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Title:

"Microdialysis as a tool to determine the local tissue concentration of dicloxacillin in man"

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Running head:

“Determining the tissue concentration of dicloxacillin using microdialysis”

Keywords:
- Dicloxacillin
- Microdialysis
- Tissue pharmacokinetics
- Prophylactic antibiotics

What is already known on the subject: Dicloxacillin is used as antibiotic prophylaxis during different types of surgical procedures. Microdialysis has previously been used to determine the tissue concentration of other types of antibiotics in humans.

What this paper adds: We use microdialysis to determine the tissue penetration of dicloxacillin in adipose and muscle tissue. We investigate whether the prophylactic dose, most commonly used, is sufficient to ensure tissue concentrations above the MIC for Staphylococcus aureus.

Word count: 4870
Number of tables: 5
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Summary

Aim – The most common pathogen in Denmark, to cause postoperative infections, is Staphylococcus (S.) aureus (1). Despite using prophylactic antibiotics, infections are still seen. Whether the tissue concentration is above the minimal inhibitory concentration (MIC) for the pathogen is unknown. Thus, the concentration of dicloxacillin in muscle and adipose tissue was measured after intravenous administration, in healthy men.

Methods – MIC for dicloxacillin against S. aureus was determined using the broth macrodilution method. A microdialysis (MD) catheter was placed in the subcutaneous tissue of the abdomen and in the lateral vastus muscle of the thigh of six healthy male volunteers. They were given 2 g dicloxacillin intravenously. Samples from blood and MD fluid were collected. The unbound dicloxacillin was isolated from plasma. Samples were analyzed with High Performance Liquid Chromatography.

Results – The maximum concentration was reached in muscle tissue after 0.5 hours and in adipose tissue after 0.8 hours. AUC_{0-6h} for the dicloxacillin concentration in adipose tissue was significantly lower when compared to the unbound dicloxacillin concentration in plasma. The dicloxacillin concentration was above the MIC for sensitive S. aureus for a minimum of 2.3 and a median of 4.1 hours in muscle tissue and a minimum of 1.8 and a median of 3.2 hours in adipose tissue.

Conclusions – The unbound dicloxacillin concentration in adipose and muscle tissue remained above the MIC for sensitive S. aureus, for a period sufficient for many orthopedic procedures. Whether this is true in patients with compromised circulation this remains to be investigated.
**Introduction**

Postoperative infections can be serious, resulting in long hospital admissions, lengthy antibiotic treatments and increased morbidity and mortality (2).

Prophylactic antibiotics are used to prevent postoperative infections. The dominant microorganism in postoperative infections is Staphylococcus (S.) aureus(1) and dicloxacillin can be used to prevent these infections(3). A standard dose of 2 g dicloxacillin is usually administered intravenously 30 minutes prior to surgery.

Dicloxacillin is a beta lactamase stable beta lactam antibiotic. The molecule is hydrophilic and distributes in the extracellular fluid. Dicloxacillin is highly bound to plasma proteins(4). Only the unbound concentration is pharmacologically active and can diffuse from plasma to the interstitial fluid (5-7). Dicloxacillin is metabolized in the liver and excreted via glomerular filtration, tubular secretion and to some extent in feces via the bile. According to the official product resume the half-life is 45 minutes(8). For a beta lactam antibiotic to be effective, the concentration at the target site should be above the minimal inhibitory concentration (MIC) for at least 40 – 50 % of the dosing interval (9, 10).

Löfgren et al(11) measured the total plasma concentration of dicloxacillin after intravenous administration of 2 g. The plasma concentration rose to 320 µg/ml in young males and females. In this study the unbound dicloxacillin concentration was not determined. In a study by Roder et al(4) healthy volunteers were given 0.5 g, 0.75 g and 1.0 g of dicloxacillin as oral doses. The total plasma concentration rose to 12.4 µg/ml after a dose of 0.5 g, 25.6 µg/ml after a dose of 0.75 g and 29.4 µg/ml after a dose of 1 g. The study also showed antimicrobial activity against S. aureus in plasma, for 4 hours after an oral dose of 0.75 g of dicloxacillin.

Despite studies that show high plasma concentrations of dicloxacillin after administration, S. aureus infections are still seen. Since the tissue penetration may be limited for antibiotics with high protein binding (6), the unbound tissue concentration could be much lower than the total plasma
concentration and possibly be lower than the MIC. This could explain the occurrence of the early postoperative infections.

To our knowledge, a method for determination of the tissue concentration of dicloxacillin has not yet been established. The aim of this study was to establish and validate a method for the measurement of the local tissue concentration of dicloxacillin in muscle and adipose tissue in humans and to determine whether the tissue concentration of dicloxacillin is sufficiently high to prevent infections with S. aureus.

Methods

Microdialysis (MD) was used to determine the tissue concentration of dicloxacillin in adipose and muscle tissue. MD has been used to determine the tissue concentration of other antibiotics in similar studies (5, 12, 13).

Study approvals

The study was approved by The Regional Committees on Health Research Ethics for Southern Denmark (protocol number: S-20130165), The Danish National Health authorities and the Danish Data Protection Agency. The study was conducted in accordance with the Helsinki declaration of 1975, as revised in 2000 and in accordance with the good clinical practice (GCP) guidelines. The study was monitored by the GCP unit at Odense University Hospital. The study was registered in the EudraCT database (EudraCT number: 2014-000826-39).

Microdialysis

The MD technique has been described in detail elsewhere (14, 15). In brief, a MD catheter with a semipermeable membrane is placed in the tissue of interest and perfused with Ringer’s solution. As the solution passes, small molecules in the extracellular fluid diffuse across the membrane. The perfusion fluid is collected in a microvial and can be analyzed.
A complete equilibrium between MD fluid and tissue is never reached. The MD fluid concentration \(C_{\text{MD fluid}}\) will almost always be lower than the true tissue concentration \(C_{\text{tissue}}\). The factor by which the concentrations differ is termed relative recovery (RR) and is defined by the following equation:

\[
RR = \frac{C_{\text{MD fluid}}}{C_{\text{Tissue}}}
\]

RR is often determined in vivo with a method such as reverse dialyses (16). In this method, an MD catheter is perfused with MD fluid containing the substance of interest in a known concentration. The concentration of the substance, that remains in the MD fluid collected, is determined. The RR is calculated using the following formula:

\[
RR = 1 - \frac{C_{\text{MD via1}}}{C_{\text{MD fluid}}}
\]

In our study RR was determined in vitro after the in vivo experiments. We chose this approach because we observed adherence and subsequent wash out of dicloxacillin when using reverse dialysis in an in vitro setting. Dicloxacillin most likely adhered to the tubes that connected the syringe, containing MD fluid, to the MD catheter. Adherence and subsequent wash out of dicloxacillin was not observed when conducting normal microdialysis in an, in vitro setting.

RR was determined in-vitro by spiking 100 mL of human plasma obtained from the local blood bank (Odense University Hospital, J.B. Winsloews Vej 4, Odense, Denmark) with analytical grade dicloxacillin (Sigma-Aldrich, Steinheim, Germany) to a concentration of 100 µg/ml in a 100 ml Bluecap bottle. The bottle was placed in a water bath kept at 37°C and continuously stirred using a magnetic stirrer. The MD catheter was placed in the bottle and flushed with Ringer’s solution for 30 minutes at a flow rate of 5 µl/min before the start of the experiment. This was done to compensate for the dead volume of the catheter. The MD fluid was then collected in a 200 µl microvial (CMA microdialysis, Kista, Sweden) continuously for 30 minutes using the same flowrate. The unbound concentration of dicloxacillin was determined in the plasma by collecting 2x1 ml plasma samples after the last 30 minutes of
microdialysis. The dicloxacillin concentration was measured in both the microvials ($C_{\text{microvial}}$) and the plasma ($C_{\text{plasma}}$). Using these values, we calculated an assumed RR using the following equation:

$$RR = \frac{C_{\text{microvial}}}{C_{\text{plasma}}}$$

Recruitment of volunteers

Six healthy men, between 25 and 27 years, volunteered in December 2014. Written informed consent was obtained from all volunteers. The Body Mass Index (BMI) ranged from 20 - 28, and none had known allergies for beta lactam antibiotics or related drugs. All volunteers completed the experiment and no adverse effects were observed.

In vivo experiments

On the day of the experiment two 63 MD catheters 40/30 (M dialysis, Stockholm, Sweden) were placed in the adipose tissue of the abdomen and in the lateral vastus muscle of the thigh of each volunteer. After placement, the catheters were allowed to equilibrate with the surrounding tissue. An equilibration time of 30 min was used as this was found to be sufficient in similar studies (10, 12, 17). After equilibration the flowrate was set to 5 µl/min.

Two peripheral venous accesses were established with Venflon® (BD, Franklin Lakes, NJ). They were placed in a cubital vein in each arm. One was used to administer the study drug, the other to take blood samples during the experiment. The volunteers were given 2 g Diclocil® (Bristol-Myers Squibb, New York, NY) over 10 minutes.

Blood samples and MD samples were drawn at the time of administration ($T = 0$ minutes). MD samples were taken every 15 minutes for 6 hours. Blood samples were drawn at $T = 10, 20, 30, 45$ minutes and at 1, 1.5, 2, 3, 4, 5 and 6 hours. Blood samples were collected in 10 ml EDTA tubes (BD, Franklin Lakes, NJ). The blood samples were immediately centrifuged at 3000 g for 10 minutes. The isolated plasma
was then frozen in polypropylene centrifugation tubes at -20°C until analysis. The MD samples were immediately frozen in the microvial at -20°C until analysis.

**Sample preparation of microdialysis fluid**

An aliquot of 30 µl of the collected MD fluid was transferred into a 300 µl autosampler glass vial. A volume of 30 µl of 10 µg/ml cloxacillin in Milli-Q water was added as internal standard (IS) as well as 40 µl of methanol. The mixture was whirl mixed for 10 seconds and a volume of 40 µl was injected into the HPLC system. Cloxacillin was purchased from Sigma Aldrich, Steinheim, Germany.

**Preparation of plasma samples**

Ultrafiltration was used to isolate the unbound dicloxacillin from the plasma samples. Two aliquots of 300 µl of the isolated plasma from each sample was placed in 2 separate wells in a 96 well filter plate with a 30 KDa molecular weight cut off (AcroPrep 30K Omega; Pall Corporation, USA). The molecular weight cut off only allowed unbound dicloxacillin to cross the membrane. The filter plate was centrifuged for 30 minutes at 1000 g at a temperature of 37°C. The duplicates of ultrafiltrate were pooled to ensure a sufficient volume for analysis. Ultrafiltration has previously been used to isolate unbound dicloxacillin and other beta-lactam antibiotics (4, 18). In our method, a lower centrifugal force was used to avoid a pressure effect that can cause a higher concentration of unbound drug to be reached in the ultrafiltrate compared to the sample(19). A longer centrifugal time was used to produce a larger amount of ultrafiltrate. According to a study by Kratzer et al. (19) the centrifugal time does not influence the concentration reached in the ultrafiltrate.

A volume of 30 µl of ultrafiltrate was collected from each pool and transferred to a 300 µl autosampler glass vial. To this was added 30 µl of 10 µg/ml cloxacillin in Milli-Q water as IS and 40 µl of methanol.

The sample vial was closed and the mixture whirl mixed for 10 seconds.
HPLC analysis of dicloxacillin

The HPLC system was a LaChrom 7000 serie system and consisted of an L-7100 pump, an L-7250 autosampler, an L-7300 column oven, an L-7400 UV-detector and a D-7000 interface module (Merck-Hitachi, Japan). The separation was performed on a 100 x 4.6 mm Onyx Monolithic C18 column (Phenomenex, Torrance, CA). The eluent consisted of 55% 0.01M NaH₂PO₄ buffer (pH 3.0) in Milli-Q treated water and 45 % Methanol. The flow rate of the eluent was 1.5 ml/min and the analytes were detected at 220 nm. All solvents and reagents were purchased from Sigma-Aldrich, St. Louis, MI.

Validation – microdialysis fluid

Linearity - The linearity of the analytical method was determined as the ratio of the area under the curve of dicloxacillin compared to the area under the curve of the internal standard. Ringer´s solution was spiked with dicloxacillin to concentrations of 0.1 µg/ml, 0.125 µg/ml, 0.5 µg/ml, 1 µg/ml, 5 µg/ml and 15 µg/ml. The samples were prepared using the method described under “sample preparation of microdialysis fluid” and analyzed as described above. The correlation factor (R²) for the linear regression was > 0.98. No interfering peaks were observed at the retention times of cloxacillin (4.60 min) or dicloxacillin (7.20 min).

Intraday variability – Samples of Ringer’s solution were spiked with dicloxacillin to concentrations of: 0.12 µg/ml, 0.25 µg/ml, 0.7 µg/ml, 2 µg/ml and 10 µg/ml. Ten samples were analyzed of each concentration. The samples were prepared and analyzed on the same day using the same methods as described above. The intraday variability was reported as coefficient of variation (CV%) (Table 1).

Interday variability – Ringer’s solution was spiked with dicloxacillin to the 5 different concentrations listed above. Twenty-five samples were made of each concentration and stored at -20°C until analysis. Five samples of each concentration were thawed, prepared and analyzed each day for 5 consecutive days. The samples were prepared and analyzed using the same methods as described above. A mean concentration was determined of each of the spiked concentrations every day. The mean
concentration of all 5 days as well as the standard deviation was determined for each concentration.

The interday coefficient of variation for each concentration is shown in Table 1.

Accuracy - Accuracy is defined as the percentage difference of the mean measured concentration compared to the spiked concentration. Using the data from the determination of the interday variability, accuracy was determined for each spiked concentration on each day using the formula:

\[
\text{accuracy} = 100\% - \left( \frac{\text{mean measured concentration}}{\text{spiked concentration}} \right) \cdot 100 \%
\]

The mean accuracy of the 5 days for each concentration was calculated and the results are shown in table 1.

Limit of quantification (LOQ) - LOQ was defined as the lowest concentration of dicloxacillin in the calibration curves which approximated a signal to noise ratio (s/n) of 10 in the chromatogram. LOQ was determined by spiking Ringer’s solution with dicloxacillin in concentrations of 0.01 µg/ml, 0.05 µg/ml and 0.1 µg/ml. 10 samples were made of each concentration. The samples were prepared and the concentration was measured using the same methods as above. LOQ was found to be 0.1 µg/ml.

Validation – plasma samples

The HPLC assay used to determine the concentration of unbound dicloxacillin in MD fluid and plasma was the same. The preparation of the MD and plasma samples differ in the ultrafiltration of the plasma samples needed to isolate the unbound dicloxacillin. The MD fluid method was validated first. Afterwards the variation imposed by the ultrafiltration process was determined.

Variability – Human plasma obtained from the local blood bank was spiked to 5 different concentrations of dicloxacillin: 12 µg/ml, 25 µg/ml, 70 µg/ml, 125 µg/ml and 250 µg/ml. The spiked samples were prepared as described under “sample preparation of plasma”. Ten samples were prepared of each concentration. The intraday variability was determined by measuring the unbound dicloxacillin concentration in each sample on the same day using the method described under “HPLC
analysis of dicloxacillin”. The intraday variability of the ultrafiltrated plasma samples are shown in Table 2.

Pooled donor plasma was spiked to the 5 different concentrations of dicloxacillin stated above. Twenty-five samples were prepared of each concentration and stored at -20°C until analysis. On 5 consecutive days, 5 samples of each of the 5 concentrations were thawed. Each sample was prepared and analyzed using the same method described above. The Interday variability can be seen in Table 2.

Accuracy - The degree of protein binding of dicloxacillin in plasma is high. The product resume lists the protein binding degree between 95 - 99 % (8) A study determined that 96 – 97 % of dicloxacillin was bound to protein(4). This variation in the degree of protein binding makes it impossible to predict the exact concentration of the unbound fraction in the spiked plasma samples. Thus, accuracy could not be determined for the concentrations measured in plasma.

Pharmacokinetic analysis

Pharmacokinetic data were analyzed by non-compartmental analysis using the R package NCAPP (20) and the concentrations of dicloxacillin in plasma, adipose tissue and muscle tissue were plotted as a function of time. From these curves the following parameters were calculated: area under the time-concentration curve from t = 0 to t = 6 hours (AUC0-6h) using the linear up-log down method; peak concentration (Cmax for adipose and tissue concentrations, C0 for the plasma concentration); time to peak concentration (Tmax); half-life (T1/2); and the time where the concentration of dicloxacillin was above MIC. Statistical inference was assessed using STATA 15® (StataCorp, College Station, TX, USA). Geometric mean ratios (GMR) with 95 % confidence intervals were calculated for AUC0-6h and Cmax. The calculations were made for plasma compared to both adipose and muscle tissue, and for muscle tissue compared to adipose tissue. The concentrations in plasma, adipose and muscle tissue over time were related to the determined MIC.
An analysis of variance (2-way-ANOVA), followed by Sidak’s multiple comparison tests was done for the time-concentration curves of unbound dicloxacillin in plasma, adipose and muscle tissue. The points where the curves differed significantly from each other were calculated. Calculations were performed using Graphpad Prism 6 (La Jolla, CA, USA).

The mean plasma, adipose tissue and muscle tissue concentrations of unbound dicloxacillin were plotted as a function of time using Excel (Microsoft, Albuquerque, NM, USA).

**MIC determination**

The minimal inhibitory concentration (MIC) for dicloxacillin against sensitive *S. aureus* (ATCC 29213) was determined using the broth macrodilution method according to the Clinical and Laboratory Standards Institute guidelines (21). A 0.5 Mcfarland dilution of the above-mentioned bacteria was made. 100 µl of the inoculum was added to each of the following dilutions of dicloxacillin: 8 µg/ml, 4 µg/ml, 2 µg/ml, 1 µg/ml, 0.5 µg/ml, 0.25 µg/ml, 0.125 µg/ml, 0.0625 µg/ml, 0.0313 µg/ml and 0.0156 µg/ml. Triplicates were made of each concentration. There was made a negative and positive control. All samples were incubated at 35°C for 22 hours.

**Results**

Dicloxacillin could be determined in all plasma samples from all 6 volunteers. Adipose tissue concentrations could be obtained for all 6 volunteers but one sample was lost due to an analytical instrument error. Muscle tissue concentrations could be obtained for 5 of the 6 volunteers as one catheter malfunctioned at the start of the experiment. No adverse events or reactions were observed.

**MIC determination**

After 22 hours, there was no bacterial growth at 8 – 0.125 µg/ml dicloxacillin in any of the samples. In all three samples, there was growth at 0.065 µg/ml dicloxacillin and below. The positive control
showed growth and the negative control did not. The MIC was determined to be 0.125 µg/ml which lies above the determined LOQ of 0.1 µg/ml.

**Pharmacokinetic parameters**

The key pharmacokinetic parameters are displayed in Table 3. The GMRs when comparing the $C_{\text{max}}$ and $\text{AUC}_{0-\text{6h}}$ for plasma, adipose and muscle tissue can be seen in Table 4.

Time until MIC is reached and time above MIC for adipose tissue, muscle tissue and plasma can be seen in Table 5. Measured concentrations displayed as a function of time can be seen in Figure 1.

The analysis of variance showed that from 0.78 hours and beyond there was no statistically significant difference between the time-concentration curve of unbound dicloxacillin in plasma, adipose tissue and muscle tissue (Figure 1).

**In vitro recovery**

RR could be determined for 5 of the 12 catheters used. This was due to 7 catheters clotting after being removed from the volunteers. RR was determined to be 64.9 %, 58.9 %, 88.7 %, 92.1 % and 75.0 % respectively. Mean RR was 75.9 %, which was used to correct the MD fluid concentrations for the catheters where RR could not be determined.

**Discussion**

The analytical method showed an excellent accuracy and an intra- and interday variability less than 11.6% for these two matrices. Determination of the free fraction of dicloxacillin in plasma after ultrafiltration showed a higher degree of variability, with an intraday variability <20%, and an interday variability ranging from 13-40%.

The higher degree of variation in the plasma measurement method could be explained by the variable degree of protein binding(22). If the degree of protein binding is close to 100 % a change in protein binding of just 1 % can greatly influence the unbound concentration of dicloxacillin. If the degree of
protein binding varies in the samples used to determine the variation of the method this will add a falsely elevated degree of variation.

Unfortunately, we did not have the capacity to measure the exact protein binding in each sample, which would have aided in understanding the variability in the plasma measurements. The variability in the plasma concentrations could also have been influenced by the ultrafiltration process where we observed a variation in yield between the wells.

We were able to determine the in vitro recovery for only 5 of our 12 MD catheters. The mean RR for the 5 MD catheters was used to correct the measured tissue concentrations for the remaining MD catheters. We observed a variation in the RR between the 5 MD catheters where the lowest RR was 58.9 % and the highest 92.1 %. If the RR in the 7 remaining MD catheters were generally lower than the mean RR we would have under corrected the measured tissue concentrations. The opposite would be true if the actual RR were higher than the mean. We recalculated our data using the lowest and highest measured RR for the 7 remaining MD catheters. When using the lowest RR all concentrations corrected with this value were higher than when using the mean RR. This increased the median $C_{\text{max}}$ and $\text{AUC}_{0-6h}$. With higher concentrations, the time above MIC was also longer. When using the highest RR all concentrations corrected with this value were lower and decreased median $C_{\text{max}}$, $\text{AUC}_{0-6h}$ and a shorter time above MIC was observed. This emphasizes the importance of determining the RR of each catheter. As this wasn’t possible for our study, using the mean RR provided the most reliable alternative. For only one volunteer we could determine RR for both the muscle and adipose tissue MD catheter. RR was higher for the adipose tissue catheter but it is impossible to determine if RR is generally higher in adipose tissue. If RR differs between adipose and muscle tissue using the mean RR would be less reliable.

We found that the peak tissue concentration of dicloxacillin was reached after 0.8 hours in adipose tissue. This can be explained by the fact that adipose tissue has a limited blood supply that could affect the distribution of dicloxacillin from plasma to the adipose tissue. The peak muscle tissue
concentration was reached after 0.5 hours. \( C_{\text{max}} \) was reached earlier in muscle than in adipose tissue showing that dicloxacillin distributes more freely into muscle than into adipose tissue, probably due to a richer blood supply in muscle. Adipose patients and patients with diabetes and cardiovascular disease are at a higher risk of acquiring postoperative infections (23, 24). Thus, repeating these measurements could be worthwhile to determine if \( C_{\text{max}} \) is also reached after 0.8 hours in these individuals.

Knowing when \( C_{\text{max}} \) is reached in muscle and adipose tissue is important when timing the administration of prophylactic dicloxacillin. An example could be surgeries where a tourniquet is used such as total knee arthroplasty. Our findings suggest that dicloxacillin should be administered at least 0.8 hours prior to inflation of a tourniquet, to ensure that \( C_{\text{max}} \) is reached in adipose tissue.

A lower maximum concentration (as seen when comparing the GMR of \( C_{\text{max}} \)) is reached in both muscle and adipose tissue when compared to plasma. This is expected as the maximum plasma concentration is reached immediately after the administration dicloxacillin where the drug is only distributed in the volume of the plasma. When the maximum adipose and muscle tissue concentrations are reached the dicloxacillin has distributed into the larger volume of the tissues reducing the maximum concentrations. The dicloxacillin concentrations in plasma, adipose and muscle tissue remained above MIC for a sufficient amount of time for most surgical procedures. Considering interindividual variation, the shortest time above MIC in muscle tissue was 1.8 hours and in adipose tissue 2.3 hours. The results show a large interindividual variability both regarding \( C_{\text{max}} \) and the time above MIC. As the tissue penetration of dicloxacillin can vary between volunteers, the results should be interpreted with caution, yet the results suggest that a second dose of dicloxacillin should be considered if surgery extends beyond 2 hours to ensure that the dicloxacillin concentration remain above MIC.

The expected function of the curve when plotting the unbound dicloxacillin concentration as a function of time is a biphasic elimination. We expected to see a rapid initial fall in the unbound plasma concentration of dicloxacillin as the drug diffuses from the plasma to the tissues. Then we would
expect a slower exponential decrease in unbound plasma concentration as dicloxacillin is eliminated.

The time-concentration curve of the unbound dicloxacillin in Figure 1 shows an exponential function and it is difficult to discriminate between the initial rapid decrease and the following exponential decrease in concentration. According to the analysis of variability, there was no statistically significant difference between the unbound plasma concentration and both adipose and muscle tissue concentrations from 0.78 hours and beyond. This indicates that an equilibrium between the plasma and tissue concentrations has been reached after dicloxacillin diffuses from the plasma to the tissues. This also indicates that once the equilibrium was reached, plasma, adipose and muscle tissue concentrations fall at similar rates. This fits well with the expected biphasic elimination of dicloxacillin.

The GMR of AUC_{0-6h} for plasma compared to muscle tissue showed no statistically significant difference. The GMR of AUC_{0-6h} for plasma compared to adipose tissue showed a statistically significant difference with a lower AUC_{0-6h} in adipose tissue. The same was found for the GMR of the AUC_{0-6h} for adipose tissue compared to muscle tissue. This difference is likely caused by the higher concentration reached in the plasma and muscle tissue before the equilibrium is reached.

Other studies have determined the tissue concentration of cefuroxime, ertapenem and tigecycline using Microdialysis (5, 9, 13) and obtained similar results regarding tissue and plasma concentrations. Barbour et al (5) also showed a lower concentration in adipose tissue than in muscle tissue. Only a few other studies have determined the pharmacokinetics of dicloxacillin. None of these studies have investigated the tissue pharmacokinetics of dicloxacillin. A study by Löfgren et al (11) where 2 g of dicloxacillin was given as an intra venous infusion showed an AUC in young males of 419 h*µg/ml. In a study by Roder et al (4) where 1 g of dicloxacillin was given orally, AUC was 297 h*µg/ml. AUC in our study was 3.9 h*µg/ml in plasma. In our study, we determined the unbound dicloxacillin concentration in plasma. In the other studies, the total serum concentration was determined. If we assume a degree of protein binding between 95 and 99 % and correct our results by this factor, the AUC would be between 78 and 390 h*µg/ml. The results of the study from Löfgren et al. and Roder et al. are close
to our results when correcting for 99% protein binding. $T_{1/2}$ was determined in the study by Löfgren et al to be 2.11 hours. In the study by Roder et al the $T_{1/2}$ was 1.4 hours. In our study $T_{1/2}$ was only 0.99 hours in plasma, 0.8 hours in adipose tissue and 0.9 hours in muscle tissue. According to the official product resume available from the Danish medicines agency (8) $T_{1/2}$ is 0.75 h when administered intravenously. This is in accordance with our results. In the study by Roder et al $C_{\text{max}}$ was 29.4 µg/ml whereas in the study by Löfgren et al. $C_{\text{max}}$ for the young volunteers was 320 µg/ml. In both studies, the total dicloxacillin concentrations in serum were determined which explain the higher values for $C_{\text{max}}$ compared to our results of 8.2 µg/ml determined as the unbound concentration. If we correct our results with the previously assumed protein binding, the interval for the total dicloxacillin concentration is 164 to 820 µg/ml. The results of the study by Löfgren et al. lies within this interval. Corrected for protein binding, the $C_{\text{max}}$ found in our study was higher than in the study by Roder et al. This would also be expected as the bioavailability is lower when dicloxacillin is administered orally, as well as a lower dose of 1 g was given. Comparison of our pharmacokinetic results with the literature is hampered by the small number of studies. No previous studies have determined the unbound dicloxacillin concentration or investigated the tissue pharmacokinetics of dicloxacillin which makes comparison difficult.

The limitations of the Microdialysis method should be considered when interpreting the results of this study. As described earlier, a complete equilibrium between the MD fluid and the interstitial fluid is never reached. Because of this, correcting for RR is necessary. Regardless of the method used to determine RR this correction adds an additional source of variation to the results. This is especially true if recovery is low.

The concentration measured in a microvial does not represent the concentration of the interstitial fluid at the exact time point where the microvial is changed. The concentration measured in the microvial represents the mean concentration of the MD fluid during the sampling interval. This means that the peak concentration and the time the tissue concentration rises above and falls below MIC lies
somewhere within the sampling interval. Using short sampling intervals reduces this inaccuracy. In our study, we used short sampling intervals of 15 minutes.

Using a short sampling interval results in a small volume of MD fluid collected in the microvial. This can be a problem if the volume is too small to be analyzed. In our analytical method 30 µl of MD fluid was required for analysis. To produce a higher volume of MD fluid in the microvial a higher flow rate can be used. In our study, we used a flow rate of 5 µl/min. Our flowrate was high compared to other studies where flowrates between 1.5 and 2 µl/min where used (5, 9, 10, 16). The consequence of using a high flowrate can be a lower recovery, as the MD fluid has less time to create an equilibrium with the tissue. Despite our high flowrate the mean recovery of dicloxacillin was 75.9 % This is comparable to a mean RR of 55 % found by Boyadjiev et al. (9) and a mean RR in adipose tissue of 58.3 % and in muscle tissue 59.4 % found Barbour et al.(5).

**Conclusion**

Microdialysis can be used as a tool to determine the local tissue concentration of dicloxacillin in healthy men. In this study, the adipose and muscle tissue concentrations of unbound dicloxacillin rose above the MIC for sensitive S. aureus and remained above MIC for a period that is sufficient for many orthopedic procedures. The duration of which dicloxacillin remains above MIC in other patient groups such as adipose patients, trauma patients and patients with peripheral circulatory problems remain to be investigated.

**Authors' contributions**

Kristian Kraft Hansen planned and conducted the experiments, analyzed the samples, interpreted the results and drafted the primary manuscript. Lasse E. Rasmussen planned the experiments, helped conduct the experiments and revised the manuscript. Flemming Nielsen has contributed to the planning of the study, has supervised the analysis of samples and revised the manuscript. Tore Bjerregård Stage conducted the statistical and pharmacokinetic analysis of the data and revised the
manuscript. Uffe Jørgensen contributed in the planning and initialization of the study and revised the manuscript. All authors contributed to the data analysis.

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Conflicts of interest and funding

The study was funded by “Overlæge Johan Boserups og Lise Boserups legat”, “Familien Hede Nielsens Fond”, “Aase og Ejnar Danielsens Fond”, “Ingeniør K. A. Rohde og hustrus Legat” and the department of orthopedics and traumatology Odense University Hospital and the University of Southern Denmark.

T.B. Stage has held unrelated paid lectures for Eisai, Orifarm, Novartis and Astellas-Pharma.

None of the authors or funds, have any association to the manufacturers of the study drug or dialysis systems.
References


List of hyperlinked key protein targets and ligands

The submitted manuscript contains no mention of key protein targets or ligand which are featured on http://www.guidetopharmacology.org/. As a result, no hyperlinks have been added to the manuscript.
Table 1 validation of the HPLC method for determining the dicloxacillin concentration in MD fluid.

<table>
<thead>
<tr>
<th>Spiked concentration of dicloxacillin</th>
<th>0.12 µg/ml</th>
<th>0.25 µg/ml</th>
<th>0.7 µg/ml</th>
<th>2 µg/ml</th>
<th>10 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intraday CV %</strong>:</td>
<td>7.79</td>
<td>4.74</td>
<td>6.60</td>
<td>2.70</td>
<td>2.67</td>
</tr>
<tr>
<td><strong>Interday CV %</strong>:</td>
<td>11.58</td>
<td>11.22</td>
<td>7.97</td>
<td>2.32</td>
<td>1.68</td>
</tr>
<tr>
<td><strong>Accuracy (%)</strong>:</td>
<td>-4.52</td>
<td>+4.69</td>
<td>+5.43</td>
<td>-1.15</td>
<td>-4.31</td>
</tr>
</tbody>
</table>

CV %: coefficient of variation defined as standard deviation / mean x 100 %.
1) CV % between 10 samples of each of the 5 concentrations measured on the same day,
2) CV % between 5 samples of each of the 5 concentrations measured 5 times on 5 consecutive days,
3) Accuracy is the percentage difference 100 % - (measured concentration / true concentration) x 100 % where the mean concentration of all the measurements from the interday CV % determination was used.
Table 2 Validation of the HPLC method for determining the dicloxacillin concentration in plasma.

<table>
<thead>
<tr>
<th>Spiked concentration of dicloxacillin</th>
<th>12 µg/ml</th>
<th>25 µg/ml</th>
<th>70 µg/ml</th>
<th>125 µg/ml</th>
<th>250 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraday CV %</td>
<td>10,49</td>
<td>4,80</td>
<td>6,85</td>
<td>19,32</td>
<td>14,16</td>
</tr>
<tr>
<td>Interday CV %</td>
<td>13,80</td>
<td>35,37</td>
<td>40,12</td>
<td>12,77</td>
<td>13,16</td>
</tr>
</tbody>
</table>

Abbreviations as per table 1. Accuracy could not be determined for measurements of dicloxacillin in plasma as the true concentration was unknown due to the variation in protein binding of dicloxacillin.
Table 3 Key pharmacokinetic parameters for unbound dicloxacillin in plasma, muscle tissue and adipose tissue. Data are presented as median +/- range

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Plasma (n=6)</th>
<th>Muscle tissue (n=5)</th>
<th>Adipose tissue (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₀/Cₘ₅₉ (ug/ml)</td>
<td>8.2 (4.5-11.1)</td>
<td>2.1 (1.8-5.8)</td>
<td>1.1 (0.4-3.3)</td>
</tr>
<tr>
<td>Tₘ₅₉ (h)</td>
<td>N/A</td>
<td>0.5 (0.5-0.8)</td>
<td>0.8 (0.5-1.1)</td>
</tr>
<tr>
<td>Tₐ⁄₂ (h)</td>
<td>1.0 (0.5-6.2)</td>
<td>0.9 (0.7-1.0)</td>
<td>0.8 (0.5-1.1)</td>
</tr>
<tr>
<td>AUCₜₐ₆ₖ (h*ug/ml)</td>
<td>3.9 (1.9-6.1)</td>
<td>3.1 (1.7-7.9)</td>
<td>1.5 (0.7-4.5)</td>
</tr>
</tbody>
</table>

Cₘ₅₉: maximal concentration reached, C₀: concentration reached in plasma immediately after intravenous administration, Tₘ₅₉: time until maximum concentration was reached, Tₐ⁄₂: elimination half-life, AUCₜₐ₆ₖ: area under the time-concentration curve measured from t = 0 until the last sample collected after 6 hours.
<table>
<thead>
<tr>
<th></th>
<th>GMR Muscle tissue/plasma¹</th>
<th>GMR Adipose tissue/plasma²</th>
<th>GMR adipose/muscle tissue³</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C&lt;sub&gt;max&lt;/sub&gt;</strong></td>
<td>0.35 [0.20-0.61]</td>
<td>0.16 [0.09-0.28]</td>
<td>0.42 [0.23-0.75]</td>
</tr>
<tr>
<td><strong>AUC&lt;sub&gt;0-6h&lt;/sub&gt;</strong></td>
<td>1.11 [0.65-1.91]</td>
<td>0.51 [0.30-0.87]</td>
<td>0.43 [0.22-0.87]</td>
</tr>
</tbody>
</table>

Abbreviation as per table 3, GMR: geometric mean ratio, 1) pharmacokinetic parameters for plasma compared to muscle tissue, 2) pharmacokinetic parameters for plasma compared to adipose tissue, 3) pharmacokinetic parameters for muscle tissue compared to adipose tissue.
Table 5 Hours after administration of 2 g dicloxacillin where the concentration is above MIC. Data are presented as median +/- range.

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>Muscle tissue</th>
<th>Adipose tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time above MIC</strong></td>
<td>3.2 (2.1-6.3)</td>
<td>4.1 (1.8-4.9)</td>
<td>3.2 (2.3-5.0)</td>
</tr>
<tr>
<td><strong>Time onset\textsubscript{1}</strong></td>
<td>N/A</td>
<td>0.1 (0.07-0.3)</td>
<td>0.3 (0.08-0.4)</td>
</tr>
<tr>
<td><strong>Time offset\textsubscript{2}</strong></td>
<td>N/A</td>
<td>4.6 (4.1-5.0)</td>
<td>3.5 (2.5-4.5)</td>
</tr>
</tbody>
</table>

MIC: Minimal inhibitory concentration for dicloxacillin when used against S. aureus (0.125 µg/ml), 1) the time in hours after the administration of dicloxacillin where the concentration rises above MIC, 2) the time in hours after the administration of dicloxacillin where the concentration falls below MIC
Figure 1: time-concentration curve of dicloxacillin in plasma, muscle and adipose tissue. Data are shown as mean concentrations ± SD at each sample time for all volunteers.

The figure shows the time-concentration curves of unbound dicloxacillin in plasma, muscle and adipose tissue on semi logarithmic axes. The minimal inhibitory concentration of dicloxacillin against sensitive S. aureus is showed as the small dotted line. The limit of quantification, defines as the lowest concentration that can be reliably determined, is showed as the large dotted line. The maximum unbound plasma concentration is reached immediately after administration and falls as an exponential function. The time until the maximum concentrations are reached in adipose and muscle tissue is reached is delayed. An equilibrium is reached with similar concentrations in plasma, adipose tissue and muscle tissue and the concentration falls with similar rates in all three compartments.