrobial therapy, relapse rates remain high suggesting that antimicrobial penetration may be insufficient(8, 9). Indeed, evidence-based treatment guidelines are currently lacking for both treatment and perioperative antimicrobial prophylaxis. This stresses the need for tissue pharmacokinetic studies of relevant antimicrobials.

Assessment of intervertebral disc (IVD) and bone penetration of antimicrobials is challenging. So far, bone biopsy and discectomy have been the predominant approaches(10-14). These methods do, however, suffer from important limitations, which may reduce applicability of the findings(11, 12, 15, 16). Recently, the well-known pharmacokinetic tool, microdialysis (MD), has been successfully applied for assessment of antimicrobial bone concentrations(17-24). MD is a catheter-based technique, which allows for continuous sampling of the unbound fraction of water-soluble drugs in the interstitial space, and as such, it offers attractive advantages compared to other methods. No previous studies have assessed IVD pharmacokinetics of antimicrobials by means of MD.

In the present study, MD was used to assess the C3/C4 IVD, C3 vertebral body cancellous bone and subcutaneous adipose tissue (SCT) pharmacokinetics of cefuroxime in a large animal model. The primary endpoints were tissue penetration and the time with concentrations above the minimal inhibitory concentration ($T_{>MIC}$), which is the main pharmacokinetic/pharmacodynamic index for cephalosporins(25).

Materials and methods

This study was conducted at the Institute of Clinical Medicine, Aarhus University Hospital, Denmark. The study was approved by the Danish Animal Experiments Inspectorate, and carried out according to existing laws. Chemical analyses were performed at the Department of Biochemistry, Aarhus University Hospital.

Microdialysis

Briefly, MD is based on diffusion of molecules across a semipermeable membrane located at the tip of a probe. Due to continuous perfusion of the probe, non-equilibrium conditions will be present during experiments. Accordingly, the concentration of the drug in the dialysate will only represent a fraction of the actual concentration in the tissue. This fraction is referred to as the relative recovery (RR). The RR can be determined by various calibration procedures, which is a prerequisite if absolute tissue concentrations are to be determined. In this study, retrodialysis by drug was applied for calibration(26). In depth description of MD can be found elsewhere(27, 28).

MD equipment from μ -Dialysis AB (Stockholm, Sweden) was used. Specifically, the catheters used were CMA 63 (membrane length 10 and 30 mm with a 20 kilo Dalton molecule cut-off), and CMA 107 precision pumps produced a flow rate of 1 μ l/min.

The RR was calculated by using the following equation:

$$RR(\%) = 100 \times \left(1 - \frac{C_{out}}{C_{in}}\right)$$

Where C_{out} is the concentration in the dialysate and C_{in} the concentration in the perfusate.

In the data analysis, the measured concentrations were attributed to the midpoint of the sampling intervals. The absolute tissue concentrations (C_{tissue}) were obtained by correcting for RR using the following equation:

$$C_{tissue} = \frac{C_{out}}{RR}$$

Individual catheter calibration was performed for all the catheters at location.

Animals, anaesthetic and surgical procedures

Ten female pigs were included in the study (Danish Landrace Breed 75-77 kg). The pigs were kept in general anaesthesia by a combination of propofol (500 - 600 mg/h by continuous infusion) and fentanyl (0.35 - 0.45 mg/h by continuous infusion). The pH was monitored and kept within a range of 7.35-7.46 throughout the entire study by regulating the ventilation. The body temperature was kept within the range of 36-38.9°C.

The surgical procedure was initiated immediately after induction of general anaesthesia. With the pigs in supine position, vertebrae C2 to C4 were exposed via an anterolateral approach. A Kirschner wire with a fixating device (PEBAX) was drilled into the distal part of the C2. At an angle of approximately 45° to the sagittal plane, a 2 mm drill hole was made in the middle of C3 (depth 22±1 mm). With the tip of the catheter protruding approximately 20 mm from the tip of the introducer, the MD catheter (membrane length: 10 mm) was attached to a splitable introducer using endo clips. In this way, the membrane was free of the splitable introducer. The MD catheter with the splitable introducer was then placed in the drill hole and fixed to the fixating device in C2 for support in order to prevent subsequent dislocation of the catheter. The IVD between C3 and C4 was used for IVD measurements. By guidance of fluoroscopy, and again at an angle of approximately 45° to the sagittal plane, a splitable introducer with a needle was introduced into the IVD parallel to, and in the middle of the adjacent endplates. When penetrating the annulus fibrosus, loss of resistance was clearly felt. The needle was then retracted, and the splitable introducer was advanced into the nucleus

pulposus until resistance from the opposite wall of the annulus fibrosus was felt. A 10 mm MD catheter was then introduced into the nucleus pulposus through the splitable introducer. Concurrently, the splitable introducer was pulled back, until the membrane was exposed in the IVD in full length. The MD catheter was attached to the splitable introducer by endo clips. Correct location of the MD catheters was verified by fluoroscopy before and at the end of the study. Fluoroscopy images were stored for documentation.

In addition to the two catheters in the spine, a 30 mm reference MD catheter was placed in the SCT of the lateral thoracic wall, according to the guidelines of the manufacturer.

Sampling procedures

Immediately after placement of the MD catheters, the catheters were perfused with 0.9% NaCl. Initially, a 30 min tissue equilibration period was allowed for. Fifteen hundred mg of cefuroxime was then administrated intravenously over 15 min starting at time 0. Cefuroxime was chosen because first or second generation cephalosporins are currently recommend for antimicrobial prophylaxis in spine procedures(29). The chosen weight of the animals resembles that of an average human being. For humans, 1,500 mg is the standard dose of cefuroxime. From time 0-180 min, dialysates were collected every 30 min, and thereafter every 60 min from 180-480 min. Blood samples were drawn from a central venous catheter every 15 min from time 0-75 min, every 30 min from 75-165 min, and every 60 min from 165-450 min. When the last dialysate was collected after 480 min, the perfusate was replaced with 0.9% NaCl containing 100 μ g/ml cefuroxime. After a 15 min equilibration period, the catheters were calibrated by collecting a 30 min sample.

Handling of samples

The dialysates were immediately frozen and stored in a -80°C freezer until analysis. Venous blood samples were stored in a +5°C refrigerator for a maximum of 24 hours before being centrifuged at 3,000 g for 10 min. Plasma from the venous blood was frozen and stored in a -80°C freezer until analysis.

Ultra High Performance Liquid Chromatography-analysis of cefuroxime

The dialysate and free plasma concentration of cefuroxime were quantified using ultra high performance liquid chromatography. This method has been described in details elsewhere(24). The intrarun (interrun) imprecisions (given in per cent coefficients of variation [%CVs]) were 5.6% (6.8%) at 0.25 μ g/ml, 4.3% (4.7%) at 2.5 μ g/ml, and 2.6% (2.8%) at 10 μ g/ml for the dialysates. For the free plasma concentration, the intrarun (interrun) imprecisions were 1.8% (6.5%) at 9.2 μ g/ml and 1.6 (6.2%) at 38 μ g/ml. The lower limit of quantification in both dialysates and free plasma was 0.06 μ g/ml.

Pharmacokinetic analysis and statistics

For each subject, pharmacokinetic parameters were determined separately by noncompartmental analysis (NCA) using Stata (version 12.0; StataCorp, USA). The area under the concentration-curve from zero to the last measured value (AUC_{0-last}) was calculated using the trapezoidal rule. The terminal half-life ($T_{\frac{1}{2}}$) was calculated as \ln_2/λ_{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 selected by inspection of the individual concentration-time profiles. By use of Microsoft Excel, $T_{\text{>MIC}}$ was estimated using linear interpolation. The number of pigs attaining targets of 40% and 65% $T_{\text{>MIC}}$ was counted manually.

Concentration measurements below the lower limit of quantification were set to be zero.

One-way analysis of variance (ANOVA) with a random animal effect was used for an overall comparison of the pharmacokinetic parameters and of the $T_{>MIC}$. Furthermore, post-hoc pairwise comparisons were made for the IVD and the vertebral cancellous bone versus the remaining compartments and for the two compartments in between. For the $T_{\frac{1}{2}}$ data, transformation to the log scale improved normal distribution, and therefore the data were analysed as such. Consequently, $T_{\frac{1}{2}}$ values are given as median (95%-confidence interval). A P-value < 0.05 was considered significant. The free tissue AUC_{0-last} to free plasma AUC_{0-last} ratio (*f*AUC_{tissue}/*f*AUC_{plasma}) was calculated as a measure for tissue penetration. Statistical analyses were performed using Stata (version 12.0; StataCorp, USA).

Results

All 10 pigs completed the study. The creatinine level was in the range of 118-166 μ mol/l. Except for one vertebral and one IVD catheter with malfunction, all other catheters were included in the analysis. Fluoroscopy confirmed correct location of all catheters, see figure 1. Mean RR (±SD) were 44.9±6.7%, 25.0±6.3% and 26.3±7.2% for SCT, vertebral cancellous bone and IVD, respectively.

Mean concentration-time profiles are shown in figure 2. Key pharmacokinetic parameters can be found in Table 1. For both the IVD and the vertebral cancellous bone, AUC_{0-last} was significantly lower than free plasma AUC_{0-last} (P<0.01 and P<0.05, respectively). Tissue penetration (95%-confidence interval) of cefuroxime was significantly incomplete for the IVD 0.78 (0.57; 0.99), while for vertebral cancellous bone 0.78 (0.51; 1.04) and SCT 0.94 (0.73; 1.15) it was not. IVD C_{max} of cefuroxime was significantly lower than those of the remaining compartments, while IVD T_{1/2} was significantly longer. Furthermore, T_{max} revealed a delayed penetration of cefuroxime from plasma to the IVD. Cefuroxime penetrated well into the SCT.

Figure 3A illustrates the relationship between the $T_{>MIC}$ and MIC for all compartments. For MICs up to 6 µg/ml, $T_{>MIC}$ was significantly longer for the IVD compared to the remaining compartments. At higher MICs no significant differences were found.

Figure 3B shows the proportion of pigs achieving targets of 40% T_{>MIC} (low target) and 65% T_{>MIC} (high target) for a range of relevant MICs. For MICs above 1 μ g/ml, the number of animals to achieve even the low target decreases rapidly for all compartments but the IVD. Accordingly, no animals achieved the high target in plasma and SCT for this MIC, while only 33% achieved it in the vertebral cancellous bone. Considering the IVD, 89% of the animals achieved the high target for a MIC of 1 μ g/ml, but for higher MICs target attainment also dropped rapidly for the combination of IVD and the high target.

Discussion

This is the first study to investigate IVD and vertebral bone pharmacokinetics of cefuroxime using the MD technique. Previously, bone biopsy and discectomy have been the predominant

approaches, but due to inherent poor temporal resolution, these methods do not allow for precise estimates of T_{>MIC}(10-16). The main finding of this MD study is indeed that IVD cefuroxime T_{>MIC} was significantly longer than the remaining compartments for a range of relevant MICs despite incomplete and delayed tissue penetration. Though tissue targets for prevention of infection is largely unknown, it is generally recommended that plasma as well as tissue concentrations of time-dependent antimicrobials like cefuroxime exceed MIC values of relevant pathogens throughout the surgical procedure and until a few hours after the incision is closed(30). Based on this recommendation, our data suggest that superior tissue exposure of cefuroxime is obtained in the IVD compared to the other compartments. The majority of isolated staphylococcus aureus exhibit MICs in the range 0.5-2 µg/ml(31). Looking at the concentration-time profiles and the relationship between the T_{>MIC} and MIC, our free plasma, SCT and vertebral cancellous bone data suggest that a single dose of 1,500 mg of cefuroxime will only provide effective concentrations in these compartments for a limited part of several spine procedures, whereas in the IVD, this dose will generally suffice. Combined with the delayed IVD penetration, the findings in this study suggest that cefuroxime should be administered in due time and repeatedly for spine procedures lasting more than 2-3 hours.

Interestingly, the half-life of cefuroxime in the IVD is approximately twice the size of those found in the other compartments. This seems to be the reason for the long IVD cefuroxime $T_{>MIC}$ despite incomplete tissue penetration. The impact of this finding is illustrated by comparing the C_{max} and the AUC_{0-last} for the IVD and vertebral cancellous bone. Despite the fact that the C_{max} value of the vertebral cancellous bone is twice the value of the IVD, AUC_{0-last} is almost equal for the two compartments. This prolonged elimination rate seems to be responsible for longer $T_{>MIC}$ despite low overall IVD cefuroxime penetration. One explanation for the prolonged elimination of cefuroxime from in the IVD may be a high diffusion coefficient in the IVD. Irrespective of the reason, however, the prolonged elimination may have important clinical implications with respect to selection of antimicrobials for both prevention and treatment of pyogenic spondylodiscitis, as time-dependent antimicrobials would appear to benefit more from prolonged elimination than concentration-dependent drugs. Assuming a dose-independent elimination rate, even a small increase of dose may improve IVD $T_{>MIC}$ significantly, while an AUC increase would be limited.

Pigs have been shown to resemble humans in terms of anatomy and physiology(32). Nonetheless, important interspecies and age-related differences should be considered(33). Firstly, in man, blood vessels are only present in the annulus fibrosus in the first decade of life(5, 34). Later, the IVD rely upon nutrition from the endplates. We used juvenile pigs (age 5 months), for which this matter remains to be investigated. Secondly, thinner IVDs are found in pigs suggesting shorter diffusion distances and therefore possibly higher drug concentrations(33). Thirdly, obese and large subjects are likely to have larger volumes of distribution than the present pigs, which will lead to lower concentrations. Based on these considerations, uncritical inference on adult dosing regimens based on the present IVD findings is unsafe. Indeed, it can be speculated that human IVD penetration may be even lower than found in this study. This may lead to sub-MIC IVD concentrations throughout the entire surgical procedure, particularly in case of high-MIC pathogens.

The present measurements where obtained in healthy tissue. In osteomyelitis, increased osseous pressure and thrombosed vessels lead to impaired blood flow(35). As such, it can be speculated that the presence of infection will reduce tissue penetration further. As mentioned, cefuroxime is a time-depend drug, for which $T_{>MIC}$ has been shown to be the best predictor of efficacy(25). While cephalosporin tissue targets for treatment of infection are unknown, *in vitro* and animal infectious model studies suggest that plasma targets of 40-70% $T_{>MIC}$ are generally effective(25, 30, 36-39). Rencently, however, clinical trials on the use of meropenem and two cephalosporins for non-orthopaedic infections have indeed suggested that aggressive plasma pharmacokinetic/pharmacodynamic targets of 100% $T_{>1-5_{xMIC}}$ are more predictive of successful outcomes compared to traditional targets. Combined with possible parallel displacement of the present concentration-time profiles due to infection, tissue $T_{>MIC}$ may be very limited or not present at all. This may partly explain the difficult management and high relapse rates for pyogenic spondylodiscitis. However, this hypothesis clearly needs further investigation.

Until now, studies assessing antimicrobial penetration into the vertebral bone and the IVD have relied on bone specimens and discectomy. These approaches, however, suffer from a number of important methodological limitations(11, 12, 15, 16). When analysing tissue

biopsy specimens, no selective measurement of the free extracellular concentration or distinction between the intra- and extracellular compartments can be made. Furthermore, concentration of the antimicrobial is given by mass and not by volume, and temporal resolution is poor or non-existing. Accordingly, pharmacokinetic parameters obtained by tissue specimens are difficult to relate to pharmacokinetic/pharmacodynamic targets. MD, on the other hand, allows for serial sampling of the free, and thus active fraction of drug in the interstitial space. As the majority of infections occur in this compartment, such features are desirable. It is, however, appreciated that both bone/IVD specimens and MD approaches suffer from the lack of a reference method to validate the findings. Finally, the mandatory correction for RR is associated with a magnification of the variations associated with preanalytical sample handling and the chemical assay. These variations will increase exponentially with a decreasing RR, and it is recommended that measures should be taken to obtain RRs of over 20%(40). The setup in our study resulted in mean RRs in the range of 25.0-44.9%, which is considered an acceptable trade-off given the anatomical limitations for increasing membrane length and the need for high temporal resolution. Indeed, the variations in our tissue pharmacokinetic parameters were comparable or only slightly larger than that of plasma.

In conclusion, this study successfully applied MD for serial assessment of the concentration of cefuroxime in the IVD and vertebral cancellous bone. Despite incomplete and delayed tissue penetration, IVD $T_{>MIC}$ was significantly longer than in the remaining compartments. Using relevant staphylococcus aureus MIC values, our data suggest that cefuroxime should be administered in due time and repeatedly if effective concentrations are to be sustained in all tissues but the IVD throughout spine procedures lasting more than 2-3 hours. In the present study, measurements were obtained in healthy tissue in juvenile pigs. The presence of infection and age-related reduction in blood supply may reduce IVD cefuroxime concentrations to insufficient levels. Future studies investigating these issues are warranted.

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Figures



Figure 1. Fluoroscopy image showing the location of the MD catheters. 1; C3 vertebral body. 2; C3/C4 IVD. 3; The gold thread within the MD catheter membrane tip in the vertebral cancellous bone. 4; The gold thread within the MD catheter membrane tip in the IVD. 5; The Kirschner wire with the fixating device in the C2 vertebral body.



Figure 2. Mean concentration-time profiles. Bars represent standard deviations.



Figure 3. Time with concentrations above the MIC versus MIC (A) and the percentage of animal achieving targets of 40% and 65% $T_{>MIC}$ versus MIC (B). Bars represent standard deviations.

Tables

Table 1 Pharmacokinetic Free plasma Vertebral Intervertebral disc ANOVA Subcutaneous parameter adipose tissue cancellous bone AUC₀ - last (min μ g/mL) 3049 (2539; 3558) 2818 (2308; 3327) 2420 (1892; 2948) 2315 (1787; 2843) P=0.03 80.4 (73.9; 86.9) 34.4 (27.9; 40.9) 27.7 (20.9; 34.5) 11.9 (5.1; 18.7)^a P<0.001 C_{max} (µg/mL) T_{max} (min) 15 (15; 15) 45 (45; 45) 45 (45; 45) 57 (45; 75) T_{1/2} (min) 45.7 (41.1; 50.4) 46.1 (41.4; 50.8) 50.7 (45.4; 56.0) 103.1 (92.2; 114.0) a P<0.001 0.94 (0.73; 1.15) 0.78 (0.51; 1.04) 0.78 (0.57; 0.99) fAUCtissue/fAUCplasma

 AUC_{0-last} , area under the concentration-time curve from 0 to the last measured value

AUC and C_{max} are given as mean (95%-confidence intervals)

 T_{max} are given as mean (ranges)

 $T_{\frac{1}{2}}$ are given as median (95%-confidence interval)

 a P < 0.05 for all post hoc comparisons between the IVD versus the other compartments

Supplementary information

Abbreviations

AUC	Area under the concentrations-time-curve
C _{max}	The highest concentration reached in the compartment of reference
IVD	Intervertebral disc
LLOQ	Lower limit of quantification
MD	Microdialysis
MIC	Minimal inhibitory concentration
PET	Positron emission tomography
РК	Pharmacokinetic
PK/PD	Pharmacokinetic/pharmacodynamic
PSO	Pyogenic Spondylodiscitis
RR	Relative recovery
SCT	Subcutaneous adipose tissue
T _{>MIC}	Time with free concentration above the minimal inhibitory concentration
UHPLC	Ultra High Performance Liquid Chromatography

2 Introduction

2.1 Overview

Pyogenic spondylodiscitis (PSO) is the main manifestation of haematogenous osteomyelitis, which is most frequent at an age over 50[1, 2]. It can, however, manifest in any age. Appropriate management of PSO requires long-lasting antimicrobial treatment where surgical intervention can be necessary[2]. Treatment of PSO is complicated, difficult and related with high relapse rates[3, 4]. Consequently, treatment of PSO can be very costly for both the patient and the health system.

Proper antimicrobial dosing is essential when treating or preventing infections. Information about how to achieve therapeutic exposure of antimicrobials at the site of infection (e.g. target site) is important for optimizing bacterial killing. Assessment of bone and intervertebral disc (IVD) antimicrobial penetration is, however, challenging. Accordingly, current strategies for treatment and prevention of PSO are largely empirically based.

The limited knowledge of IVD and bone antimicrobial penetration is predominantly based on bone specimens and discectomy[5-9]. These methods do, however, have limited usability due to methodological limitations[6, 7, 10, 11]. To overcome some of these limitations, our research group has applied the well-known pharmacokinetic (PK) tool, microdialysis (MD), for measurements of antimicrobials in bone[12-14]. In this research year project, MD was used to obtain PK parameters for cefuroxime in IVD, vertebral cancellous bone and subcutaneous adipose tissue (SCT) in a large animal model. The primary endpoints were tissue penetration and the time with free concentrations above the minimal inhibitory concentration (T_{MIC}).

2.2 Antimicrobial pharmacokinetics/pharmacodynamics

Pharmacokinetics refers to the study of the bodily absorption, distribution, metabolism and excretion of drugs. Pharmacodynamics, on the other hand, refers to the relationship between exposure and the pharmacologic and toxicologic effects of drugs[15, 16]. In the particular case of antimicrobial pharmacodynamics, measured antimicrobial concentrations are linked to clinical and microbiological effects[16]. The ultimate goal of antimicrobial treatment is to

administer a dose of drug that maximise the probability of attaining an effective response balanced with minimised unwanted side effects. Both minimal inhibitory concentration (MIC), which refer to the lowest drug concentration that results in stasis, and minimal bactericidal concentration, which is defined as the lowest drug concentration required to kill a specific microorganism, are commonly used parameters. However, these two parameters do not include time-course and magnitude of bacterial killing. A Study of time-kill-curves has identified two primary patterns of bacterial killing[17]. For *concentration-depend drugs*, a linear relationship between concentration and bacterial killing is characteristic. Both flouroquinolones and aminoglycosides are examples of concentration-depend drugs. The best pharmacokinetic/pharmacodynamic (PK/PD) parameters to describe the efficiency of this group of drug are C_{max}/MIC (C_{max}; the highest concentration reached in the compartment of reference) or AUC/MIC (AUC; area under the concentration-time-curve)[15-18]. *Time-depend drugs*, in the other hand, exhibit very limited concentration depend killing. For this group of antimicrobials, bacterial killing is largely determined by the time of exposure. Beta-lactams, like cefuroxime, is an example of a drug belonging to this group. For this group of drugs, T_{>MIC} is the best predictor of efficiency. Depending on the drug, it is recommended that $T_{>MIC}$ is achieved for 30-70% of the dosing interval for treatment of infection[19]. However, if the objective is to prevent a infection it is generally recommended that plasma as well as tissue concentration of time-depend drugs exceed MIC values of relevant pathogens throughout the surgical procedure and until a few hours after the incision is closed[20]. This is, however, only a general recommendation and targets for prevention of infections is largely unknown.

2.3 Tissue distribution of antimicrobials

Bacterial infections of solid tissues are primary located in the interstitial space. Free plasma concentration was, and in some situations it still is, considered to reflect the free tissue concentration[21-24]. Thus, treatment of infections in solid tissues is commonly based on plasma concentrations[23-25]. During the last decades, however, a growing number of studies have found both incomplete and increased tissue penetration of a number of different antimicrobials[26-34]. These studies clearly indicate that the perception of a homogeneous tissue-plasma distribution may be mistaken. Furthermore, incomplete tissue penetration may be taken into count as a reason for therapeutic failure. Clinical studies on beta-lactams have

suggested that aggressive plasma targets of 100% T_{>1-5xMIC} are more predictive of a successful outcome than traditional targets[35-37]. Application of aggressive targets, like these, obviously reduces the risk of insufficient exposure at the target site due to incomplete tissue penetration. With the knowledge of a heterogeneous tissue-plasma distribution, it seems reasonable to characterize not only the pharmacokinetics of drugs in a specific tissue, but also for specific conditions that may affect tissue penetration.

The importance of antimicrobial tissue penetration has let to a number of studies applying different techniques in order to determine antimicrobial tissue concentration. Until now, studies assessing antimicrobial penetration into bone and IVD have relied on bone specimens and discectomy. These approaches do, however, suffer from a number of important methodological limitations. When analysing tissue specimens, no selective measurement of the free extracellular concentration or distinction between the intra- and extracellular compartments can be made. Furthermore, concentration of the antimicrobial is given by mass and not by volume, and temporal resolution is poor or non-existing, due to the inherent invasiveness which only makes it possible to harvest a rather limited number of specimens. Accordingly, PK parameters obtained by tissue specimens are difficult to relate to PK/PD targets.

PSO is difficult to treat. The antimicrobial treatment time is prolonged and the relapse rates are high[3, 4]. Both circumstances suggest incomplete tissue penetration of antimicrobials in the vertebral cancellous bone and in the IVD. Additionally, this clarifies the importance of an effective prophylactic antimicrobial treatment throughout surgical spine procedures. Accordingly, identification and validation of new techniques to increase our currently limited knowledge on vertebral and IVD pharmacokinetics of antimicrobials are needed. The well-known pharmacokinetic tool, MD, represents a potential candidate. MD will be described in 3.1.

2.4 Cefuroxime

In Denmark, cefuroxime is widely used as antimicrobial prophylaxis in orthopaedic surgery. Cefuroxime is a second-generation cephalosporin, which are a class of beta-lactams that exerts its bactericidal effect by disrupting the synthesis of the peptidoglycan layer forming the bacterial cell wall[38]. Cefuroxime is effective against a broad spectrum of both gram-positive and gram-negative pathogens. Ninety five percent is excreted unchanged in the urine, and as a consequence of the importance of the kidney excretion, dose reduction is recommended when creatinine clearance is less than 20 ml/min. Protein binding is in the range of 33-50%. The half-life of cefuroxime is approximately 60-90 min[39].

Like other beta-lactams, cefuroxime's bactericidal activity is time-dependent. Findings from *in vitro* studies and animal infection models have formed the $T_{>MIC}$ targets for cephalosporins. $T_{>MIC}$ targets are recommended to be in the range of 30-70%[19, 40-43]. While approximately 40% $T_{>MIC}$ leads to bacteriostatic effect, 60-70% $T_{>MIC}$ leads to bactericidal effect.

2.4 Objectives and Hypotheses

The overall objective of this research year was to apply MD for *in vivo* measurements of cefuroxime in vertebral cancellous bone and IVD in order to obtain estimates of key PK parameters and $T_{>MIC}$ in the these anatomical compartments and compare these to SCT and plasma PK parameters. The specific hypotheses of the study were:

- The IVD penetration of cefuroxime is incomplete.
- For clinical relevant MICs, T_{>MIC} is longer in plasma, SCT and vertebral cancellous bone compared to that of the IVD.
- A single dose of 1,500 mg of cefuroxime will provide effective perioperative antimicrobial concentrations in all compartments but the IVD throughout most spine procedures.

3 Materials and methods

3.1 Microdialysis

MD is a minimal-invasive probe based technique that allows for continuous sampling of nonprotein-bound water-soluble molecules in the interstitial space of different tissues[28, 42, 44-47]. Accordingly, MD allows for serial assessment of the pharmaceutical active fraction of antimicrobials. The sampling of molecules occurs as the molecules diffuse along the concentration gradient across the semipermeable membrane at the tip of the probe.

Briefly, the MD technique is performed by placing the membrane in the tissue of interest. Driven by a small precision pump, the MD catheter is continuously perfused with physiologically compatible solution. The solute that has passed trough the catheter, referred to as dialysate, can be collected in vials for subsequent analysis (see figure 1). Due to the continuous perfusion, equilibrium will never occur. Consequently, the concentration of molecules in the dialysate will only represent a fraction of the concentration in the examined tissue. This fraction is referred to as the relative recovery (RR). In some studies, relative changes from baseline and ratios of concentrations may provide adequate information, however, in PK studies, determination of absolute tissue concentration is crucial. Absolute tissue concentration can only be calculated by use of the RR, which can be determined by use of various calibration methods[23, 48, 49].



Figur 1. Schematic illustration of the basic principle of microdialysis. Reprinted from Wikipidia (https://en.wikipedia.org/wiki/Microdialysis).

RR depends on various factors such as chemical conditions of the compound being collected, diffusion coefficient, perfusion rate, MD membrane length and tissue structure etc.[23, 48, 49]. Consequently, an individual MD catheter calibration is needed. Frequently used calibration methods are the low-flow-rate method, the no-net-flux method and retrodialysis by calibration of drug[23, 48, 49]. In the present study, retrodialysis by drug was used in the end of the trial. Additionally, retrodialysis by drug can be performed in the beginning of the trial. However, this requires a washout period to prevent spill over of drug to the actual experiment.

In the present research year project, CMA 107 precision pumps (μ -Dialysis AB, Stockholm, Sweden) and CMA 63 catheters (membrane length 10 and 30 mm, molecular cut-off 20 kilo Daltons) were used throughout the study.

3.2 Ultra High Performance Liquid Chromatography

MD is only a sampling technique that has to be linked to an appropriate analytical assay. Due to low volumes of dialysate a highly sensitive, precise and accurate analytical assay is essential. In the present research year project, cefuroxime was quantified using an Ultra High Performance Liquid Chromatography (UHPLC) method with UV-detection.

The particular steps in the analysis are described elsewhere[13]. The intrarun (interrun) imprecisions (given in per cent coefficients of variation [%CVs]) were 5.6% (6.8%) at 0.25 μ g/ml, 4.3% (4.7%) at 2.5 μ g/ml, and 2.6% (2.8%) at 10 μ g/ml for the dialysates. For the free plasma concentration, the intrarun (interrun) imprecisions were 1.8% (6.5%) at 9.2 μ g/ml and 1.6 (6.2%) at 38 μ g/ml. The lower limit of quantification (LLOQ) in both dialysates and free plasma was 0.06 μ g/ml. Accordingly, the UHPLC method is sensitive, precise and accurate, which makes it reliable for quantification of cefuroxime in both dialysates and blood samples.

3.3 Alternative techniques

Monitoring radiolabelled drug molecules, using positron emission tomography (PET), is worth mentioning as an alternative or supplement to bone biopsy, discectomy and MD. The method has been applied to investigate bone pharmacokinetics of trovafloxacin, fleroxacin and sparfloxacin in both healthy volunteers as well as in an experimental infection model[50-52].

Like bone biopsy and discectomy this method does not distinguish between intra- and extracellular antimicrobial concentration. Consequently, only total antimicrobial concentration per bone mass can be determined. Furthermore, involvement of PET scan for every measurement is related to both practical challenges as well as ethical considerations. However, like MD this method applies for serial assessment of the concentration of antimicrobials.

Other examples of techniques applied over the years in order to estimate tissue concentrations of antimicrobials include the skin blister method[53, 54], concentrations measurements in wound exudates[55, 56] and tissue[57, 58] and fibrin clots[59]. These methods are considered to share notable methodological limitations, and they are often lacking a pathophysiological counterpart[23, 60]. A major disadvantage of the skin blister technique is that the drug concentration has been shown to vary with the size and surface area to the volume ratio[61, 62].

3.4 Animal model

Danish Landrace Breed female pigs were chosen in the present research year project. With a weight of 75-77 kg and an age of 5 month, this was a large juvenile pig model. A large animal model was chosen because of the anatomical size of vertebra and IVD, which made implantation of MD membranes possible. Additionally, pigs have been shown to resemble humans in terms of anatomy and physiology[63]. The pigs were kept in general anaesthesia during the entire measurement period, and were euthanised at the end of the trial.

4 Methodological considerations

4.1 Microdialysis

Advantages

MD offers attractive advantages compered to other methods for determination of tissue pharmacokinetics. Only the unbound fraction of drug, and thereby the pharmaceutically active drug, is measured by MD[6]. Accordingly, drug pharmacokinetics can be compared directly to relevant PK/PD indices. Additionally, MD allows for continuous sampling of the drug of interest, which provides relatively high-resolution concentration-time profiles compared to other approaches like biopsy.

Weaknesses and limitations

When performing MD-studies, the outcome should always be interpreted with the possible limitations of the method in mind. An important factor that may be compromised by experimental needs is the RR. The correction of the measured concentration for RR will lead to magnification of the variations associated with the pre-analytical handling and the chemical assay. This magnification will increase exponentially with decreasing RR. Consequently, it is recommended to achieve a RR above 20%[48]. We found a mean RR in the range of 25.0-44.9%. Experimental factors, that contribute to lower the RR, are short membrane length and high perfusion flow. Due to anatomical factors, a longer membrane length in the IVD was not possible. To compensate for the risk of a low RR, due to the short membrane, a relatively low perfusion flow of 1 μ l/min was chosen. Even though MD precision pumps allows us to select perfusion rates down to 0.1 μ l/min, and thereby gives the opportunity to obtain an even higher RR, this would, however, prevent high-resolution sampling, which the short half-life of cefuroxime calls for.

In this study, individual catheter calibration was performed for all catheters at location. However, the individual catheter RR was calculated based on only one recovery sample. With only one recovery sample the variation associated with the pre-analytical sample handling and the chemical assay will have bigger impact at the RR. A RR based on two or even three recovery samples would reduce this impact. In both SCT and IVD the MD probe can be introduced using a standard introducer. However, standard introducers cannot be used to introduce the MD probes into bone due to its solid nature. This obstacle has been overcome by introducing the MD probes into drill holes in the bone[12-14, 31, 34, 42, 45, 47, 64-66]. This approach raises the question whether MD measurements obtained from the drill holes reflect the bone or the surrounding tissue concentration. In a study comparing PK parameters for sealed and unsealed drill holes it was, however, not possible to find any difference in the PK parameters for sealed and unsealed drill holes[13].

It is appreciated that the present study does not validate MD for use in vertebral cancellous bone and IVD, but only demonstrates that MD can be used to obtain cefuroxime measurements in these compartments. Nonetheless, MD has been validated for use in a variety of other tissues[23, 48]. For the particular case of bone, previous studies have also suggested that MD can be used to estimate antimicrobial bone concentrations[12-14]. Given the lack of a validated reference method, these attempts do, however, remain indirect. No studies have previously applied MD for measurements of antibiotics in the IVD. Nevertheless MD offers significant advantages compered to bone biopsy and discectomy approaches, particularly because MD only samples the unbound pharmaceutically active fraction of the drug, and that serial measurements can be obtained even after surgery has been completed.

4.2 Ultra High Performance Liquid Chromatography

Advantages and weaknesses

The UHPLC method is sensitive, precise and accurate, which makes it reliable for quantification of cefuroxime. However, this method does not come without weaknesses. The volume of the dialysates, obtained by MD, is always low. The volume is, however, depending on the sampling interval and the perfusion rate. In the present study, dialysate volumes ranged from $30-60 \mu$ l. Given the standard volume demand of 15μ l, and the fact that not all the dialysate from the vial can be collected, only a limited number of analysis can be conducted.

Even small variations in the pipetting of volumes will affect the results significant. Accuracy of pipetting is therefore vital when the standard volume is 15μ l.

4.3 Animal model

Advantages

It has been shown that pigs, to some extent, resemble humans in terms of anatomy and physiology[63]. Moreover, the similarities of pig and human bone and IVD size made the surgery and the implantation of MD probe possible. Clinical studies on humans, based on the method used in the present study, are not feasible due to the invasiveness of the method used in this study. The best alternative, from our point of view, was the large porcine model. Additionally, the conditions and traditions of conducting pig experiments at the local research laboratory are excellent.

Limitations and weaknesses

Even though pigs to some extent resemble humans with respect to physiology and anatomy, the major limitation of this study is the fact that pigs remain as a species different to humans. Important interspecies differences must therefore be taken into account[63, 67].

The method used in this study is highly invasive. For that reason and for the convenience of the study, the pigs were kept in general anaesthesia during the entire measurement period. To execute this study at unanaesthetised pigs would be both unethical and an impossible task. General anaesthesia is known to cause physiological alterations, which may affect pharmacokinetics. This must be taken into account when evaluating data. Additionally, general anaesthesia limits the length of the experiments. However, for a short half-lived drug like cefuroxime no different steady state is likely to be achieved after multiple doses.

An animal weight resembling that of the average human being was chosen. However, this animal weight corresponds to juvenile pigs. Blood vessels are presented in the annulus fibrosus in the first decade of life in man[2, 68]. This may be the same for juvenile pigs. Additionally, IVD is thinner in pigs than in humans. Both facts may affect the diffusion of antimicrobials to and from the IVD.

5 Perspectives

Studies evaluating antimicrobial distribution to different tissues by means of MD, will contribute with knowledge to an area of science, where information currently is sparse. In the present study MD was successfully applied for evaluation of cefuroxime IVD concentrations. Thus, MD can become a very useful method for assessment of antimicrobial IVD pharmacokinetics in the future.

At the moment, antimicrobial prophylactic treatment throughout surgical spine procedures and antimicrobial treatment of PSO are based on tradition rather than evidence. MD has the potential to overcome the tradition-based antimicrobial dosing. With greater knowledge, therapeutic concentrations of antimicrobials at the target site can be achieved. Thus, if accomplished, it is reasonable to expect that we in the future can provide the patients with optimized 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 administrated to the patients. If the grade of bone and IVD penetration, time course of bone and IVD concentration and clinical outcome can be evaluated, the most suitable PK-target for treatment of a bone or an IVD infection can be derived[6]. Larger evidence-based knowledge will ultimately result in better antimicrobial treatment and thereby lower risk of orthopaedic-related infections, shorter hospitalization time, lower relapse rates and lower mortality rates for the patients. This is in great interest for the patients and the health system.

The present study is only an experimental study, which cannot replace clinical studies, and particularly not randomised trials with clinical endpoints. Moreover, this was only performed on healthy bone and IVD. Thus, it seems relevant to explore the IVD and vertebral cancellous bone pharmacokinetics of various antimicrobials in clinical studies, in infected IVD and vertebral bone 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.

6 Conclusion

In this study we successfully applied MD for serial assessment of the concentration of cefuroxime in the IVD and vertebral cancellous bone. As hypothesized, IVD cefuroxime penetration was incomplete and additionally delayed, while for SCT and vertebral cancellous bone it was not. However, $T_{>MIC}$ was not longer in plasma, SCT and vertebral cancellous bone as thought. Instead we found a significantly longer IVD cefuroxime $T_{>MIC}$ than for the remaining compartments for a range of relevant MICs. Thus, 1,500 mg of cefuroxime provide more effective perioperative antimicrobial tissue concentration in the IVD compared with the remaining compartments. Using relevant *staphylococcus aureus* MIC values, our data suggest that cefuroxim should be administered in due time and repeatedly if effective concentrations are to be sustained in all tissues throughout spine procedures lasting more than 2-3 hours.

As MD know has successfully been applied for evaluation of cefuroxime IVD and vertebral cancellous bone concentrations, MD may become a very useful alternative method to discectomy and bone biopsy for assessment of antimicrobial bone and IVD pharmacokinetics. Future studies, investigating a variety of antimicrobials in both healthy and infected IVD, seem relevant.

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