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The Pharmacodynamic Effects of Amoxicillin Against *Staphylococcus aureus* in an *In-vitro* Kinetic Model

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Abstract: The pharmacodynamic effect of amoxycillin against *Staphylococcus aureus* was evaluated in an *in-vitro* pharmacodynamic model. For amoxycillin peak concentrations of 0.4, 1.0, 1.6 and 3.2 mg mL^{-1} and half-lives of 2 and 5.5 h were examined. The bactericidal effect was measured as the reduction in colony count (log CFU mL^{-1}) during the experimental period and the overall pharmacodynamic effect as the area under the bacterial growth versus time curve (AUBC). In an *in vitro* model with elimination half-life of 2 h, only 16 MIC amoxicillin exhibited persistent killing of *Staphylococcus aureu* with a bacterial number reduction of 2.51 log10. in an *in vitro* model with simulative elimination half-life of 5.5 h, 5, 8 and 16 MIC amoxicillin exhibited persistent killing of *Staphylococcus aureu* with a bacterial number reduction of 2.42, 2.72 and 2.53 log10 noted during the 12 h, respectively. These data indicate that amoxicillin reach the maximum killing at 5-folds of their MIC values and the antimicrobial maintaining time is mainly correlated with T>MIC. But during the whole experimental period, bactericidal effect and bacterial elimination were not achieved on *Staphylococcus aureu* strains for which the MIC was 0.2 μg mL^{-1}, even with a Cmax/MIC ratio of 16.

INTRODUCTION

In veterinary drug development procedures, Pharmacokinetic (PK) and Pharmacodynamic (PD) data have generally been established in separate, parallel studies to assist in the design of dosage schedules for subsequent evaluation in clinical trials. However, systemic drug action is mediated via the time course of drug concentration at the site of action which bears a proportional relationship with plasma concentration there is the realization over the last decade that the relationship between the concentration of an antimicrobial in an animal and its effect on the target pathogen is not simple. Most of the current dosing regimens of antibiotics in clinical practice are based on relationships between the drug's serum concentration and the respective Minimum Inhibitory Concentration (MIC) for the micro-organism of interest. Although, MIC is a good predictor of potency for antibacterial activity, it is not an ideal PD parameter because it is a threshold value with poor precision and

antibacterial efficacy is a dynamic concentration- and time dependent process. Many additional factors need to be studied in order to increase knowledge about optimal dosing regimens, e.g., the relation between Pharmacokinetic and Pharmacodynamic parameters (PK-PD) interactions with the immune system of the host and pharmacological determinants for selection of resistance at the site of infection and in the normal flora. Thus, dosage strategies have been developed which may increase the efficacy or reduce the selection pressure for resistance associated with the antimicrobial for which they are tailored. The utility of PK/PD information is demonstrable in the development of new antimicrobials, the more specific selection of appropriate antimicrobials from formularies, the design of optimal dosage strategies and the reduction in selection of antimicrobial resistance. PK/PD modeling has major potential for rational dosage regimen determination as it considers and quantifies the two main sources of interspecies variability (PK and PD). In recent years, there have been several important reviews on PK-PD integration of antimicrobial drugs used in veterinary medicine (Toutain and Lees, 2004; Mckellar *et al*., 2004). PK-PD experiments with Danofloxacin have been conducted in calves, goats, sheep, camels, turkeys and rabbits (Aliabadi and Lees, 2001, 2003; Sarasola *et al*., 2002; Aliabadi *et al*., 2003a, b; Haritova *et al*., 2006; Fernandez-Varan *et al*., 2007). PK-PD investigations with moxifloxacin has also been described in rabbits (Fernandez-Varan *et al*., 2005). Although there have been reports about pharmacodynamic effects of amoxicillin against *Streptococcus pneumoniae* and *Actinobacillus pleuropneumoniae* in *in-vitro* kinetic nodel (Gustafsson *et al*., 2002; Lindecrona *et al*., 2002) there is no pharmacodynamics of amoxicillin against *Staphylococcus aureus* in *in-vitro* kinetic nodel. The aim of this study was to study the effect of amoxicillin against *Staphylococcus aureus.* In an *in-vitro* one-compartment kinetic model which simulated PK profiles of Amoxicillin in animals with a view to describe the relationship between PK data and PD data and to predict the likely efficacy in clinical use of recommended regimen.

MATERIALS AND METHODS

Drugs and chemical reagents: Amoxicillin reference standard (86.2%, Lot #130409-200608) was purchased from National institute for the control of pharmaceutical and biological product. Acetonitrile from Fisher Scientific was high performance liquid chromatography (HPLC) grade. Other agents were A.R. grade and purchased in China. Mueller-Hinton broth and nutrient agar were also purchased in China.

Bacteria: *Staphylococcus aureus* ATCC®25923 was used as test strain which was obtained from National institute for the control of veterinary pharmaceutical and biological product.

Determination of Minimum Inhibitory Concentrations (MICs): The amoxicillin minimum inhibitory concentrations for the bacterial strains was determined using the broth macrodilution method. The minimum inhibitory concentration was defined as the lowest concentration of drug inhibiting visible macroscopic growth of an inoculum of approximately 1×10^5 colony forming units per milliliter (CFU mL^{-1}) after an appropriate incubation period (24 h). The MIC determinations were made in triplicate on separate occasions.

Bacterial quantification: Bacterial counts were determined by plating three serial 10-fold dilutions of the samples on nutrient agar plates. The dilutions were made in sterile normal saline. Aliquots (100 μL) of each dilution were plated in triplicate. The plates were incubated at 37 for 24 h before reading. Following incubation, colonies were counted in all readable plates. The number of colonies in each dilution tube at each time was determined by averaging the counts obtained.

***In-vitro* kinetic model:** A simple one-compartment *in vitro* model which has previously been described by Lindecrona *et al*. (2002) was developed to study the PD effect of concentrations of amoxicillin against *Staphylococcus aureus*. The *in-vitro* model consisted of a 500 mL magnetic stirred cell filled with 300 mL broth. The broth was continuously stirred during the experiment. Elimination of the antimicrobial agents was achieved using a peristaltic pump to supply fresh antibiotic-free medium to the compartment and a pump to withdraw the medium continuously from the compartment at a constant rate, resulting in the simultaneous displacement of antibiotic-containing medium. The flow rate of the pump was set according to the half-life being obtained by us in previous work (Zeng, 2002). Elimination of the bacteria was prevented by a filter (pore size 0·2 μm, Millipore) and a prefilter placed in the bottom of the compartment. Samples were taken through a silicone membrane. Hence in this model, the antibiotic concentration was diluted continuously and different elimination half-lives of an antibiotic were simulated by changing the flow rate of the pump. The antibiotic was diluted according to first-order kinetics: $C = C_0 e^{-kt}$ where *C* is the achieved concentration after a constant elimination rate (k) of the C_0 during the course of time (t). The AUC at 24 h (AUC_{24}) was calculated as follows: $AUC_{24} = C_0/k\text{-}C_{24}/k$ where C_{24} is the concentration after 24 h, depending upon the elimination constant k, determined from $k = \ln 2/t_{1/2}$. In the experiments, the degradation was included in the flow rate. The complete system (including auto-dilution system, pump and magnetic stirring plate) was placed in a large incubator at 37°C during the experiments.

Experiments design: The intention during the experiments was to keep the $t_{1/2}$ to be 2 and 5.5 h over 12 h with the initial drug concentration ranged from 2 MIC, 5MIC, 8MIC-16 MIC. The flask was prepared with appropriate broth and bacteria suspension from logarithmic growth phase were inoculated into the model and incubated for 0.5 h before addition of the antibiotic in the thermostatic room (37°C). Then the drug were delivered as a bolus into the compartment in doses to get the desired initial antibiotic concentration. The flow rate of the pump was set to obtain the different $t_{1/2}$ s of the drugs. Samples were withdrawn at 0, 0.5, 1, 2, 3, 4, 6, 8 and 12 h for viable bacterial number counts and drug concentration determination. Appropriate dilutions were plated (0.1 mL) on nutrient agar and incubated overnight and viable counts were determined. The limit of detection of bacterial numbers was 10 CFU mL^{-1}.

HPLC analysis: Amoxicillin concentrations in broth were determined with an HP 1100 HPLC system using a method adapted from Zeng (2002). Briefly, an aliquot of 1.0 mL sample was deproteinized with 1.0 mL 20% trichloroactic acid solution in a centrifuge tube, centrifuge for 5 min at 6000 rpm, decant supernatants into a second centrifuge tube, add 1 mL pH 2.0 buffer solution and 0.5 mL 7% formaldehyde solution to the supernatant., vortex the tube contents and place in a mineral oil bath at 95 for 2 h, cool tubes in an ice water bath for 5 min, add 5 mL diethyltether to each tube and shake very gently for 20 min, centrifuge for 10 min at 3500 rpm using a glass syringe, aspirate the bottom(aqueous) layer of each tube and discard. Evaporate tube contents under a stream of nitrogen at 60. Reconstitute with 0.25 mL mobile phase for HPLC analysis. Twenty microlitres of the supernatant was injected into the HPLC system (Hewlett Packard 1100, PaloAlto, CA, USA) for analysis. Chromatography was carried out using a Hypersil BDS C18 Column (5 μm, 4.6×250 mm); the mobile phase consisted of phosphate buffer solution (pH 5.6) and Acetonitrile (80:20, v/v) at 1 mL^{-1} min flow rate. The fluorescence detector operated at an excitation wavelength of 362 nm and an emission wavelength of 435 nm. Chromatogram peak areas were quantitated by the external standard technique using standard solutions of amoxicillin. For calibration, both 1.0 mL blank broth was spiked with 20 μL of a series of diluted amoxicillin working standard solutions and analyzed as above. The concentration of amoxicillin in the prepared standard samples were 0.03, 0.1, 0.2, 0.5, 1, 2, 5, 10 μg mL^{-1}. The limit of quantitation, quantitation linearity and recovery of amoxicillin from plasma were determined in pigs and broilers. Coefficients of Variation (CV%) within and between HPLC runs were also calculated.

Pharmacokinetic and pharmacodynamic analysis: Pharmacokinetic parameters were calculated for amoxicillin in broth using a MCPKP program which was described elsewhere (Xia and Chen, 1988). Minimum Akaike Information Criterion Estimates (MAICE) were applied to discriminate the best fitting model. Quantitative evaluation of bactericidal activity was expressed as the Area Under the Bacterial Killing-Curve (AUBKC) and log10 difference between bacterial count (CFU mL^{-1}) after 12 h incubation and the initial inoculum bacterial count ($\triangle$log10 CFU mL^{-1}). AUBKC was calculated according to the trapezoidal rule up to the last sampling point (Gibaldi and Perrier, 1982).

Statistical analysis: The effect of peak concentration and half-life on the AUBKC and the absolute change in log CFU mL^{-1} during the experiment period was evaluated by a two-way analysis of variance. $p<0{\cdot}05$ was considered significant.

RESULTS

Pharmacokinetics of amoxicillin *in-vitro*: The MIC of amoxicillin against *Staphylococcus aureus* was 0.2 μg mL^{-1}. In this study, the method refined in this study was selective for the substance analyzed; no endogenous interference was observed on chromatograms. The limit of quantitation was 0.03 μg mL^{-1} for amoxicillin in broth. Amoxicillin quantitation was linear within a range of 0.03-4 μg mL^{-1}. The recoveries of amoxicillin from broth samples were >75%. Coefficients of variation were <5% for within runs and <10% between runs.

The broth concentration vs. time curves of amoxicillin *in vitro* model are shown in Fig. 1 for simulative $t_{1/2}$ 2 h and Fig. 2 for simulative $t_{1/2}$ 5.5 h. with different initial concentration. A one-compartment model best described the drug concentration-time data *in vitro* model. The initial amoxicillin concentration determined in the vitro model was 0.47±0.07, 1.00±0.11, 1.75±0.06 and 3.08±0.22 μg mL^{-1}, respectively. The elimination half-life of amoxicillin *in vitro* model were 2.02±0.12 and 4.92±0.17 h, respectively.

Pharmacodynamics of amoxicillin *in-vitro*: The bactericidal kinetic curve of amoxicillin against

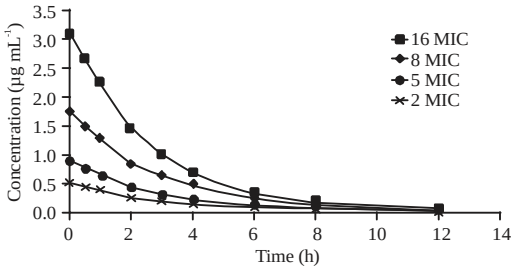

Fig. 1: The concentration-time curve of amoxicillin with different initial concentration *in vitro* model with elimination half-life of 2 h

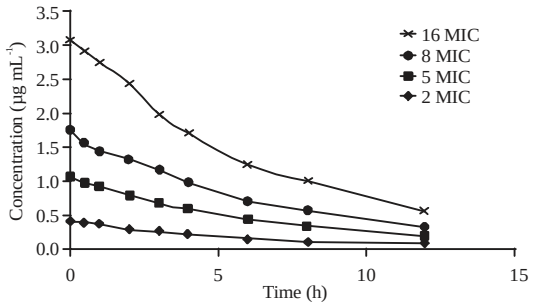

Fig. 2: The concentration-time curve of amoxicillin with different initial concentration *in vitro* model with elimination half-life of 5.5 h

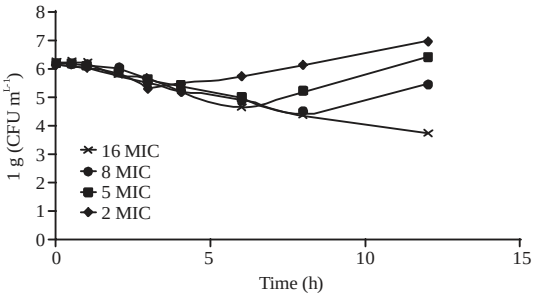

Fig. 3: The bactericidal kinetic curve of amoxicillin against *Staphylococcus aureu* in an *in vitro* model with simulative elimination half-life of 2 h

Staphylococcus aureu in an *in vitro* model with simulative elimination half-life of 2 h is shown in Fig. 3. In a series of experiments with the simulative initial amoxicillin concentration ranging from 2-16 MIC, only 16 MIC amoxicillin exhibited persistent killing of *Staphylococcus aureu* with a bacterial number reduction of 2.51 log10 noted during the 12 h. 2, 5 MIC and 8 MIC amoxicillin only produced 3, 6 and 8 h inhibiting or killing effect for *Staphylococcus aureu* in the model, respectively.

The bactericidal kinetic curve of amoxicillin against *Staphylococcus aureu* in an *in vitro* model with simulative elimination half-life of 5.5 h is shown in Fig. 4. In a series of experiments with the simulative initial amoxicillin concentration ranging from 2-16 MIC, 5, 8 and 16 MIC amoxicillin exhibited persistent killing of *Staphylococcus aureu* with a bacterial number reduction of 2.42, 2.72 and 2.53 log10 noted during the 12 h, respectively. 2 MIC amoxicillin only produced 8 h inhibiting or killing effect for *Staphylococcus aureu* in the model.

PK-PD integration parameters: Some pharmacokinetics-pharmacodynamics parameters are summarized in Table 1 and 2. AUC_{0-24}/MIC (the area under the concentration-time curve over 24 h divided by

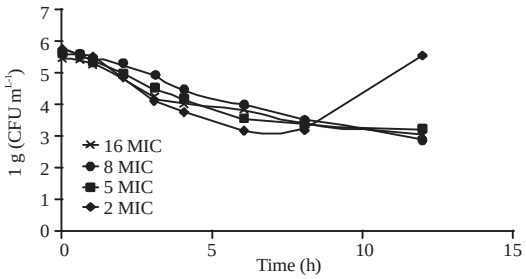

Fig. 4: The bactericidal kinetic curve of amoxicillin against *Staphylococcus aureu* in an *in vitro* model with elimination half-life of 5.5 h

Table 1: Some parameters of different concentration of amoxicillin *in vitro* model with elimination half-life of 2 h ($\bar{X}$ ±SE n = 3)

Initial drug concentration (μg mL^{-1})	(PK-PD parameters) AUC_{0-24}/MIC (h)	C_{max}/MIC	T>MIC (h)	AUBKC (lg CFU·h mL^{-1})
2MIC	7.85±0.85	2.58±0.30	2.84±0.35	71.98±0.57
5MIC	12.98±0.58	4.46±0.30	4.34±0.10	65.69±2.28
8MIC	26.27±1.06	8.75±0.21	6.51±0.19	62.84±0.65
16MIC	42.43±2.36	15.80±0.89	7.40±0.16	58.34±2.32

Table 2: Some parameters of different concentration of amoxicillin *in vitro* model with elimination half-life of 5.5 h ($\bar{X}$ ±SE n = 3)

Initial drug concentration (μg mL^{-1})	(PK-PD parameters) AUC_{0-24}/MIC (h)	C_{max}/MIC	T>MIC (h)	AUBKC (lg CFU·h mL^{-1})
2MIC	14.19±0.27	2.02±0.02	6.88±0.12	49.90±2.18
5MIC	36.02±1.82	5.26±0.17	11.76±0.47	46.92±0.71
8MIC	59.41±0.42	8.65±0.09	15.35±0.18	49.13±0.93
16MIC	104.14±3.44	15.60±0.77	18.91±0.18	46.61±1.00

MIC) and C_{max}/MIC (the peak level divided by MIC)are commonly recognized as indices of efficacy for concentration-dependent antibacterial agents and T>MIC (the time that the drug concentration exceeds the MIC) is recognized as indices of efficacy for time-dependent antibacterial agents. AUBKC (Area under the bacterial kill curve) are regarded as an antibacterial effect of a drug in *in-vitro* mode. It can be seen from Table 1 and 2 that in the in vitro model with simulative elimination half-life of 2 h, there is no significant difference between the AUBKC of the 5, 8 and 16 MIC initial amoxicillin concentration. In the in vitro model with simulative elimination half-life of 5.5 h, there is no significant difference between the AUBKC of the 2, 5, 8 and 16 MIC initial amoxicillin concentration.

DISCUSSION

Conventionally, dose and drug selection in antimicrobial therapy is based on a static in vitro parameter, the Minimal-Inhibitory-Concentration (MIC). However *in-vivo*, bacteria are not exposed to constant but to constantly changing antibiotic concentrations with peaks and troughs, a fact that is largely ignored by static MIC determinations. Therefore, the aim of the present

study was to employ dynamic *in-vivo* PK data of amoxicillin obtained from animals, to simulate the target site PD-profile of amoxicillin.

Although, many investigators have confirmed that the behavior of bacteria in an *in-vitro* environment is not equivalent to that *in-vivo* (e.g., changes in bacterial growth characteristics, micro-organism viability, effects of protein binding on antimicrobial activity, immune defense system, etc.) *in vitro* models permit direct study of the interaction between the bacteria and the antibiotic in a controlled and reproducible way and allow direct comparisons of different antibiotics and different dosing regimens in a more convenient and faster way. Antimicrobial agents with desirable in vitro antibacterial efficacy will still stand a good chance of succeeding in clinical trials.

In this study, the 2 MIC initial amoxicillin provided a poor antimicrobial effect both *in-vitro* model with 2 and 5.5 h elimination half-life when the initial concentration of amoxicillin exceeded 5 MIC or higher The bactericidal kinetic curve suggested a better antimicrobial effect and there was no significant difference between the AUBKC values in 5.5 h elimination half-life vitro model. When the T>MIC was 7.40, 11.76, 15.35 and 18.91 in this study, there was also no significant difference between the reduction of bacteria (2.51, 2.42, 2.72, 2.53 log 10 CFU mL^{-1}, respectively) which indicated that when the initial concentration of amoxicillin exceeded 5 MIC or higher increased concentration did not provided increased antimicrobial effect. From Fig. 3 and 4, it can be seen that only 16 MIC amoxicillin exhibited persistent killing of *Staphylococcus aureu* in 2 h $t_{1/2}$ vitro model (with the T>MIC 7.40 h), while in 5.5 $t_{1/2}$ vitro model, 5, 8 and 16 MIC amoxicillin exhibited persistent killing of *Staphylococcus aureu* during the 12 h experimental period (with the T>MIC 11.76, 15.35 and 18.91 h, respectively). These findings are consistent with the statement that β-lactams reach the maximum killing at 4-5-folds of their MIC values and the antimicrobial maintaining time is mainly correlated with T>MIC (Craig and Ebert, 1990). During the experimental period, bacterial regrowth occurred regularly. in the in vitro model with simulative elimination half-life of 2 h, for 2, 5, 8 MIC initial amoxicillin, bacterial regrew after 3, 6, 8 h incubation respectively. in an in vitro model with simulative elimination half-life of 5.5 h, regrowth was observed only with 2 MIC initial amoxicillin. The data obtained in this study indicated that, to prevent the bacterial regrowth, the T>MIC should exceed 7.40 h.

However, in this vitro model, significant bactericidal effect (99.9% reduction of the original inoculum count after 12 h incubation) and bacterial elimination (the maximal antibacterial effect, that is a reduction in bacterial count to the limit of detection (10 CFU mL^{-1}) (Aliabadi and Lees, 2001) were not achieved on *Staphylococcus aureu* strains for which the MIC was 0.2 mg L^{-1}, even with a Cmax/MIC ratio of 16 . But in vitro studies of amoxicillin against penicillin-susceptible pneumococci and the penicillin-intermediate strain showed maximal killing by amoxicillin at a T>MIC of 50% with the reduction of bacteria count varying from about 3.5-4.0 log 10 CFU mL^{-1} (Gustafsson *et al*., 2002). In an *in-vitro* pharmacodynamic model study of amoxycillin against *Actinobacillus pleuropneumoniae* (Lindecrona *et al*., 2002), the initial bactericidal effect of amoxycillin was maximal at peak concentrations of two to four times the MIC. Peak concentration and half-life only influenced the pharmacodynamic effect of amoxycillin if the antibiotic concentration fell below the MIC during the experiments which is consistent with T>MIC as the most important parameter of pharmacodynamic effect of β-lactam drugs. But the reduction of bacteria count from 0 to 3 h varied from about 0.3-2.9 log 10 CFU mL^{-1}, <3.0 log 10 CFU mL^{-1} which is considered as bactericidal effect. The discrepancy between these results may be explained by the fact that pharmadynamics of antimicrobials may differ with different drugs and different bacterial and furthermore research should be carried out to integrate the pharmacokinetics and pharmacodynamics for amoxicillin against *Staphylococcus aureus* such as with >12 h incubation period and with the higher initial drug concentration. In this study, we attended to describe the pharmacodynamics of amoxicillin against *Staphylococcus aureus* in *in-vitro* kinetic model under simulative pharmacokinetic profile of amoxicillin in pigs. So, the concentration of amoxicillin was not hold constant for the duration above MIC and it is difficult to apply the inhibitory Sigmoid Emax model to describe the antimicrobial efficacy ($\triangle$log10 CFU mL^{-1}) as a function of the T>MIC. The main reason is that only 5MIC or higher amoxicillin concentration provided good antibacterial effect which resulted in only 6 pairs data of $\triangle$log10 CFU mL^{-1} and T>MIC, not enough for PK-PD integration.

CONCLUSION

One important limitation of the present approach relates to the fact that in vitro, experiments are usually performed in a milieu devoid of cells and humeral factors with potential antimicrobial activity. Thus *in-vivo*-PK *in-vitro*-PD approaches solely reflect drug-related antimicrobial activity in an immunocompromised host and may underestimate an overall antimicrobial effect in an immunocompetent host. However, the same is true for any MIC-based noncompartmental PK/PD method. Another limitation is the well-known fact that the *in-vitro* growth rate of bacteria in the luxurious broth conditions is much faster than the actual growth rate at the site of infection. Hence, results from *in-vitro* June 3, 2020 studies should therefore only be extrapolated to *in-vivo* situations in a relative way, i.e., for comparisons of different dosing regimens but not in absolute numbers.

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