Bioassay of Amoxicillin in Rats

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Few reports are available about tissue concentration of amoxicillin. The techniques used to measure tissue concentration usually require rupture and are expensive. The objective of the present study is to assess the utility of an animal model to predict tissue concentration of amoxicillin using induced granulomatous tissue. We used 160 rats with four polyurethane sponges previously implanted in their backs. At 7, 14, 21 and 28 d after sponge introduction, groups of eight animals each received 3.5, 7.0, 40.0 or 80.0 mg/kg of amoxicillin (*p.o.*) or 1 ml of 0.9% NaCl solution (control group). One hour after drug administration, 10 μl of serum and granulomatous tissue were obtained. Tissue and serum were placed on different plates containing Mueller Hinton agar inoculated with 10^8 cfu (colony forming unit) of *Staphylococcus aureus* (ATCC 25923), and the diameters of the inhibition zones were measured after 18 h of incubation. Analysis of variance showed no statistically significant differences ($p>0.05$) among time periods for the same dose of amoxicillin. These results suggest that the pharmacokinetics of amoxicillin did not change in relation to the development of granulomatous tissue; therefore this method is valid to measure the tissue concentration of amoxicillin.

Key words *in vivo* model; amoxicillin; pharmacokinetics

Some *in vivo* models such as implanted fibrin clots or dialysis sacks allow the diffusion of antimicrobial agents into the site of infection but can limit cellular and humoral defense. Granulomatous or fibrinous tissues are formed as a result of the implantation of porous or hollow devices. These models may be valuable for determining the capacities of antibiotics to penetrate a specific site.[1)]

Many different factors are known to affect antimicrobial efficacy *in vivo*. Some of these factors, such as the infectious microorganisms, the site of infection, the intrinsic activity of an antimicrobial agent, and its pharmacokinetic behavior, have been recognized and investigated in experimental infection models.[2)]

When the antibacterial activity, pharmacokinetics and pharmacodynamics of an agent are considered, the treatment time should be more specifically defined as the time during which the concentration of the agent is greater than the minimum inhibitory concentration (MIC) of the pathogen at the site of infection.[3)] In fact, the MIC should be considered in relation to tissue concentration, and not in relation to blood serum concentration, because the development of an infection generally requires superficial adherence to some biological barriers. Furthermore, most bacteria have no pathogenic effects on body fluids; they are found in such fluids as a result of diffusion from the infection site, contamination by instrumentation or due to tissue rupture.[4)]

Few infection models utilize endogenous infection in experimental chemotherapy because the administration of an infectious agent, usually in a large inoculum, is required to establish a reproducible infection in animals. This large inoculum may be needed to overcome host defenses, but may result in a fulminant course of infection owing to the immediate introduction of large numbers of infectious organisms.[5)]

The disadvantages of animal infection models include unnaturalness, possibility of fulminant infections and an artificial mode of infection. Chronic or progressive infections may result in an ever-increasing pain level owing to continuous deterioration of the host. The pain and suffering of the animals should be minimized.[5)]

Amoxicillin is a widely prescribed aminopenicillin, mainly administered orally.[6)] Ninety percent of the administered dose is absorbed without molecular modification.[7)] It provides serum concentration ranging from 7.6 to 10.8 μg/ml when 500 mg/*p.o.* is used;[8,9)] 15.1 μg/ml when 15.4 mg/kg/*p.o.* is used,[6)] and 14.5 μg/ml when 40 mg/kg/*p.o.* is used.[10)]

The amoxicillin has plasmatic protein binding ranging from 17 to 20%.[7)] Protein binding has no clinical importance if less than 70% of an antimicrobial agent is bound.[11)] Food does not interfere with either absorption or plasmatic concentration.[10)]

Mercury nitrate titration,[12)] microbiological method, iodometric assay, optical method,[13)] spectrophotometric assay,[14)] and HPLC assay[15)] are some of the possible methods, currently used to quantify amoxicillin. A comparison between the HPLC and microbiological methods showed no differences.[15,16)]

Amoxicillin is metabolized into penicilloic acid to a limited extent, which is then excreted in urine. About 60% of an oral dose of amoxicillin is excreted, in an unchanged form, in the urine in 6 h by glomerular filtration and tubular secretion.[17)] The penicilloic acid has no antimicrobial activity.[7)]

In the present experimental study we assessed the utility of an animal model in predicting tissue concentration of antimicrobial agents in induced granulomatous tissue.

MATERIALS AND METHODS

Pharmacological Agents Amoxicillin trihydrate was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and was used in aqueous suspensions of 1.75, 3.5, 20.0, and 40.0 mg/ml. Physiological saline solution (0.9% NaCl) was used in all the suspensions.

Animals A total of 160 adult male Wistar rats (Rattus norvegicus–albinus), 60 d of age and weighing 175±25 g were obtained from CEMIB/UNICAMP (Centro de Bio-

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terismo–ICLAS Monitoring/Reference Center, Campinas, Brazil), where they were maintained under aseptic conditions. The Pharmacology Graduate Committee scientifically and ethically approved this study, and the ethical guidelines issued in the Declaration of Helsinki were applied.

Bacterial Strain *S. aureus* (ATCC 25923) was used in the *in vitro* test microorganism to determine the MIC (minimal inhibitory concentration) by using the susceptibility test in Mueller–Hinton broth[18] and in the regression line.

Granulomatous Tissue and Groups Granulomatous tissue was induced in all rats by subcutaneous implantation of 4 sterilized polyurethane sponge discs (density=35 kg/m^3) into their backs. This sponge (PRO301–Proespuma Com. e Ind. Ltd.) is similar to that used in mattresses. It was cut in ring-shaped pieces 6.2 mm in diameter and 2 mm in height, weighing 2.1±0.1 mg.

After 7, 14, 21 or 28 d of sponge implantation, eight animals for each time period received amoxicillin (*p.o.*) in a single dose of 3.5, 7.0, 40.0 or 80.0 mg/kg, 1 h (C_{max} between 1 to 2 h[8]) before the removal of tissue and serum. The administered quantities ranged from 0.3 to 0.4 ml/animal. The control group (n=8) received 0.4 ml/animal physiological saline (0.9% NaCl) one hour before the removal of tissue and serum samples.

Regression Line Solutions with concentrations of 0.03, 0.05, 0.1, 0.3, 0.5, 0.7, 1.0, 3.0, 5.0, 7.0, 10.0, 13.0, and 15.0 μg of the antimicrobial agent in 10 μl of distilled and deionized water were placed on dry 6.25 mm paper disk filters. These discs were placed in triplicate on 150 mm Mueller–Hinton agar plates inoculated with 10^8 cfu of *S. aureus* and the resulting zones of inhibition were measured after 18 h of incubation at 37 °C.[19]

The correlation line was obtained by plotting the log of the concentrations used against the linear measurement of the zone of inhibition. A formula was obtained to facilitate data conversion using computer software (Excel 97® for Windows®, Microsoft Corporation).

Surgical Procedures and Samples After rapid anesthetic induction with ethyl ether, the carotid plexus of each animal was cut. Blood samples were collected from the 7-d group of animals, centrifuged, and 10 μl of serum was placed on three 6.25 mm sterilized paper discs and allowed to dry at room temperature.

Two samples of these paper discs with serum were placed on Mueller–Hinton agar plates inoculated with 10^8 cfu of *S. aureus*. Granulomatous tissue was delimited and removed. Two samples were placed onto other Mueller–Hinton agar plates inoculated with 10^8 cfu of *S. aureus*. After 18 h of incubation at 37 °C, the zones of inhibition were measured. The other two tissue samples were weighed and analyzed by a routine histological technique.

The diameters of the inhibition zones were submitted for analysis of variance (ANOVA) using computer software (Jump® for Windows®–SAS Institute).

RESULTS

MIC The MIC for amoxicillin against *S. aureus* in the present study was 0.1 μg/ml.

Wet Weight and Histology The mean (±S.E.M.) wet weights that were used to obtain the tissue concentration of the antimicrobial agent are shown in Table 1.

Table 1. Mean (±S.E.M.) Granulomatous Tissue Wet Weight

	Wet weight (g) ± S.E.M.
7 d (n=56)	0.0230±0.00121
14 d (n=56)	0.0216±0.00056
21 d (n=56)	0.0213±0.00070
28 d (n=56)	0.0210±0.00081

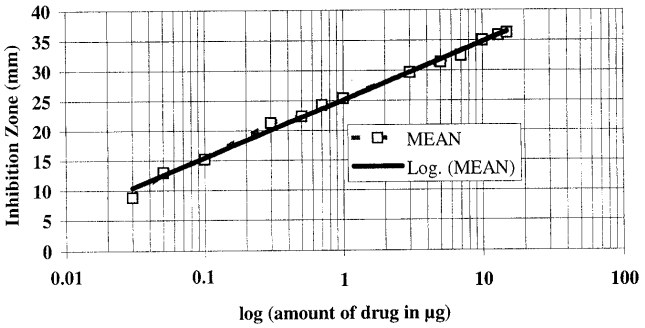

Fig. 1. Graphic Representation of the Diameter of the Zone of Inhibition (Mean in mm) Plotted *versus* the Log of Amount of Drug (μg) and the Log of the Mean (Regression Line)

Histological technique showed no qualitative differences among granulomatous tissue of the control or treated groups. The findings of the 7-d groups showed a fibrous capsule involving the sponge that was delimited very well. Fibroblasts, mesenquimal cells and capillary formation was noted in large scale. After 14 d we found more fibrous tissue involving the sponge, but with a reduced number of cells in the periphery and cell proliferation at the center of the tissue. Macrophages were observed at 21 d of tissue formation. At 28 d the granulomatous tissue was completely formed, showing fibrous fibers involving fewer cells and blood vessels in the center of the tissue.

Regression Line The detection limits used in the regression line were 8.8 mm (obtained with 0.03 μg) to 36.2 mm (obtained with 15.0 μg). Figure 1 shows the regression line.

The data were plotted with computer software (Microsoft Excel 97 for Windows) using the equation $y=4.2009\times Ln(x)+25.083$ or Zone (mm)=4.2009×Ln(μg of antimicrobial agent)+25.083 with an R-square value of 0.994.

It was possible to calculate the antimicrobial concentration in serum and granulomatous tissue by using the mean inhibition zone diameter calculated and the wet weight for each group.

Tissue and Blood Figure 2 shows the mean tissue and blood serum concentrations obtained with the equation conversion for all study periods. The results of inhibition zone diameter for all study periods of the control group were zero.

Analysis of variance did not show significant differences among the groups in inhibition zone diameter or in wet weight when the same dose was considered.

DISCUSSION

The present MIC data were similar to those obtained by other studies, using similar conditions.[18,19]

Mean wet weights did not vary significantly within or between groups (p>0.05), probably due to the fact that only

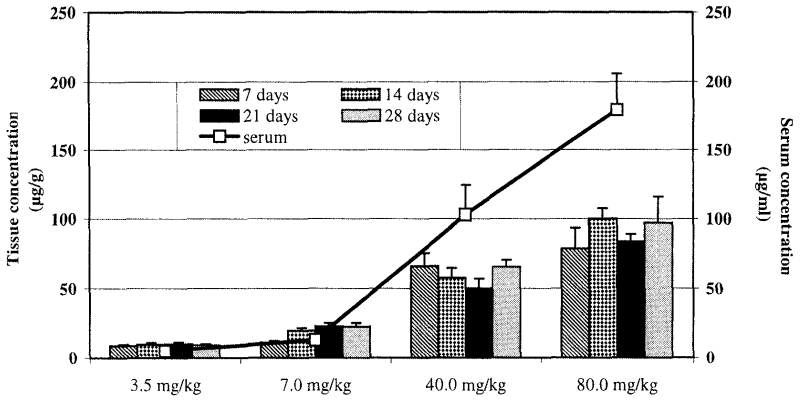

Fig. 2. Mean Amoxicillin Concentrations in Tissue (μg/g ± S.E.M.) and Serum (μg/ml ± S.E.M.) at the Different Time Periods

one operator was responsible for the surgical procedure. The observed histological findings were very similar to results of other authors when they used the same material implanted in the back of rats.[20]

The results for the control group (serum and tissue) indicate that the host defense system did not produce inhibition zones *in vitro*, and therefore, the antimicrobial agent only caused the inhibition zones detected in the other groups. The use of tissue samples on agar in sensitivity tests has been described in other studies.[21—23] The present model was able to detect serum concentrations with results similar to those reported in the literature, in which the concentrations ranged from 7.6[19,24] to 10.8 μg/ml.[9]

The administration of an infectious agent to establish infection was not utilized in this model, permitting us to determine the action of a free antimicrobial agent present in granulomatous tissue in a very large inoculum without a fulminant death. Since in the present model the animals were not directly submitted to infection, problems associated with this type of procedure, such as pain, contamination, septicemia or differences in reproducibility,[5] were not observed. The present model also shows other desirable characteristics of an infection model, as described in another study,[5] such as simplicity of the technique, reproducibility, measurability and tissue involvement.

The evolution of granulomatous tissue did not affect its amoxicillin concentration, so it was possible to detect the tissue concentration of the drug at all periods tested. This model could be applied to assays involving amoxicillin, other antimicrobial agent or drug interactions.

We conclude that this methodology is valid to evaluate the diffusion and concentration of amoxicillin in serum and tissue. The development of this type of tissue did not interfere with the diffusion of the antibiotic, and the concentration of the antibiotic was measurable and larger than the MIC at all tested time periods.

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