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Pharmacokinetic – Pharmacodynamic Considerations for Bovine Mastitis Treatment

Nora Mestorino and Jorge O. Errecalde
Department of Pharmacology and Toxicology,
Faculty of Veterinary Science,
Universidad Nacional de La Plata,
Buenos Aires,
Argentina

1. Introduction

Bovine mastitis is a disease that affects dairy herd production, characterized by considerable economical loss due to diminished milk secretion, potential productive cow damage, increase in production costs and milk contamination. Intramammary infection (IMMI) is the most common reason for the use of antimicrobials in dairy cows. Antimicrobials (ATMs) have been used to treat mastitis for more than fifty years, but consensus about the most efficient, safe, and economical treatment is still lacking.

Staphylococcus aureus is considered one of the main bacteria causing bovine mastitis, which is widely distributed in different countries. *S. aureus* is Gram-positive cocci, catalase-positive and facultative anaerobe. The *Staphylococcus* genus comprises more than thirty species which are able to colonize many environments and are part of the cutaneous or mucousal flora of various animals and humans. The intracellular survival of *S. aureus* is believed to contribute to the recurrence of some infections such as mastitis. Some publications reported the ability of this pathogen to colonize multiple cell types. However the precise fate of intracellular *S. aureus* is still poorly understood.

The general lack of therapeutic success against subclinical mastitis caused by *S. aureus* has prompted a reevaluation of treatment strategies. Despite the availability of several antibiotics with good in vitro activity, cure rates are poor, suggesting that inadequate concentrations of active antibiotic are coming into contact with the infecting bacteria for sufficient time and /or adequate concentrations to be effective.

Over the last few years, much concern has been raised regarding the optimization of antibiotic use, owing to the worrying increase of bacterial resistance. In this context, progress in the field of anti-infective pharmacology has led to the emergence of a new discipline, referred to as pharmacokinetics/pharmacodynamics (PK/PD) of antibiotics, the discipline that strives to understand the relationships between drug concentrations and effects, both desirable (bacterial killing) and undesirable (bacterial resistance). Over the past 15 years, three key PK/PD parameters have been elaborated, which determine how

antibiotic concentrations reached in body fluids over time (as predicted from the PK profile of the drug) compare with potentially effective antibiotic concentrations (as deduced from the minimal inhibitory concentration (MIC) or minimal bactericidal concentration (MBC) of antibiotics in vitro). The first parameter is the time during which concentrations of the ATM are above the MIC ($t > MIC$), it links bactericidal effects to time and is critically dependent on the half-life of the drug, dosage and frequency of administration over a given period of time. The second parameter is the peak plasma concentration divided by the MIC (C_{max}/MIC), it relates bactericidal effects to concentration, and is primarily dependent on the dose. The third parameter is the area under the concentration-time curve divided by the MIC (AUC/MIC), and it combines both types of effects, since it corresponds to the total amount of drug to which bacteria are exposed over the time period, and is directly related to the total dose given during that period and inversely proportional to the drug clearance (Van Bambeke et al., 2006).

These parameters are critical in predicting antibiotic activity and, therefore, in establishing dosages on a rational basis. The application of these parameters, however, have so far been limited to extracellular infections in well-vascularized tissues, because they are all based on serum antibiotic levels.

Antimicrobials exhibit three major patterns of ATM activity. The first pattern is characterized by concentration dependent killing and moderate to prolonged persistent effects. Higher concentrations would kill organisms more rapidly and more extensively than lower levels. The prolonged persistent effects would allow for the administration of large doses with long inter-dose periods. Microorganism regrowth is not immediate at the time in which the drug concentrations fall below the MIC. This called post antibiotic effect is variable between different drug types but always present for ATMs exhibiting this kind of killing. This pattern is observed with aminoglycosides, fluoroquinolones, daptomycin, ketolides, and amphotericin B. The goal of a dosing regimen for these drugs would be to maximize concentrations. The peak level and the AUC should be the pharmacokinetic parameters that would determine in vivo efficacy (Andes et al., 2001; Craig, 2001).

The second pattern is characterized by time-dependent killing and minimal to moderate persistent effects. High drug levels would not kill organisms better than lower concentrations. Furthermore, organism regrowth would start very soon after serum levels fall below the MIC. This pattern is observed with β -lactams, macrolides, clindamycin, and oxazolidinones. The goal of a dose regime for these drugs would be to optimize the duration of exposure. The duration of time that serum levels exceed some minimal value such as the MIC should be the major parameter determining the in vivo efficacy of these drugs (Andes et al., 2001; Craig, 2001).

The third pattern is also characterized by time-dependent killing, but the duration of the persistent effects is much prolonged. This can prevent any regrowth during the dosing interval. This pattern is observed with azithromycin, tetracyclines, quinupristin-dalfopristin, glycopeptides, and fluconazole. The goal of a dose regime is to optimize the amount of drug administered to ensure that killing occurs for part of the time and there is no regrowth during the dosing interval. The AUC should be the primary pharmacokinetic parameter that would determine in vivo efficacy (Andes et al., 2001; Craig, 2001).

Antimicrobial PK/PD relationship reflects a correlation between the drug concentrations in the blood, the concentration of the biologically active drug at the site of the infection, and the microbial or clinical outcome (Levison, 2004). The PK component describes the processing of the drug by the host (absorption, distribution, metabolism, and elimination). The PD component describes the effect of the drug on the bacterial pathogen. By identifying an association between PK and PD for specific host-drug-microbe combinations, the PK/PD approach provides a valuable guide for estimating the doses and dosage regimens that can optimize the bacteriological or clinical outcome.

The extent to which a drug has access into milk when given systemically, or is absorbed and distributes throughout the udder when given intramammarily, depends on its properties: lipid solubility, degree of ionization, and extent of binding to serum and udder proteins. High lipid solubility, poor degree of ionization and less plasma protein binding contributes to a better transfer into milk. With IMM preparations, the type of vehicle is also important.

Weak organic bases like macrolides and sulfonamides tend to accumulate in milk in the ionized form after parenteral administration, and attain concentrations higher than those in blood. On the other hand, concentrations of weak acids like penicillins and cephalosporins in milk are significantly lower than those in blood. In the concentration-dependent group of ATMs (e.g. aminoglycosides and fluoroquinolones) concentration of several times the MIC for the target organisms at the infection site increases the efficacy. In the time dependent group (e.g. penicillins, cephalosporins and classical macrolides) the efficacy depends on the time during which the concentration of the drug exceeds the MIC, but high concentrations do not increase efficacy.

While there are a number of factors that contribute to the PK/PD indexes (e.g. *in vitro* MIC of the drug, its post-antibiotic effects -PAE-, and sub-MIC effects), there are also several factors that the traditional PK/PD approach does not describe. For example, the *in vitro* MIC, which is the basis for the PD component of these indices, does not provide information on time to kill, time to maximum kill, log change within a fixed time, or the maximum reduction in viable bacterial counts. There are also many others *in vivo* factors that influence ATM effectiveness (e.g. anti-inflammatory effects, presence of bacterial biofilms, the drug's ability to interfere with bacterial colonization on epithelial surface, and influence of the drug on toxin production and release). Furthermore, plasma drug concentrations do not necessarily reflect a compound's ability to diffuse into the site of infection and into the bacterial cell.

An ideal drug for mastitis therapy should have a low MIC for mastitis pathogens. As treatment should be efficient and targeted towards specific infections, Gram-negative and Gram-positive infections in fact would require different ATMs. Antimastitic drugs should preferably have bactericidal action, as phagocytes act normally immediately after milking, but as time elapses they incorporate fat globules becoming "engorged" thus diminishing its phagocytic capacity. As a consequence milk phagocytes are less effective than plasma ones. Milk should not interfere with ATM activity.

The objective of this study was to make a bibliographic compilation on the PK of different ATM agents in milk, based in our experience and some other relevant publications, and to establish the relationship between the PK and the PD interaction with the target bacteria. We consider that non prudent use of ATMs in dairy farms can be fought through the implementation of rational therapeutical procedures based on the following items:

1. Knowledge on the microbiological profile of mastitis. Clinical and laboratory diagnosis (isolation, typification and antibiogram) is one of the basic pillars for the rational use of ATM agents.
2. Knowledge on the PK and PD of antibiotics in milk producing animals, both healthy and mastitic. Understanding the relation PK/PD in these animals will result in an increase of ATM efficiency and a decrease in the selection of resistant strains, two important aspects leading to the success of the therapy.
3. More efficient and safer prescription of antimastitic drugs. The development of new products should involve more than just the discovery of a new substance; it should deal with the full utilization of the effects of the agents in the organism. The way a drug is transported to the target location should be considered; once there, it should be available in the appropriate concentration and time to be effective.
4. Establishment of the correct withdrawal periods for the antibiotics tested, following internationally agreed protocols.

2. General pharmacokinetic considerations

An important question regarding the treatment of mastitis is whether the ATM should accumulate in milk or in the udder tissue (Erskine et al., 2003). The target site may depend on the causative agent: streptococci are known to remain in the milk compartment, but *S. aureus* penetrates udder tissue and causes deep infection (Table 1). The most common route of administration of ATMs in mastitis is the IMM route. Efficacy of IMM treatment varies according to the pathogen, with the best therapeutic response being shown for mastitis caused by streptococci, coagulase-negative staphylococci, and *Corynebacterium* spp.

	Milk/ducts	Udder tissue	Cow
<i>Streptococcus agalactiae</i>	+++	---	---
Other streptococci	+++	+	---
<i>Staphylococcus aureus</i>	+	+++	---
Coagulase-negative staphylococci	+++	---	---
<i>Arcanobacterium pyogenes</i> (summer mastitis)	---	++	+++
Coliforms	+	--	+++

Table 1. Where to target ATM therapy in clinical mastitis due to different pathogens (Erskine et al., 2003)

2.1 Parenteral antimicrobial treatments

Systemic administration for treatment of mastitis was first used in the 70's (Ziv, 1980a). In acute cases, the IMM administration often fails due to poor and uneven distribution of the drug, either by the growth of breast parenchyma or blockage caused by the products of inflammation. In these circumstances, parenteral therapy is preferred (Mestorino, 1993a).

From the clinical point of view, the success of parenteral therapy depends mainly on the ATM passage from blood to milk (Ziv, 1980a). The ATM concentrations in highly vascularized tissues are equivalent to those determined in blood plasma. By contrast, in places where the irrigation is poor or those which are separated from the central compartment by biological membranes, drug levels are not equivalent (Baggot, 1986). The time during which concentrations in the mammary gland are effective depends largely on

the drug characteristics, the dose, the bioavailability of the molecule, the ability to penetrate the mammary gland and the microorganism susceptibility (Ziv, 1980b; Mestorino, 1993a).

The ability to penetrate the mammary gland or milk bioavailability (F_{milk}) is determined by the ratio $AUC_{0-\infty} \text{ milk} / AUC_{0-\infty} \text{ plasma}$, as shown in the following equation:

$$F_{milk} = \frac{AUC_{0-\infty(milk)}}{AUC_{0-\infty(serum)}} \quad (1)$$

This equation determines the relationship between the amount of ATM that is absorbed to the central compartment and the amount of ATM that passes through the mammary gland for reaches the milk compartment (Mestorino, 1993a).

Those antibiotics that have a high volume of distribution penetrate better into the mammary gland. However, differences in the degree of penetration blood: milk occur even among compounds that are chemically and structurally related. These differences can be explained by the principle of passive diffusion (Ziv, 1980b). ATMs cross biological membranes by passive diffusion or specialized transport.

Since the surface of the lipid portion of the membrane is extremely high, passive diffusion through membranes can be considered synonymous of diffusion through membrane lipids (Errecalde, 2004). The transfer in this case is directly proportional to the concentration gradient and the lipid-to-water partition coefficient of the ATM (Ziv, 1980b).

Weak organic acids and bases are found in milk and plasma as ionized or nonionized forms. The nonionized fraction is generally more soluble than the ionized one and diffuses better through the biological membrane (Ziv, 1980b; Errecalde, 2004; Mestorino, 1993a). The proportion of the drug in the nonionized form depends on the pKa of the molecule and the pH of the medium in which it is dissolved. When the molecules pass through the membrane by simple diffusion, are distributed according to their degree of ionization, the charge of their ionized form and the extent of protein binding. This is because the molecules bound to proteins or tissues are not able to cross membranes (Ziv, 1980b).

The theoretical relationship between the drug concentrations on both sides of a biological membrane can be calculated according to the Jacobs equation, that for organic acids such as penicillin G (pKa = 2.8) is the following:

$$\text{Ratio milk:plasma} = \frac{1 + 10^{pH_{milk} - pK_a}}{1 + 10^{pH_{plasma} - pK_a}} \quad (2)$$

And in the case of organic bases, such as spiramycin (pKa = 8.2) is:

$$\text{Ratio milk:plasma} = \frac{1 + 10^{pK_a - pH_{milk}}}{1 + 10^{pK_a - pH_{plasma}}} \quad (3)$$

The serum pH is 7.4 and the milk has a pH between 6.6 - 6.8. The organic bases administered by the parenteral route tends to accumulate in milk and remain there in its ionized form (ion trapping), thus achieving milk concentrations which exceed those in plasma. Instead, the concentrations of weak acids in milk are lower than those found in plasma (Erskine, 2002b).

That is to say that the majority of the drugs can be ionized or nonionized according to its pKa and the pH of the surrounding environment. Nonionized compounds have a higher lipid-water partition coefficient than the ionized ones and thus it is easier for them to diffuse through lipidic membranes. The amphoteric molecules, such as danofloxacin (pKa = 6.2 to 9.4) does not depend on the relationship pK/pH and therefore its distribution is essentially determined by the degree of lipid solubility of the molecule and consequently by its lipid-water partition coefficient.

Table 2 shows different ATMs with its theoretical and experimental milk: plasma ratios. Observing this table we can conclude that the diffusion of organic acids into milk is highly predictable, but the diffusion of organic bases can be predicted only when these are largely nonionized in plasma and have a moderate degree of lipid solubility.

Antimicrobial agent	Chemical nature	PK _a	Lipid solubility	Milk to serum concentration ratio Theoretical - Experimental
Sulfanilamide	Acid	10.4	Moderate	1.00 - 0.97
Sulfathiazole	Acid	7.1	Moderate	0.37 - 0.35
Sulfadiazine	Acid	6.5	Moderate	0.28 - 0.21
Penicillin G	Acid	2.8	Moderate	0.16 - 0.20
Cloxacillin	Acid	2.8	High	0.16 - 0.22
Ampicillin	Acid	2.8; 7.2	High	0.26 - 0.26
Amoxicillin	Acid	2.8; 7.2	High	0.26 - 0.26
Cephacetrile	Acid	2.4	Moderate	0.12 - 0.15
Cephapirin	Acid	2.6	Moderate	0.14 - 0.18
Rifamycin SV	Acid	2.8; 6.7	Moderate	0.25 - 0.25
Rifampicin	Acid	7.9	High	0.85 - 1.10
Novobiocin	Acid	4.3	High	0.30 - 0.33
Penethamate	Base	8.5	High	5.7 - 6.1
Streptomycin, neomycin	Base	8.9	Low	7.5 - 0.5
Polymyxin B, colistin	Base	10.0	Very low	8.0 - 0.3
Erythromycin	Base	8.8	High	6.5 - 8.5
Tylosin	Base	7.1	High	5.0 - 4.5
Spiramycin	Base	8.2	High	4.8 - 4.6
Lincomycin, clindamycin	Base	7.6	High	4.2 - 4.4
Tetracyclines	Amphoteric	-	Moderate	0.4; 0.8 - 0.6; 1.4

Table 2. Partition of ATMs in plasma and milk in lactating animals. From Ziv G. (1980b)

In the case of a weak base, when administered parenterally, e.g. intramuscularly, the rapid penetration from blood to milk is characterized by early appearance of measurable concentrations in this fluid, a ratio $C_{\max}(\text{plasma}) / C_{\max}(\text{milk})$ less than or equal to 1, a t-lag between the $C_{\max}(\text{plasma})$ and $C_{\max}(\text{milk})$ short and a ratio $AUC(\text{plasma}) / AUC(\text{milk})$ less than or equal to 1 (Ziv, 1980b).

During the mastitis process, the chemical composition of milk presents changes consequence of the inflammatory process. There are ions, proteins and inflammatory cells passage from blood into the gland lumen, because of a great increase in the vascular permeability. The physical properties of milk are also affected and there is an increase of pH, conductivity and viscosity, while the density and redox potential decrease (Korhonen & Kaartinen, 1995).

In mastitic milk it is expected that the passage of organic bases is diminished as the pH increases in detriment to the ion trapping in milk. However, the lipid solubility of this kind of molecules suggests that the relationship milk:plasma is always greater than 1 (Ziv, 1980b).

In the case of organic acids, the penetration is favored by the increase of milk pH. However, these compounds will reach milk: plasma ratios above 1 only when the milk pH exceeds 7.4. This indicates that lipophilic weak bases parenterally administered may have a certain advantage in distribution into milk in comparison with acids (Ziv, 1980b).

2.2 Intramammary antimicrobial treatments (IMM)

The IMM infusion is the more used administration route in the ATM treatment of mastitis. However, many IMM products have been released to the market without the necessary scientific support about its PK behavior and studies about its clinical and bacteriological efficacy. The benefits of IMM administration are the high concentrations reached in milk and less loss due to drug absorption and transfer processes through biological membranes. While the disadvantages of this route may be the uneven distribution of various compounds within the udder, the risk of mammary contamination by bacteria inoculation through the teat canal and the possible irritation of tissue breast by the formulation (Gruet et al., 2001). Even in vitro studies have shown that ATMs administered by IMM route can negatively affect the phagocytic process in the mammary environment (Nickerson et al., 1985; 1986).

After administration of an IMM infusion, the contact between the ATM agent and the pathogen within the mammary gland is subject to a series of successive events (Ziv, 1980c; Mestorino, 1993a):

1. Pharmaceutical Phase: begins after drug administration including the following steps:
 - Disintegration of the formulation
 - Drug dissolution
 - Liberation of the drug in milk
2. Pharmacokinetic Phase: Assumes the presence of the drug in milk (drug availability) and includes the following events:
 - Absorption (milk: plasma)
 - Distribution (local and systemic)
 - Metabolism (systemic)
 - Excretion (local and systemic)
3. Pharmacodynamic Phase: The effect of the drug against bacteria in the infection site.

The kind and proportions of the pharmaceutical excipients contained in the formulation are which determines the rate of drug release. They define the pharmaceutic phase which governs the initial shape of the ATM milk concentration versus time curve.

IMM formulations used for mastitis treatment during lactation contain pharmaceutical excipients that favors quick release of the active components, because the objective is to eradicate the infection of glandular tissue and minimize the withdrawal time (Ziv, 1980c).

On the other hand, when the IMM treatment is during the dry period, the process of release of the active component must be slow and necessarily has great influence on the initial shape of the concentration versus time curve. The excipients used, although very variable, may include vegetable or mineral oils. The mineral oil, such as paraffin or glycerine oils, has the advantage of delaying the release of the active compound and prolonge the permanence of the ATM inside the gland (Ziv, 1980c; Mattie et al., 1997).

In some cases the pharmaceutical excipients contain other agents that increase the retention, such as aluminum monostearate in an oily base, hydroxystearin (Ziv, 1980c). Mercer et al. (1974) studied the absorption in blood and the persistence in milk of penicillin G, after IMM infusion of 400000 IU in mastitic quarters in a rapid (aqueous formulation) and a slow release base (3.6% aluminum monostearate in peanut oil). Penicillin G administered in the slow release formulation could still be detected in the milk 120 h after infusion compared to only 56 h for the aqueous base formulation. These figures must be considered in relation to the MIC of penicillin G for *S. aureus* in milk which is around 0.1 IU.mL^{-1} ; this means that active levels were maintained above the MIC for 72 h for the oil-based formulation compared to 32 h for the aqueous-based formulation. After the administration of IMM syringes, part of the formulation will be lost with the milk in the first milking post-treatment. This loss is negligible in the case of treatment at the start of the dry period, because the cows are removed from the milking routine. However, the disruptions of the milking routine trigger physiological changes in the mammary gland that may affect the pharmaceutical and PK phases.

The start of the PK phase presupposes the existence of available drug in the milk secretion and depends on the disintegration of the formulation and the dissolution of the drug. These processes determine the drug availability.

The amount of drug recovered in milk is influenced by several factors, among which we can mention the kind of excipient used in the formulation, the milk: plasma passage rate, the size of the udder and the volume of milk contained in the gland (Ziv, 1980c).

The passage of the antibiotic from milk to serum involves the movement of drug across a biological membrane. The factors influencing this passage are the same that affect their transfer from the blood into the milk: the molecule pKa, the lipid solubility of the nonionized fraction, the percentage of udder tissue binding and the binding to milk proteins (Mestorino, 1993a). Hydro soluble drugs pass through the membrane mainly through the protein channels. Lipid soluble drugs cross the membrane through the lipoproteic region .

The ability to cross the milk: plasma barrier is expressed as lipid solubility coefficient for nonionized fraction percentage (Mattie et al., 1997) and the real rate of passage can be quantified by the $C_{\max(\text{plasma})}/C_{\max(\text{milk})}$ or by the $AUC_{(\text{plasma})} / AUC_{(\text{milk})}$ ratios (Ziv, 1980a).

The milk: plasma absorption phenomenon results in the presence of the ATM agent in blood plasma. Once in the systemic circulation, the drug will be object of the PKs phenomena taking place after absorption (distribution, metabolism and excretion) (Ziv, 1980b).

The ATM distribution inside the gland occurs by passive diffusion of the molecules through the lipophilic and hydrophilic components of the glandular secretion. This phenomenon is affected by drug binding to breast tissue and / or components of milk (Mestorino, 1993a). Once the drug establishes contact with the glandular cells, its penetration into tissue continues depending on lipophilicity. As will be detailed later, cell penetration is a critical point in certain infections. The excretion process of the ATM from the mammary gland is governed by the kind of excipient used, the quantity of milk produced, the molecule characteristics, the health of the mammary gland, the number of daily milkings made (Ziv, 1980c), the dose and total volume of formulation among other factors.

After the IMM treatment at cow drying, the ATMs elimination from the mammary gland can be expressed by a monoexponential or biexponential curve. The elimination rate is affected by the dose, the nature of the vehicle, the drug characteristics, and the extent of binding of the antibacterial agent to the gland content and to the mammary tissue (Ziv, 1980c; Mestorino, 1993a; Mattie et al., 1997).

3. Antimicrobial pharmacodynamic concepts

Successful ATM chemotherapy depends on a correct diagnosis, selection of the appropriate ATM agent, and its administration with an adequate dosing scheme.

When considering the choice of ATM agent and dosage regimen, we need to consider the PKs of the chosen drug in the target animal species and the PD indices that drive its clinical effectiveness. For example, penicillins, like all β -lactam ATMs (penicillins, cephalosporins, carbapenems and monobactams), exhibit time-dependent killing. This means that maximum clinical effectiveness is achieved by ensuring that the free serum concentration of the selected β -lactam exceeds the MIC of the pathogen for the appropriate percentage of the dosing interval. If the pathogen is a Gram-positive organism, the targeted duration is usually $\geq 40\%$ of the dosing interval. Instead, concentrations of most β -lactams should exceed the MIC of the pathogen by $\geq 80\%$ of the dosing interval when the infectious agent is a Gram-negative organism. In other words, for drugs exhibiting time-dependent killing, increasing the concentration of the drug in excess of the MIC of the pathogen does not increase the killing rate. Rather, the extent of killing is dictated by the duration of time that bacteria are exposed to the drug.

On the other hand, other bactericidal ATM agents, as fluoroquinolones and aminoglycosides, exhibit concentration-dependent killing. In this situation, the rate of killing increases as the drug concentration increases above the MIC of the bacterial pathogen. Thus, ATM agents may be classified as those that exhibit time-dependent killing with null or brief post-antibiotic effect (e.g. β -lactams), time-dependent killing with prolonged post-antibiotic effect (e.g. glycopeptides), concentration dependent killing (e.g. fluoroquinolones and aminoglycosides), and those that are generally considered to be bacteriostatic (e.g. tetracyclines, macrolides, lincosamides and phenicols).

Mouton et al. (2005) published an attempt to standardize the interpretation of these various PK/PD parameters (Fig. 1). Some of the basic definitions are as follow:

- AUC (area under the concentration versus time curve). Should be expressed in terms of unbound drug. If multiple dosing regimens are applied, AUC should be measured over a 24 hour dosing interval at steady state. It should be noted that for compounds exhibiting linear PK, the AUC over a single dosing interval at steady state ($AUC_{0-\tau}$) is equal to AUC extrapolated to infinity ($AUC_{0-\infty}$) following single administration.
- AUC/MIC (AUC divided the minimum inhibitory concentration). Although sometimes given the dimension of time (generally 18 to 24 hours), this ratio can be more conveniently expressed as a dimensionless value.
- $T > MIC$ (period of time during which the drug concentrations exceed the MIC). The cumulative percentage of a 24-hour period that the free drug concentration exceeds the MIC at steady-state pharmacokinetic conditions.
- In vitro PAE (post-antibiotic effect). The period of suppression of bacterial growth after short exposure of an organism to an ATM compound (unit = time).
- In vivo PAE. The difference in time for the number of bacteria in a tissue of treated versus control animals to increase 1 log₁₀ over values when drug concentration in serum or at the infection site fall below the MIC (unit = time). The in vivo PAE includes any effect associated with sub-MIC concentrations. Sub MIC effect. Any effect of an ATM on a microorganism at concentrations below the MIC (unit = time)
- Post-antibiotic sub-MIC effect. The effect of sub-MIC drug concentrations on bacterial growth following serial exposure to drug concentrations exceeding the MIC (unit = time)

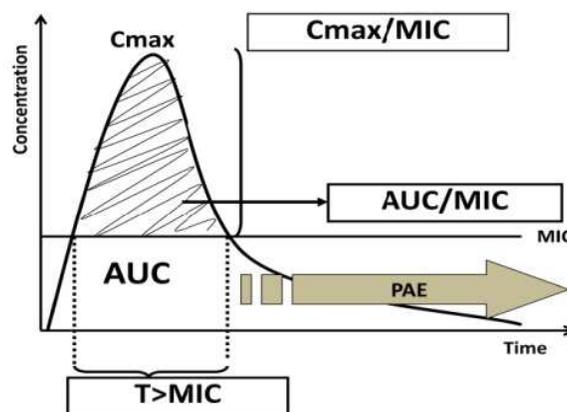


Fig. 1. Illustration of the main PK/PD parameters that correlate with efficacy against extracellular infections.

The units associated with the AUC/MIC ratio are hours, by dividing this value by the dosing interval (e.g. 24 hour), we obtain the average plasma concentration over the steady-state 24-hour dosing interval relative to MIC, which may be far more informative than the traditional method for expressing this value.

The PD parameter providing the most appropriate surrogate for drug effectiveness is dependent on several factors. This includes: mechanism of action of the different drugs, whether its effects are time or concentration dependent, and the PAE duration (Table 3).

Drug	Mechanism	Activity	Bacterial effect	Duration PAE	PD Parameter
Macrolides Erythromycin,	Binds to 50S ribosomes (inhibits proteins synthesis)	Static	Time-dependent	Brief*	T>MIC
Azalides Azithromycin	"			Prolonged	AUC ₂₄ /MIC
Lincosamides	"			Brief	AUC ₂₄ /MIC
Ketolides (telithromycin)	Binds to 50S ribosomes and some 30S ribosomal unit activity	Static and cidal (S.pneumoniae, S.pyogenes)	Time-dependent	Prolonged	AUC ₂₄ /MIC
B-lactams (e.g. penicillins, cephalosporins, carbapenems, monobactams)	Inhibits cell wall synthesis	Cidal	Time-dependent	Gram- bacteria: null or brief, Gram+: may be prolonged	T>MIC
Glycopeptides (e.g.vancomycin)	Inhibits cell wall synthesis	Cidal(slower than β -lactams)	Time-dependent	Prolonged	AUC ₂₄ /MIC
fluoroquinolone (e.g. enrofloxacin, ciprofloxacin, danofloxacin)	Inhibit DNA gyrase, prents transcription and replication	Cidal	Concentration -dependent	Prolonged	AUC ₂₄ /MIC C _{max} /MIC
Aminoglycosides (e.g.gentamycin, streptomycin)	Binds to 30S ribosome (inhibits protein synthesis) and disrupts biofilms	Cidal	Concentration -dependent	Prolonged	AUC ₂₄ /MIC C _{max} /MIC
Tetracyclines	Inhibits protein synthesis (30S)	Static	Time-dependent	Prolonged	AUC ₂₄ /MIC
Phenicols (e.g.chloramphenicol, florfenicol, thiamphenicol)	Inhibits protein synthesis by inhibition peptydyl-transferase (50S)	Static	Time-dependent	Prolonged	AUC ₂₄ /MIC
Trimethoprim	Inhibits folic acid synthesis by inhibiting dihydrofolate reductase	Static alone Cidal with sulfonamides	Time-dependent	Brief	T>MIC
Sulfonamides	PABA analogue interferes with folic acid synthesis	Static	Time-dependent	Brief	T>MIC
Oxazolidinones (linezolid)	Inhibits initiation of protein synthesis (50S)	Static (Staph. and Enterococcus) Cidal (most Strep.)	Time-dependent	Brief	T>MIC

Table 3. Relationships among drug, drug effects, and the PD surrogate most closely aligned to its clinical response (Martinez et al, 2006). *Brief: less than one hour. Prolonged: up to six hours.

Whether a drug exhibits concentration-dependent or time-dependent killing is largely a function of the shape of its concentration-effect curve, the steeper the curve, the less will be the impact of increasing drug concentrations on the ATM response. Conversely, the more shallow the curve, the greater the relationships between the rates of bacterial kill versus the ATM drug concentration. This relationship can be described using a sigmoidal Emax model, also known as the Hill model, which can be described as follows (Toutain, 2002):

$$E(t) = E_0 \frac{E_{max} \times C^h(t)}{EC_{50}^h + C^h(t)} \quad (4)$$

Where:

$E(t)$ is the effect observed for a given concentration at time t ($C(t)$);

E_{max} is the maximal effect attributable to the drug;

EC_{50} is the plasma concentration producing 50% of E_{max} ;

h is the Hill coefficient, which adjust the degree of sigmoidicity in the curve; and

E_0 describes the rate of spontaneous cure.

When $h=1$, the Hill model reduces to the E_{max} model, which corresponds to a hyperbolic function.

While there are certain characteristics common to all ATMs within a given drug class, there can be important differences in the PK/PD ratios needed to achieve a desired effect. Within the fluoroquinolones (FQ), it has been demonstrated that the rate of kill and the duration of the in vitro PAE (Finberg et al., 2004; Firsov et al., 1998b) can be markedly different across compounds and microbial species. In some cases, the PK/PD relationship necessary to achieve a 2-log kill can also vary as a function of the microbial strain (Andes and Craig, 2002). Similarly, the AUC/MIC ratio of 100-125 frequently quoted as a target for FQ ATM activity may be an appropriate predictor of success for many Gram-negative infections, but lower AUC/MIC ratios (e.g. 35 to 40) may be appropriate for infections due to Gram positive organisms (Wright et al., 2000). With regard to the β -lactams, while there tends to be a substantial in vivo PAE for *S. aureus*, a substantially shorter PAE is associated with Gram negative organisms (Craig, 1993).

Within any given bacterial population, the possibilities of bacterial subpopulations that are less susceptible to the ATM agent exist. As demonstrated by Drusano (2004), unless these less susceptible pathogens are killed, succeeding microbial generations will re-populate the infection site with pathogens whose MIC values are higher than those found within the initial infection.

Accordingly, ensuring adequate exposure following an initial dose of a FQ is as important as insuring that high drug concentrations occur after repeated administration. Drug concentrations need to be adequate to either destroy the existing bacterial population at the site of the infection or to reduce its size to the point where the host defense mechanism can successfully control and eliminate the remaining pathogens.

For drugs exhibiting concentration-dependent killing, C_{max}/MIC ratios may be particularly important when the pathogen has a high MIC value or is rapidly proliferating (Craig and Dalhoff, 1998). Rapidly proliferating bacteria have a greater likelihood of undergoing a

mutational event that could lead to the genesis of a less susceptible population. In infectious disease processes where there is a high bacterial burden (inoculum effect), the risk of a mutational event is increased due simply to the laws of probability (Craig and Dalhoff, 1998). In these cases, to ensure maximum killing, the targeted C_{\max}/MIC ratios are approximately 10 to 12 (Drusano et al., 1993). Such ratios ensure increased killing of susceptible organisms and an increased killing or inhibition of organisms with higher MICs. The goal in these situations is to reduce bacterial numbers to a level where the host can effectively handle those pathogens not killed by the ATM agent. While a high C_{\max}/MIC ratio (e.g. 10) is correlated with a high rate of bacterial kill for compounds exhibiting concentration-dependent killing, there are conditions under which AUC/MIC may be as or more predictive of a sustained ATM activity. AUC/MIC could be considered a major PK/PD parameter when the infection is caused by relatively slow growing bacteria, when there is little or no PAE that will contribute to inhibition of bacterial re-growth or when the MIC for the pathogen is relatively low.

3.1 Post-Antibiotic Effects (PAE)

High drug concentrations relative to the MIC may contribute to an increase in the duration of the *in vitro* and *in vivo* PAE. For those bacteria/drug combinations that exhibit a PAE, *in vivo* PAEs have been shown to be longer than *in vitro* PAEs for most organisms. Thus, optimizing the C_{\max}/MIC ratio will delay the re-growth of the pathogen, sometimes by several hours. This type of dosing schemes results in fewer organisms remaining that can evolve into a resistant subpopulation and can be managed by the host defenses.

For many compounds, the duration of the *in vivo* and *in vitro* PAE is substantially greater for Gram-positive than for Gram-negative pathogens. Because the duration of the *in vitro* and *in vivo* PAE of β -lactams tends to be negligible for Gram-negative species, it is recommended that concentrations of drug remain above the MIC of the pathogen for >80% of the dosing interval to combat this type of organisms. While a $T > \text{MIC}$ of about 40% is sufficient for staphylococcal species.

This difference in the duration of the *in vitro* and *in vivo* PAE may also be one of the reasons why the *in vivo* AUC/MIC for FQ tends to be less for Gram-positive than for Gram-negative pathogens. For Gram-negative organisms, the estimated AUC/MIC ratios needed to ensure effective treatment and prevent the selection of resistant strains is estimated to be approximately 100 to 125 (Forrest et al., 1993). In contrast, the AUC/MIC ratio for Gram-positive bacteria is considerably lower, approximately 30 to 50 for a number of drug-pathogen combinations (Wright et al., 2000). Studies involving the third and fourth generation FQ suggest that for Gram-positive organisms AUC/MIC values are substantially lower when C_{\max}/MIC values are ≥ 10 (Nightingale et al., 2000).

Blood concentrations and MIC data alone cannot predict drug effectiveness. For example, using human and bovine estimated breakpoints for cephapirin and oxytetracycline, Constable and Morin (2002) showed that the MIC values predicted that the causative pathogens would be susceptible to both agents. However, these compounds were not effective in the treatment of acute bovine mastitis. In the same line, compounds effective in the treatment of acute bovine mastitis may be ineffective in the treatment of chronic bovine mastitis (Owens et al., 1997).

Drug potency is often considered in terms of MIC, which is a measure of a drug's static effect on microbial growth. The MIC may not be the same as a compound's minimum bactericidal concentration (MBC). Since both the MIC and MBC values are in vitro estimates, they do not reflect the killing rate of a drug, the effect of serum on ATM activity, PAE, or post-ATM sub-MIC (Craig and Dalhoff, 1998). Firsov et al. (1999) demonstrated that even with comparable AUC/MIC ratios, different FQ can have marked differences in in-vitro time-kill profiles. Such differences cannot only influence the selection of an AUC/MIC target value, but also the potential for selecting for resistant bacterial strains.

Traditional in vitro susceptibility data reflect the impact of therapeutic agents on bacteria that are in the active growth phase. These tests do not describe the differential activity across the different life phases of bacteria (Cerca et al., 2005). As an example, in the case of bacteria from biofilms, entering a non-growth phase, many compounds begin to lose their ATM effects. This very important facts cannot be predicted when MIC alone is used as the PD component of the PK/PD relationship.

There are some examples of traditional susceptibility tests failing to adequately reflect in vivo drug activity. For instance, β -lactams are inactivated by purulent material due to the accumulation of bacterial β -lactamases; gentamicin can be inactivated by reversibly binding to DNA released from lysed neutrophils; netilmicin and amikacin are inactivated by disrupted leukocytes (Labro, 2000). Owens et al. (1997) noted that the bacteriologic cure rate for newly (less than two weeks duration) acquired *S. aureus* IMMI, was 70% when treated with penicillin and novobiocin combination. However, the cure rate dropped to less than 35% for chronic infections (those lasting longer than four weeks). Accordingly, they observed that while the successful treatment of acute infections could be predicted on the basis of in vitro susceptibility test results, this was not the case for chronic *S. aureus* infections. It is not know if this outcome is due to the driving of *S. aureus* infections from extracellular to intracellular sites, to biofilm formation, or to some other bacterial pathogen-host interaction (Brouillete et al., 2004).

Staphylococcal bovine mastitis exemplifies a persistent infection that is difficult to treat and where frequent relapses commonly lead to a chronic disease (Sandholm et al., 1990). Antibiotic treatment of this disease is problematic (as mentioned in the preceding paragraphs) partly because in vitro antibacterial susceptibility is a poor predictor of efficacy in chronic cows (Owens et al., 1997). Failure to eliminate the infection from dairy herds is likely due to a combination of an adaptive response of the pathogen to survive in the mammary gland despite the presence of antibiotics (Sandholm et al., 1990), and the inability of host defenses to clear the pathogen. Infectious bacteria in chronic mastitis may survive intracellularly and remain quiescent and protected from the action of antibacterials and host defenses. Although reinfections that follow attempts to treat acute mastitis can be due to newly acquired strains, it is equally possible that the reinfection results from persistence of the original infective organism (Sandholm et al., 1990). It is possible that *S. aureus* having the small-colony variants (SCV) phenotype contribute to chronic mastitis. SCVs have been isolated from cases of bovine mastitis (Ziv & Sompolinsky, 1976; Sompolinsky et al., 1969).

Both lactational and dry cow therapy are part of *S. aureus* control programs. Reported cure rates for *S. aureus* mastitis vary considerably in a range from 4 to 92% (Owens et al., 1988, 1997). The probability of cure depends on cow, pathogen, and treatment factors. Cure rates

decrease with increasing age of the cow, increasing somatic cell count, increasing duration of infection, increasing bacterial colony counts in milk before treatment, and increasing number of quarters infected. *S. aureus* mastitis in hind quarters has a low cure rate compared with front quarters. ATM treatment of IMMI with penicillin-resistant *S. aureus* strains results in a lower cure rate for treatment with either β -lactam or non- β -lactam antibiotics. The most important treatment factor affecting cure is treatment duration. Increased duration of treatment is associated with increased chance of cure. Economically, extended treatment is not always justified, even when indirect effects of treatment such as prevention of contagious transmission are taken into consideration.

The β -lactams (penicillins and cephalosporins) have become the first line of ATM agents used for treatment of bovine mastitis in Argentina. Within this class, penicillin, amoxicillin, cloxacillin and ampicillin are the most used agents. In the Nordic countries penicillin is used as the first-line antibiotic treatment of bovine mastitis, because of its potency, low resistance rate and narrow spectrum. This is an important tool to limit the development of antibiotic resistance as much as possible. In a one study performed for us 34% of *S. aureus* were classified as penicillin resistant, while 100% were classified as sensible to the combination amoxicillin/clavulanic acid (Lucas, 2009a). This prevalence of resistance to penicillin was similar than those obtained by others authors in different regions of Argentina (Gentilini et al., 2000; Russi et al., 2008). And this was the same comparing the proportion of resistance (47 %) as obtained by Giannechini et al. (2002) in Uruguay. The comparison between these results obtained over the years demonstrated that the situation in general has not changed during the last 25 years in relation to penicillin resistance. However, it results higher than in Norway, 4.2% from clinical cases and 18% from sub-clinical cases (Hofshager et al., 1999), and Sweden, 6% (Franklin, 1998). In the Table 4 we present in vitro susceptibility results of strains of *S. aureus* obtained by different studies performed in different countries.

4. Specific antimicrobial agents

4.1 Beta-lactam agents

4.1.1 Amoxicillin

The use of β -lactam antibiotics in the treatment of mastitis remains one of the first elections. Within this group, any penicillin (PEN) such as amoxicillin (AMX) provides a number of advantages. AMX is a β -lactam antibiotic of wide spectrum with a chemical structure and antibacterial activity similar to ampicillin (AMP) (Hunter et al., 1973).

AMX is rapidly converted to AMP in the body. AMX pharmacokinetics is essentially similar to other β -lactam antibiotics. The half-life is short (about 1 hour) and the volume of distribution low. The first AMX pharmacokinetic study was conducted in ruminants after IV and IM administration in cattle and after IM administration in goats by Ziv and Nouws (1979).

Since then AMX disposition was studied after its administration by different routes in production animals (Archimbault et al, 1981; Baggot, 1988, Nouws et al, 1986, Wilson et al, 1988). According to Erskine et al. (2002) about 60% of *S. aureus* isolates are susceptible to AMP. The susceptibility to AMP among mastitis causing streptococci is 100% and for *E.coli* 85%. Therefore, significant improvement by AMX/clavulanic acid treatment compared to AMX or AMP alone can be expected only for *S. aureus*.

Antimicrobial agent	MIC ($\mu\text{g.mL}^{-1}$)			Resistance Breakpoint	References
	MIC ₅₀	MIC ₉₀	Range		
Penicillin	0.5 0.06 0.25 0.06	>8 0.5 1 4	0.12 - > 8 0.06-0.5 0.062-2 0.03-8	≥ 0.25	Giannechini et al., 2002 Rubin et al., 2011 San Martín et al., 2002 Russi et al., 2008
Ampicillin	0.5 0.125	8 1	≤ 0.12 ->16 0.06-4	≥ 0.5	Giannechini et al., 2002 San Martín et al., 2002
Amoxicillin	0.125	1	0.06-4	≥ 0.5	San Martín et al., 2002
AMX/CLA	2	2	2 - 16	≥ 8	Rubin et al., 2011
Oxacillin Cloxacillin	≤ 0.5 0.25 0.25	≤ 0.5 2 1	≤ 0.5 -1 0.12-0.5 0.125-16	≥ 4	Giannechini et al., 2002; Russi et al., 2008 Lucas, 2009 San Martín et al., 2002
Cephalotin	≤ 4	≤ 4	4-64	≥ 32	Giannechini et al., 2002; Rubin et al., 2011
Cephoperaz.	0.5	2	0.25-16	≥ 8	San Martín et al., 2002
Gentamicin	≤ 1 2 1 0.25	≤ 1 2 4 0.5	≤ 1 -4 2-16 0.25-32 0.03-2	≥ 16	Giannechini et al., 2002; Rubin et al., 2011 San Martín et al., 2002 Russi et al., 2008
Neomycin	≤ 1	≤ 1	≤ 1 -64	≥ 64	Giannechini et al., 2002
Oxitetracycline	2 2 2	>64 2 4	≤ 0.25 ->64 2-32 0.5-64	≥ 16	Giannechini et al., 2002 Rubin et al., 2011 San Martín et al., 2002
Erythromycin	≤ 0.5 0.25 0.125	≤ 0.5 0.25 0.25	≤ 0.5 ->4 0.25-16 0.125-0.5	≥ 8	Giannechini et al., 2002 Rubin et al., 2011 Russi et al., 2008
Azythromycin	0.5	1	0.25-2		Lucas, 2009
Spiramycin	4	4	0.5-4		Lucas, 2009
Clindamycin	≤ 1 0.25	≤ 1 0.25	≤ 1 0.25-16	≥ 4	Giannechini (2002) Rubin et al., 2011
Chloramph.	4	8	4-32	≥ 32	Rubin et al., 2011
Florfenicol	0.5	2	0.25-128	≥ 32	San Martín et al., 2002
Enrofloxacin	≤ 0.25 ≤ 0.25 0.125	≤ 0.25 ≤ 0.25 0.5	≤ 0.25 -0.5 0.25-16 0.06-8	≥ 4	Giannechini (2002) Rubin et al., 2011 San Martín et al., 2002
Danofloxacin	0.25	1	0.25-2		Lucas, 2009
Trimethoprim/ Sulfamethox	0.25 19	0.5 19	<0.006-8 19-76	≥ 4 76	Giannechini (2002) Rubin et al., 2011

Table 4. In vitro susceptibility of strains of *S. aureus* obtained from clinical and sub-clinical bovine mastitis cases by different authors.

We performed some studies with the objective of evaluating the PK behavior of AMX trihydrate in serum and milk after its IM administration at therapeutic dose in healthy lactating cows (Mestorino et al., 1997) (Fig. 2). AMX was absorbed relatively fast after its administration ($T_{1/2ab}$ 0.92 ± 0.10 h). Because AMX trihydrate was used, and that this is not a water soluble salt, the formulation to be administered was a suspension. This formulation allows a fraction of the AMX dose to be rapidly liberated and absorbed whereas another fraction, less soluble, is precipitated at the site of administration, and absorbed more slowly, leading to the slow elimination profile described, which allows the estimation of dosing regimens with long intervals. It should be emphasized that the profile of long-acting medication is not due to slow elimination, but a slow absorption. The drug is eliminated at normal speed, but no molecules are available at a higher rate of absorption; therefore it is the absorption process that commands the elimination half-life.

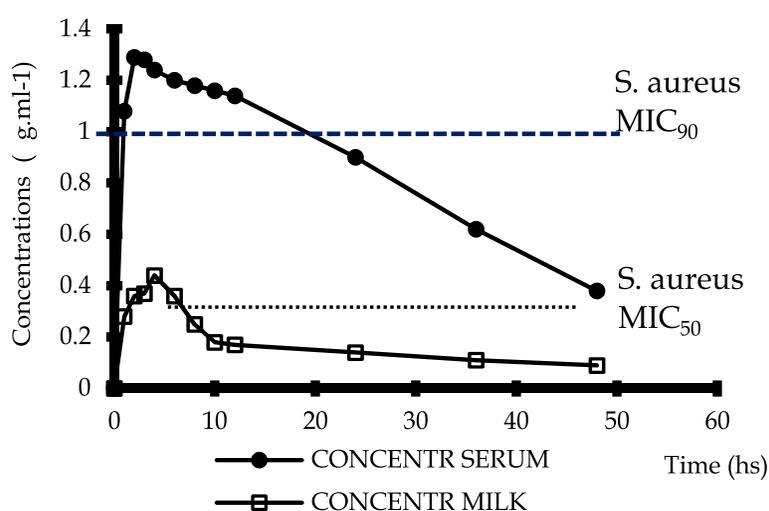


Fig. 2. AMX concentrations in serum and milk after IM administration of 15 mg.kg^{-1} to four healthy dairy cows.

The average time of penetration into milk ($T_{1/2p}$) of 1.24 ± 0.09 h, was considered fast enough for an antimastitic agent. The ratio $AUC_{\text{milk}}: AUC_{\text{serum}}$ was 0.18, which is a low level of milk availability (Table 5). Beta-lactam antibiotics, due to their acidic character are more dissociated in plasma than in milk, so milk penetration is severely restricted. Serum and milk concentrations were determined until 48 h post-administration, which, considering the mentioned MIC_{90} of *S. aureus*, does not represent a good therapeutic tool because C_{max} in milk was only $0.44 \pm 0.13 \mu\text{g.mL}^{-1}$. It has to be remarked that the mentioned parameter was obtained in healthy animals. In mastitic animals, on the other hand, it appears logical to expect a greater penetration of AMX in the gland, because raising the pH, would permit greater dissociation of AMX in the cistern, and a process of ion sequestration with higher drug concentrations in milk. In view of the influence of the factors mentioned above, PK studies should always be performed not only on healthy animals but on sick animals too.

The most significant factor affecting the cure rates for clinical *S. aureus* mastitis is the ability of the isolate to produce β -lactamase. This has also been shown by other authors (Sol et al. 2000), and could indicate either that penicillin resistant strains are more virulent than penicillin-susceptible strains, or that the antibiotics used to treat mastitis caused by

penicillin-resistant strains are less efficient, due to PK or PD factors, as we explained above. In the study by Sol et al. (2000), clinical *S. aureus* mastitis caused by β -lactamase positive or negative isolates was treated intramammarily with 5 different ATM treatments. All the isolates were found to be in vitro susceptible to the drugs used.

Parameter	Serum	Milk
K_{abs} (h ⁻¹)	0.75 ± 0.09	0.56 ± 0.08
$T_{1/2 abs}$ (h)	0.92 ± 0.10	1.24 ± 0.09
C_{max} (µg.ml ⁻¹)	1.29 ± 0.14	0.44 ± 0.13
T_{max} (h)	2.00 ± 0.00	4.00 ± 0.00
β (h ⁻¹)	0.025 ± 0.07	0.03 ± 0.001
$T_{1/2 \beta}$ (h)	27.61 ± 0.09	22.59 ± 0.91
AUC (µg.h/ml)	57.72 ± 3.87	10.34 ± 1.28
R C_{max_S}/C_{max_M}		2.93
R AUC_M/AUC_S		0.18
F_{Milk} (%)		18

Table 5. PK parameters (mean ± SD, N = 4) obtained in serum and milk after IM administration of AMX at dose of 15mg.kg⁻¹ in dairy cows

No difference in the bacteriological cure rates between the different ATM treatments in each group was found, but major differences in bacteriological cure rates between mastitis due to β -lactamase positive and negative strains was significant. *S. aureus* is known to possess many virulence factors, like capsule and slime formation, which make it more resistant to ATMs (Sandholm et al. 1990; Taponen et al., 2003).

Information about the PKs of systemic amoxicillin-clavulanic acid (AMX-CLA) suspension in dairy cows is almost totally lacking. However AMX-CLA is widespreadly used in veterinary medicine. Injectable formulations (IM) have been used in cattle, pigs and sheep, and oral formulations for the treatment of pre-ruminant animals. Like other β -lactams, AMX alone or combined with CLA is commercially available for IMM use in bovine mastitis.

There are few antecedents of combining an IMM treatment with a systemic one, and, in general these studies compare clinical efficacy without PK determinations (Ziv 1980a; 1980c). As this is an interesting strategy to treat mastitis, we decided to compare the pharmacokinetic behaviour of AMX-CLA after their IMM infusion vs the combination therapy IMM-IM in lactating Holstein cows with subclinical mastitis caused by *S. aureus* (Lucas et al., 2009a). The experimental animals were allocated by production level (high and low production level) and the quarters were grouped by health state. In the IMM alone assay, each quarter of all cows received 3 IMM infusions of AMX-CLA (200-50 mg) with a 12 h interval. Individual quarter milk samples were collected until 96 h after 3rd administration (Fig 3). The purpose was to evaluate the effects of the health status of the quarters (SS): mastitic quarters vs. healthy ones and the level of milk production (LP): quarters of high-producing cows vs quarters of low-producing cows. LP had a significant effect on $T_{1/2\beta}$ and

MRT, which were higher in quarters of low producing-cows ($P < 0.05$). The MIC_{90} for AMX-CLA 4:1 was $8 \mu\text{g}\cdot\text{mL}^{-1}$ and the $T > MIC_{90}$ was higher in quarters of low-producing cows. The LP modified the AMX-CLA PK profile and it could be a determinant of efficacy.

It resulted remarkable that levels of AMX were higher and remained for a longer period of time in the mammary quarters of low production cows than in the quarters of high production cows. These differences were not such in the case of CLA, as calculated parameters were similar in both groups. We determined also the $T > MIC_{90}$ in the milk of each quarter ($MIC_{90} = 6.4:1.6 \mu\text{g}\cdot\text{mL}^{-1}$) (See Table 6). The health status of the mammary quarters had no statistically significant effect on any of the PK parameters. The level of milk production, however, had a significant effect on the AMX $T_{1/2\lambda}$ ($P = 0.0000$), $AUC_{0-\infty}$ ($P = 0.0057$), CL/F ($P = 0.0057$) and MRT ($P = 0.0019$).

IMM treatment given to supplement systemic administration of ATMs increases drug concentration in the milk compartment, and higher concentrations throughout the mammary gland will follow (Ziv 1980a; 1980c). In theory, combination treatment thus should improve cure rates for deep infections such as *S. aureus* mastitis (Sandholm et al. 1990). On the other hand, β -lactam ATMs are time-dependent drugs, and very high concentrations at the infection site do not increase efficacy (Craig 2001).

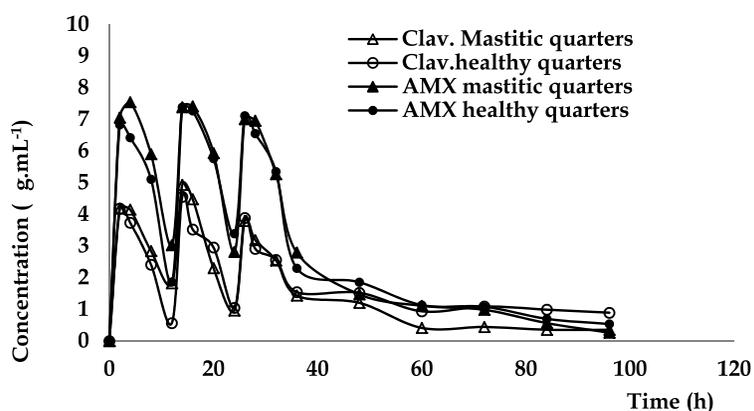


Fig. 3. Mean concentrations of AMX-CLA in milk of healthy and mastitic quarters after administration of 3 IMM syringes (200 mg-50 mg) every 12 h

	$X_{\text{mastitic}} \pm \text{SD}$	$X_{\text{healthy}} \pm \text{SD}$	$X_{\text{M high prod.}} \pm \text{SD}$	$X_{\text{M low prod.}} \pm \text{SD}$
$T > MIC_{90}$ (%)	52.78 ± 30.71	48.60 ± 31.65	46.30 ± 35.80	53.69 ± 26.72

Table 6. $T > MIC_{90}$ (%): percentage of the period between 0 and 12 h post-administration during which the concentration was \geq the MIC_{90} ; Amx:Cla 4:1, the MIC_{90} was considered as the ratio 4:1 ($MIC_{90} = 6.4:1.6 \mu\text{g}\cdot\text{mL}^{-1}$)

We performed another study where the effect of the combined IMM treatment with systemic administration was evaluated (Lucas et al., 2009a). The experimental animals received 3 IMM infusions of AMX-CLA (200-50 mg) in each quarter in combination with $3.5 \text{ mg}\cdot\text{kg}^{-1}$ of 15% AMX-CLA by IM route every 12 h. Individual quarter milk samples were collected until 96 h after 3rd administration. The incidence of the health status of the quarters (SS): mastitic quarters vs. healthy ones, and the level of milk production (LP): quarters of high-producing cows vs quarters of low-producing cows in the PKs of the drugs was evaluated (Fig. 4).

We determined once again the $T > MIC_{90}$ in the milk of each quarter ($MIC_{90} = 6.4:1.6 \mu\text{g}\cdot\text{mL}^{-1}$) (See Table 7). It was observed that concentrations achieved in cows receiving combination therapy (IM + IMM) were higher than those in cows treated intramammarily alone, which was a logical finding.

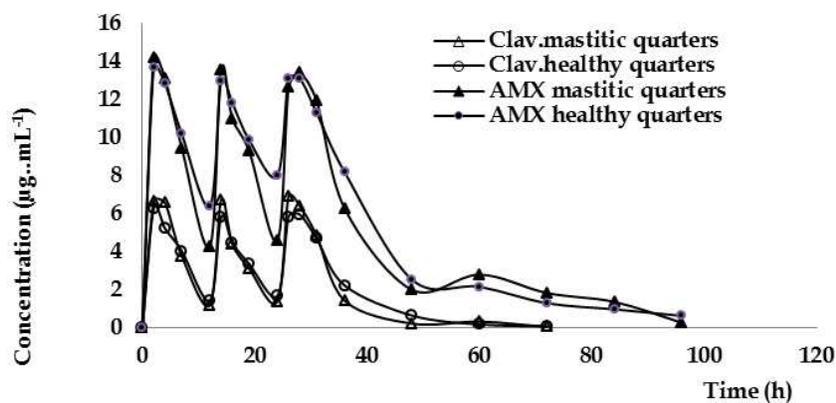


Fig. 4. Mean concentrations of AMX-CLA in milk of healthy and mastitic quarters after administration of 3 IMM syringes (200 mg-50 mg each) in combination with three 3.5 mg.kg⁻¹ of 15% AMX-CLA IM administrations every 12 h

Significant effects of quarter health status on the PK parameters were found. The C_{max} resulted higher in milk from sick quarters ($P = 0.0384$). The level of production had significant effect over milk AMX C_{max} and T_{max} , these were higher in the mammary quarters of low production cows. The level of production also affected the PK profile of CLA, the C_{max} , T_{max} and $AUC_{0-\infty}$ were higher in animals of low production. The differences observed between CL/F_{mam} suggest that the ATMs removal rate was higher in the quarters of high production cows. Significant effect of the mammary quarter health status and production level on the $T > MIC_{90}$ was found. It should be emphasized that the $T > MIC$ determined after the combined treatment (IM + IMM) exceeded those calculated after IMM infusion alone.

	$X_{mastitic} \pm SD$	$X_{healthy} \pm SD$	$X_{M \text{ high prod.}} \pm SD$	$X_{M \text{ low prod.}} \pm SD$
$T > MIC_{90}$ (%)	62.64 ± 8.87	78.25 ± 23.16	65.31 ± 20.33	79.48 ± 18.67

Table 7. $T > MIC_{90}$ (%): percentage of the period between 0 and 12 h post-administration during which the concentration was \geq the MIC_{90} ; Amx:Clav 4:1, the MIC_{90} was considered as the ratio 4:1 ($MIC_{90} = 6.4:1.6 \mu\text{g}\cdot\text{mL}^{-1}$)

Owens et al. (1988) found higher cure rates in *S. aureus* mastitis with combined treatment as compared with IMM treatment only. Recently, the therapeutic effects of parenteral, IMM and combination treatments with AMX-CLA have been compared by Perner et al. 2002 too. They found the combination treatment to be superior than parenteral or IMM treatment only. In this paper, the bacteriological cure rate for all causing agents and mastitis types (acute, subclinical and chronic), was 75.3%. We found also low bacteriological cure rates (62.5%) after AMX-CLA IMM infusion alone, but after the combined treatment the cure rate was complete (100%) (Lucas et al., 2009a, b).

4.1.2 Penethamate

Penethamate hydriodide (PNTM) is a diethylaminoethyl ester of penicillin which, unlike salts of penicillin, is unionised and so exists in a neutral state. It is only weakly water soluble forming a suspension in an aqueous environment. After its intramuscular administration, is rapidly absorbed from the site of injection and on entering the blood, partially dissociates by hydrolysis into penicillin G and diethylaminoethanol. At the blood pH (7.4), equilibrium is established where 90% of the active drug is present in its hydrolyzed form (penicillin G) with the remainder persisting as PNTM. As PNTM leaves the circulation due to its neutral and lipophilic properties and its high affinity to milk, this equilibrium is maintained by re-association of penicillin G and diethylaminoethanol until excretion is complete. PNTM easily passes the milk-blood barrier due to the pH gradient between milk (pH 6.6-6.8) and plasma (pH 7.2-7.4) and its weakly basic properties (pKa = 8.4). This is further facilitated by its highly lipophilic characteristics which facilitates its passage across the lipo-protein blood-milk barrier. PNTM starts to dissociate as it passes over the barrier and this process continues during diffusion of the drug through the udder, releasing increasing quantities of penicillin G (PEN_G). PEN_G is rapidly ionised in the udder (pKa = 2.8) so limiting its return to the circulation. It therefore becomes "trapped" in the udder in increasing concentrations.

The same pH gradient between blood and milk presides in the case of mild to moderate udder inflammation such as in sub-clinical mastitis, the pH gradient between blood and milk is the same than in healthy animals thus generating similar PK behaviors to those which take place in the healthy udder. In acute mastitis, although the pH of milk is nearer that of blood due to a breakdown of the blood-milk barrier, higher concentrations of PNTM are still found in mastitic milk than in blood due to its lipophilic properties. Not only does undissociated PNTM rapidly and easily penetrate the udder whether inflamed or not, but its liposoluble nature gives it advantage, compared with other beta-lactam antibiotics such as amoxicillin and aminoglycosides to diffuse through the parenchyma of the udder, pass into the milk and penetrate the lactogenic cells. This diffusion through the udder is supported by the mechanism of "ion trapping" mentioned above and so explains the different penetration of PNTM compared with PEN_G (Friton et al., 2003).

It must be remembered, however, that *S. aureus* survives in acidic media, including phagolysosomes. Controversial in vitro/in vivo data exist on its susceptibility to antibiotics in such environments. We performed some studies to evaluate the effect of the pH variation on the antibacterial activity of penicillin against strains of *S. aureus* isolated from mastitic quarters (Moncada Cárdenas et al., 2009). MIC of *S. aureus* field strains and *S. aureus* ATCC 25923 were tested at pH 7.4, 6.5 and 5.0, in order to simulate the conditions of acidity of subcellular structures which are commonly associated with *S. aureus* intracellular persistence. The PEN MIC₉₀ at pH 7.4 was consistent with those reported by CLSI 2007 (0.5 µg/mL) but at pH 5.0 (phagolysosomes) the activity of PEN_G increased markedly and almost linearly (~10 fold decrease in MIC -0.06 µg/mL) (Figure 5).

4.1.3 Cloxacillin

Cloxacillin (CLX) is used in the treatment or prevention of staphylococcal bovine mastitis. Its ATM activity against *S. aureus* is higher than that of PEN_G. There are strains of *S. aureus* resistant to isoxazolilpenicillins (oxacillin or methicillin resistant *S. aureus* -MRSA). These strains are a menace to public health.

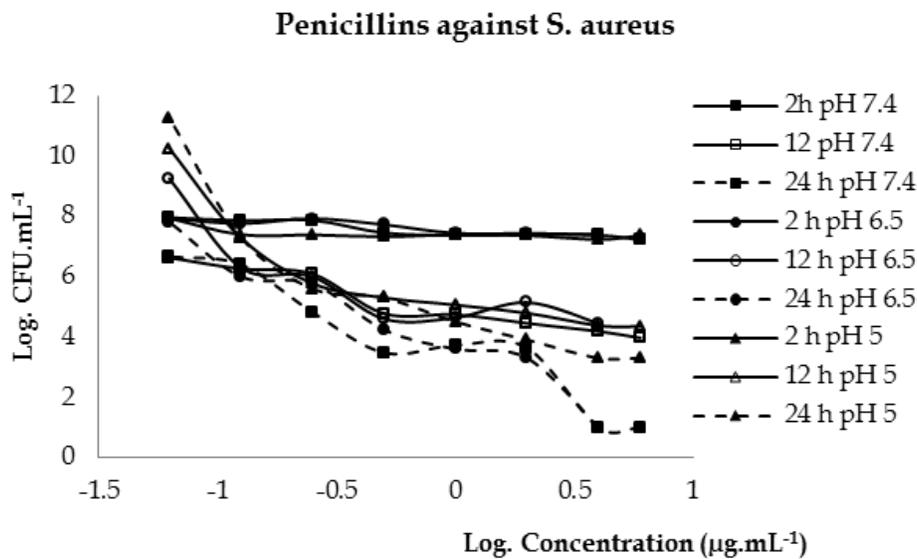


Fig. 5. Inhibition of *S. aureus* exposed to different concentrations (expressed as Log₁₀) of PEN (0.25 MIC; 0.5MIC; 1MIC; 2MIC; 4MIC and 8 x MIC) in function of the time and the medium pH (5; 6.5 and 7.4).

Benzathine CLX is used to treat bovine mastitis by IMM route at drying off and may be associated to other compounds, eg antiinflammatory agents (prednisolone) or other ATMs (eg. AMX, AMP, streptomycin, cephalosporins, etc.). Intramammary products with combinations of two or even three ATMs were introduced due to suggested synergistic action and broad spectrum. The evidence of their efficacy against clinical mastitis is many times lacking and synergistic action has never been proven *in vivo* (Taponen *et al.* 2003; Ødegaard & Sviland, 2001). For mastitis treatment during lactation the formulations contain CLX as sodium salt and, in general, the drug is not combined with other compounds. After IMM administration, sodium CLX binds scarcely to the mammary tissue (25%), with a moderate passage milk:plasma.

We evaluated the pharmacokinetic behavior of sodium CLX in milk after its IMM administration in healthy lactating cows of low production. The experimental cows received syringes containing 250 mg sodium CLX IMM, three times at 12 hour intervals in each quarter. Milk samples were obtained at different post administration times (Fig. 6).

The PK analysis was performed after the last administration. Milk elimination half-life was rather prolonged ($T_{1/2\beta} = 4.37 \pm 0.458$ h), with levels in milk at 60 h post-last administration ≥ 0.35 µg.mL⁻¹. In low production animals the elimination of the ATM is even slower, with longer milk half life (Table 8). Various benzathine CLX containing IMM suspensions are registered for use in cattle as antibiotics for IMM use at drying off. These formulations are recommended for routine use in cows at drying off to treat existing IMM infections and to provide prolonged protection against new infections during the next lactation.

The excretion through the mammary gland depends on the characteristics of the pharmaceutical excipient, production level (high vs. low), molecule characteristic and udder health status (Mestorino, 1993a). The elimination rate is affected also by the binding level to dry udder secretion, which is very high (80%) (Ødegaard & Sviland, 2001).

In a recent study, we obtained an MIC₉₀ of 2 µg.mL⁻¹ against *S.aureus* (isolated from cattle with subclinical mastitis) (Lucas et al., 2009a).

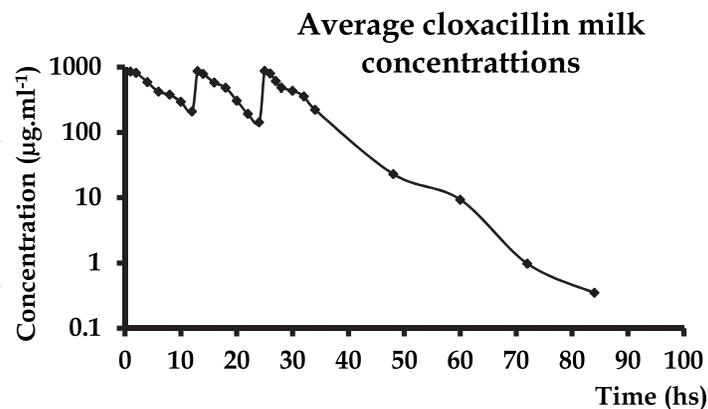


Fig. 6. Mean CLX concentrations in milk of dairy cows after IMM infusion of 250 mg CLX three times at 12 hour intervals in each mammary quarter.

PARAMETER	MEAN	SD
B (µg.mL⁻¹)	1288.557	464.800
β (h⁻¹)	0.160	0.016
T 1/2β (hs)	4.370	0.458
AUC_{0-all} (µg.h/ml)	7372.005	2062.654
MTR (hs)	6.982	1.469

Table 8. Mean pharmacokinetic parameters obtained in milk after IMM infusion of 250 mg CLX in each quarter three times at 12 hour intervals.

An experiment was conducted in cows with staphylococcal subclinical mastitis at drying off for the purpose of studying the pharmacokinetics behaviour of CLX benzathine in the dry udder secretion after a single IMM infusion (Lucas et al., 2009b) (Fig. 7).

In Table 9 the CLX PK parameters in udder dry secretion are presented. We determined the T>MIC₉₀ in the mammary dry secretion of each quarter. Normally, T>MIC₉₀ is expressed as the percentage of time of the inter-dose interval during which the concentration remains above the MIC. Since we used a single dose of benzathine CLX in a slow-release formulation, there was no inter-dose interval. Therefore, with the objective of evaluating the PK behaviour based on the MIC, we estimated T>MIC₉₀ as hours post-treatment during which the ATM concentration was above the MIC.

The milk:plasma passage rate was very low and the CLX concentrations in plasma could not be measured. For mastitis treatment by IMM route it is desirable the permanence of the ATM in the mammary compartment or glandular tissue for a long time.

Therefore, these findings are acceptable considering that the formulation used is indicated for the IMM treatment of mastitis at drying off. The level of binding of CLX to dry udder secretion is high ($86 \pm 6.5 \%$), acting as a reservoir and increasing the persistence of the ATM inside the udder. The experimental animals maintained concentrations above the limit of quantitation by microbiological methods ($0.03 \mu\text{g}\cdot\text{mL}^{-1}$) during the first 24-31 days. Oliver et al. (1990) found that after IMM administration of CLX at drying off, the secretion samples had detectable levels until 28-35 days post-treatment in some animals and until 42-49 in others. This finding shows a marked variability in CLX concentrations at drying off. The determinants of this variability are varied and complex: udder size, seasonal effects, body condition of cows, physiological changes in body condition at the start of the dry period and other factors affecting gland and body.

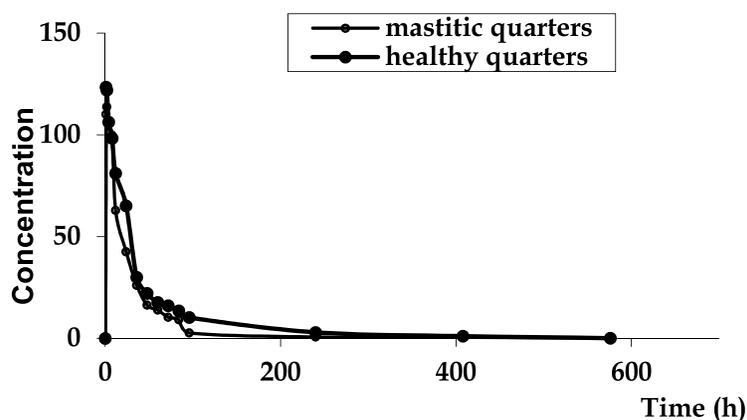


Fig. 7. Average CLX milk concentrations in dry udder secretion (mastitic vs. healthy) after the administration of one IMM syringe in each quarter at drying off (600 mg)

Parameter	Unit	Mean _{mastitic} \pm SD	Mean _{healthy} \pm SD
$T^{1/2\lambda}$	h	48.13 ± 51.27	39.53 ± 27.29
ABC_{0-24h}	$\mu\text{g}\cdot\text{h}\cdot\text{mL}^{-1}$	2130.24 ± 797.31	2144.09 ± 850.06
$ABC_{0-\infty}$	$\mu\text{g}\cdot\text{h}\cdot\text{mL}^{-1}$	5003.07 ± 3292.88	5358.52 ± 3398.00
TMR	h	62.98 ± 47.49	57.42 ± 35.07
$T > CIM_{90}$		257.14 ± 188.45	231.54 ± 153.82

Table 9. CLX PKs in dry udder secretion in mastitic quarters vs. healthy quarters after its administration at dose of 600 mg in each quarter at drying off. $T > CIM_{90}$: post-administration hours during which CLX concentrations remained above the MIC_{90} ($2 \mu\text{g}\cdot\text{mL}^{-1}$)

4.1.4 Cephalosporins

Several IMM products for the IMM treatment of dairy cattle contain cephalosporins, most commonly as a single product (e.g., cefquinome; cephapirin), but also in combination (cephalexin and kanamycin). The cephalosporins are semisynthetic antibiotics derived from cephalosporin C (produced by *Cephalosporium acremonium*). There are currently 4 generations of cephalosporins, which vary from the narrow-spectrum first generation through the expanded-spectrum fourth generation (Hornish and Kotarski, 2002), with all generations being used in veterinary medicinal products. When using a first-generation cephalosporin in an IMM preparation, there is a strong rationale for combining it with an

additional antibiotic to extend the spectrum of the product. There are cephalosporins that, when administered systemically penetrates in significantly low levels to the mammary gland of the producing cow. Is the case of ceftiofur. Ceftiofur is an interesting tool because it can be used in cows that are producing milk to treat infections that are located out of the mammary gland (metritis, foot infections), without discarding milk.

4.2 Phenicol

The toxicity of chloramphenicol (CAP) has been the cause of its use being banned or strictly regulated worldwide. This has accentuated the need for an effective broad-spectrum antibiotic to be used in food producing animals. Florfenicol (FLF) is a synthetic fluorinated CAP analogue that is exclusively used in food animals for treatment of infectious diseases. PK studies showed that high concentrations, both in serum and milk, are obtained following IMM administration in cows, which led to propose its use for the treatment of bovine mastitis. The FLF MIC₉₀ among *S. aureus* obtained by San Martin was 2 µg.mL⁻¹ (2002). We obtained a thianphenicol (TAP) MIC₉₀ higher than FLF (>16 µg.mL⁻¹) (Lucas, 2009b).

TAP is a structural analogue of CAP with marked toxicological differences (Mestorino et al., 1993b). TAP has a greater in vitro activity against some bacteria that are resistant to CAP. Although TAP has similarities in its antibacterial spectrum with that of CAP, there are marked pharmacological differences between the two drugs. TAP is more stable in solution, is not appreciably protein bound in the body (16%) and does not undergo significant biotransformation. The drug diffuses into intracellular spaces, the central nervous system and the aqueous humour (Mestorino et al., 1993a; b). We performed a complete study about the TAP PK behavior in lactating healthy Holstein cows after its administration by intravenous, intramuscular, subcutaneous routes (Fig. 8) and after IMM infusion in a unique quarter and in the complete gland (in the four quarters) (Mestorino et al., 1993a; 1993b; 1995). Serum PK after IV administration was described following a bicompartamental model. The antibiotic showed a rapid distribution, with a half life of 8.84 ± 4.34 min. Half life of elimination was 1.95 ± 0.55 h, indicating that TAP is rapidly eliminated in the bovine. Volume of distribution was high, 1404.18 ± 428.19 ml.kg⁻¹. Milk concentrations were high, when the drug was administered systemically, with a mean time of gland penetration (T^{1/2p}) rather fast of 36.58 ± 9.72 min and a half life of elimination from the gland of 3.62 ± 0.97 h, indicating that accumulation of TAP takes place in the mammary gland. A milk permanency time of 4.13 ± 1.13 days was determined (Table 10).

A monocompartmental model was used to describe the PKs of TAP after its IM and SC administration. After IM administration, TAP was rapidly absorbed, with a half life of 6.53 ± 6.25 min and a half life of elimination of 2.9 ± 0.50 h. Maximum concentration was 29.38 ± 6.90 µg.mL⁻¹ and was found at 0.32 ± 0.09 h. Area under the curve was 68.45 ± 16.27 µg.h/mL and bioavailability 86.33 ± 19.08%. Milk therapeutic levels were maintained between 0.5 and 8 h with a C_{max} of 17.05 ± 2.73 µg.mL⁻¹ reached at 3.42 ± 0.19 h. Mammary gland half life of penetration was 51.11 ± 6.10 min and half life of elimination 5.31 ± 4.27 h. Ratio C_{maxS}:C_{maxM} was 1.81 and the ratio AUC_M:AUC_S 1.19. Theoretical milk permanency time was 4.79 ± 1.40 days.

After SC administration a maximum serum concentration of 19.83 ± 3.57 µg.mL⁻¹ was obtained at 0.72 ± 0.12 h with a half life of absorption of 10.09 ± 5.37 min. Half life of

elimination was 2.60 ± 0.60 h. AUC of 56.17 ± 7.33 $\mu\text{g}\cdot\text{h}/\text{mL}$ was calculated. Bioavailability was of 82.33 ± 13.36 %. Milk concentrations after SC administration were maintained above the MIC between 1 and 8 h. The C_{max} was of 13.19 ± 3.47 $\mu\text{g}\cdot\text{mL}^{-1}$ at 3.42 ± 0.19 h. The half life of penetration was 41.97 ± 4.55 min and the half life of elimination 7.57 ± 4.47 h. ratio $C_{\text{maxS}}:C_{\text{maxM}}$ and $\text{AUC}_M:\text{AUC}_S$ were 1.57 and 1.03 respectively.

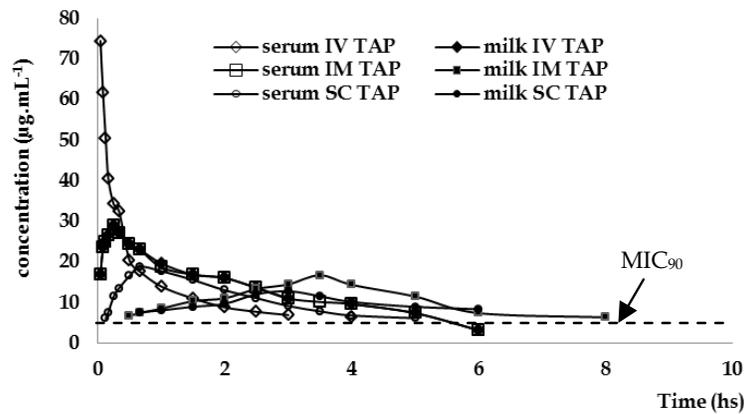


Fig. 8. Averaged serum and milk TAP concentrations for six lactating cows following intravenous, intramuscular and subcutaneous TAP administration at dose of $25 \text{ mg}\cdot\text{kg}^{-1}$

Parameter	Serum IV	Serum IM	Serum SC	Milk IV	Milk IM	Milk SC
β (h^{-1}) - K_e (h^{-1}) _M	0.40 ± 0.16	0.24 ± 0.05	0.28 ± 0.06	0.21 ± 0.067	0.18 ± 0.07	0.15 ± 0.10
$T^{1/2\beta}$ (h) - $T^{1/2E}$ (h) _M	1.95 ± 0.55	2.88 ± 0.50	2.60 ± 0.60	3.62 ± 0.97	5.31 ± 4.27	7.57 ± 4.47
K_{ab} (h^{-1}) - K_P (h^{-1}) _M		14.26 ± 8.72	5.06 ± 2.00	1.23 ± 0.37	0.83 ± 0.10	1.00 ± 0.10
$T^{1/2ab}$ - $T^{1/2PM}$ (min)		6.53 ± 6.25	10.09 ± 5.37	36.58 ± 9.72	51.11 ± 6.10	41.97 ± 4.55
AUC ($\mu\text{g}\cdot\text{mL}/\text{h}$)	50.59 ± 7.56	68.45 ± 16.27	56.17 ± 7.33	85.77 ± 21.58	75.31 ± 9.85	52.85 ± 17.50
C_{max} ($\mu\text{g}\cdot\text{mL}^{-1}$)		29.38 ± 6.90	19.83 ± 3.57	23.09 ± 3.12	17.05 ± 2.73	13.19 ± 3.47
T_{max} (h)		0.32 ± 0.09	0.72 ± 0.12	2.50 ± 0.29	3.42 ± 0.19	3.42 ± 0.19
Lag time (h)		0.05 ± 0.02	0.15 ± 0.02	1.08 ± 0.51	0.78 ± 0.23	1.42 ± 0.34
F (%)		86.33 ± 19.08	82.33 ± 13.36			
$C_{\text{max-S}}/C_{\text{max-M}}$					1.81 ± 0.64	1.57 ± 0.39
$\text{AUC}_M/\text{AUC}_S$				1.72 ± 0.47	1.19 ± 0.41	1.03 ± 0.26
Withdrawal (d)				4.13 ± 1.13	4.79 ± 1.40	3.23 ± 0.86

Table 10. TAP PKs in serum and milk after its administration at dose of $25 \text{ mg}\cdot\text{kg}^{-1}$ by IV, IM and SC routes in lactating Holstein cows. Withdrawal is the withdrawal time needed to obtain milk with TAP levels of less than $20 \text{ ng}\cdot\text{mL}^{-1}$.

As a conclusion, TAP is a highly bioavailable antibiotic, independently of the administration route, it arrives with ease to the mammary gland, reaching high levels there. That is why it can be considered an excellent option to use in mastitis therapeutics, especially against Gram negative pathogens. It has a bacteriostatic action against a broad range of microorganisms, although it may be bactericidal for some species under some conditions, and in concentrations 3 to 5 times higher than the bacteriostatic ones. Among the bacteria inhibited *in vitro* by relatively low concentrations of TAP are *Clostridium*, *Corynebacterium*

diphtheriae, *Diplococcus pneumoniae*, *Staphylococcus albus*, *Streptococcus pyogenes*, *Streptococcus viridans*, *Bacteroides*, *Fusobacterium*, *Bordetella*, *Brucella*, *Haemophilus*, *Neisseria*, *Pasteurella*, *Shigella* and some vibrio strains. Some Bacilli, *Erysipelothrix*, *Staphylococcus aureus* and *Streptococcus faecalis* are sensitive to moderate concentrations of TAP (FAO: JECFA 47, 1997).

When TAP was administered by IMM route in an individual quarter high concentrations in the nonmedicated quarters and in serum were determined. Although the experimental animals were milked every 12 h, TAP levels above the minimum inhibitory concentration for the majority of pathogens were maintained during 36 h in the treated quarter and between 8 and 12 h in the nontreated quarters and during less than 4 h in the case of blood (Mestorino et al., 1995). TAP passes easily and completely from the treated quarter to the homolateral quarter by simple diffusion, to the heterolateral quarters, on the other hand, it arrives through circulation. Time elapsed between the administration and the first measured concentration (Lag Time) was shorter in the case of the homolateral quarter (rear-left -RL-: 0.25 h), intermedial in the case of serum (0.54 h) and rear-right -RR- (0.58 h) and longer in the case of the front-right quarter -FR- (1.92 h). In the homolateral quarter, the main C_{max} and the highest bioavailability were recorded. Maximum TAP concentrations of 75.30, 15.06, 19.03 and 15.79 $\mu\text{g}\cdot\text{mL}^{-1}$ in the RL, FR, RR and serum respectively, and the ratios of $AUC_{\text{treat}}:AUC_{\text{nontreat}}$ of 10.61, 1.81, 2.78 and 1.35 in the RL, FR, RR and serum respectively, suggests that the largest passage takes place from the administered quarter to the homolateral one, and that the arrival of TAP to the contralateral quarters is via the blood due to that the longitudinal medial intermammary septum is a highly impermeable (Fig. 9).

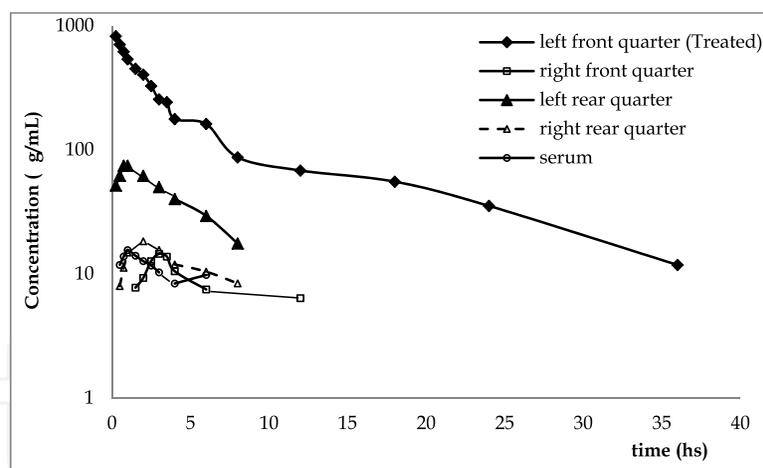


Fig. 9. Averaged milk and serum TAP concentrations for six lactating cows following IMM infusion in the front-left mammary quarter at dose of $25 \text{ mg}\cdot\text{kg}^{-1}$.

4.3 Macrolides

The prototype of the macrolide class of antibiotics was introduced in 1952 under the name of erythromycin. Macrolides are frequently used in Argentina for bovine mastitis treatment, since high concentrations in milk are obtained following parenteral administration. Erythromycin, oleandomycin, tylosin and the modern tilmycosin, azithromycin and tulathromycin have been extensively used in bovines. The last three are drugs with substantial improvements respect the older ones.

4.3.1 Spiramycin

Spiramycin (SPM) is a macrolide antibiotic that is active against most of the microorganisms isolated from the milk of mastitic cows. SPM is a very soluble weak base (pKa 8.2) which crosses lipid membranes with ease.

Our team investigated the disposition of SPM in plasma and milk after intramuscular administration. Lactating Holstein cows with subclinical mastitis caused by *S. aureus* were given two injections of SPM adipate at a dose of 16,000 IU/kg (3.75mg.kg⁻¹) with 24 hours of intervals. The experimental animals were allocated by production level (high vs. low production) and the quarters were grouped by health state (Fig. 10 A and B).

In susceptibility studies, usually erythromycin is used as a representative of the macrolide group of compounds. However, when evaluating SPM susceptibility, it is not recommended to use erythromycin disks, because it has been shown that proteins involved in macrolide-ribosome interactions are different. The binding site of erythromycin is the L22 protein while for SPM the protein is the L27 (Lucas et al., 2007; 2009b). The MIC₉₀ calculated for *S. aureus* field isolates was 4µg.mL⁻¹(17 IU.mL⁻¹) (Lucas et al., 2009b). Other authors have reported MICs of 3.25 µg.mL⁻¹ (15 IU.mL⁻¹) (Renard et al., 1996) and 8 µg.mL⁻¹ (Friis et al., 1988). After IM SPM administration, milk concentrations were higher than those in plasma. The maximum concentration in milk (C_{maxM}) was 3.91µg.ml⁻¹ at 9 hours post-first administration and 3.46 µg.ml⁻¹ 12 hours post-second administration. Whereas in plasma the measured concentrations were far below those in milk (0.12µg.ml⁻¹ at 0.5 h and 0.097µg.ml⁻¹ at 8 h post first and second administration respectively). An average milk-to-plasma ratio of 46.35 ± 11.09 was calculated by comparison of the areas under the concentration vs time curves. Our results were coincident with other authors (Renard et al., 1996). The level of production factor exert a significant effect on C_{max}, T_{1/2λ}, AUC₀₋₂₄, MRT and F_{milk} (Table 11). SPM in milk of high production animals reached higher concentration with AUC_{0-24h} also higher in this group. However, T_{1/2λ} was longer in the milk of low production cows. The average milk-to-plasma ratio was higher in high producing cows. Since the milk production level has a significant effect on efficacy predictors such as T>MIC₉₀ (41.60) and AUC₀₋₂₄/MIC₉₀ (15.87) milk SPM concentrations obtained were insufficient to achieve optimal PK/PD relationships.

4.3.2 Azithromycin

Azithromycin (AZT) is a semisynthetic compound in which the lactone ring has been expanded to a 15-member structure and is considered the prototype of the new macrolide structures identified as azalides (Lucas et al., 2007; 2009c). AZT formulations are not available for use in production animals but could possess advantages for the treatment of certain bovine infections such as those produced in the mammary gland. We performed a complete study about its pharmacokinetic behaviour in blood and milk following administration by different routes, both in healthy cows and cows with mastitis. The results observed after its administration by any of the analyzed routes was typical of the macrolides, with low plasma concentrations and very high concentrations in milk and soft tissues; a great volume of distribution and a prolonged terminal half-life both in blood and milk. The fact that AZT tends to accumulate in inflammatory cells, has a kinetic incidence in the results, especially after IMM administration.

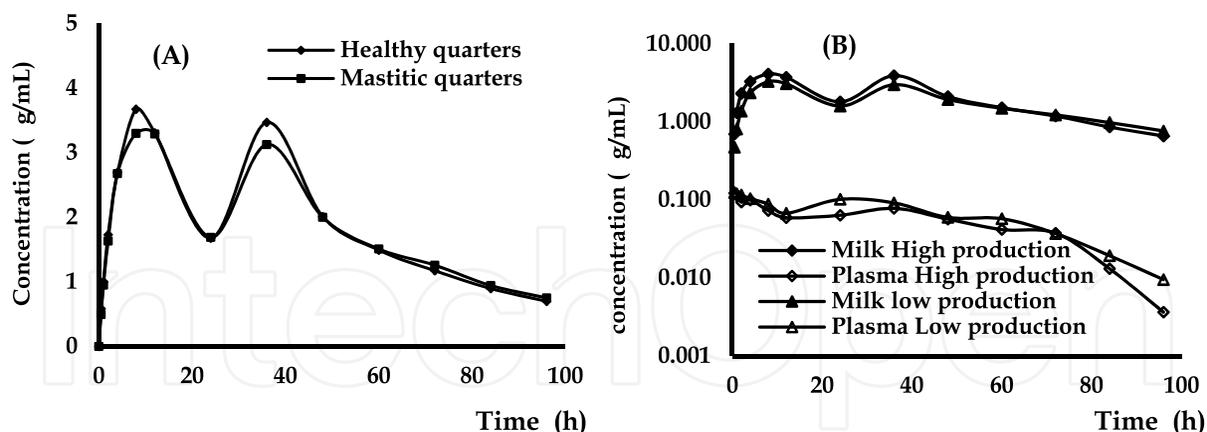


Fig. 10. (A) Mean concentrations of SPM in mastitic quarters and healthy quarters after two doses of 3.75 mg.kg^{-1} IM with a 24-h interval. (B) Mean concentrations of SPM in plasma in high-producing and low-producing cows; and quarters of high-producing cows and low-producing cows after two doses of 3.75 mg.kg^{-1} IM with a 24-h interval

Parameter		Milk High-Pr	Milk low-Pr	Plasma High-Pr	Plasma Low-Pr
$C_{\text{m}\acute{\text{a}}\text{x}1}$	$\mu\text{g.mL}^{-1}$	4.26 ± 0.66	3.57 ± 0.61	0.12 ± 0.01	0.12 ± 0.01
$T_{\text{m}\acute{\text{a}}\text{x}1}$	h	8.67 ± 2.31	9.33 ± 1.97	0.50 ± 0.00	0.50 ± 0.00
$C_{\text{m}\acute{\text{a}}\text{x}2}$	$\mu\text{g.mL}^{-1}$	3.92 ± 0.71	3.01 ± 0.71	0.08 ± 0.02	0.11 ± 0.04
$T_{\text{m}\acute{\text{a}}\text{x}2}$	h	36.00 ± 0.00	36.00 ± 0.00	32.00 ± 6.93	32.00 ± 6.93
$T^{1/2}\lambda_1$	h	12.26 ± 2.11	13.64 ± 4.20	27.83 ± 18.38	25.19 ± 11.03
$T^{1/2}\lambda_2$	h	21.17 ± 8.65	36.60 ± 7.61	7.70 ± 2.06	14.07 ± 5.56
$ABC_{0-24\text{h}}$	$\mu\text{g.h/mL}$	70.54 ± 10.62	56.44 ± 8.41	1.77 ± 0.30	2.14 ± 0.42
$ABC_{0-\infty}$	$\mu\text{g.h/mL}$	169.76 ± 30.66	171.80 ± 25.61	3.10 ± 1.14	4.04 ± 0.83
TMR	h	43.54 ± 10.93	55.96 ± 9.83	22.66 ± 0.99	28.78 ± 5.83
F_M		57.73 ± 13.61	43.91 ± 10.30	-	-

Table 11. Milk and plasma PK parameters obtained after two doses of SPM IM administration (3.75 mg.kg^{-1} every 24h) in high and low producing lactating cows.

Due to the described PK behaviour, AZT proved to be an interesting tool with potential for infections in soft tissues, although this should be backed by efficacy trials. Classically, macrolides have been considered drugs of choice to treat IMM infections not only because of their antibacterial efficacy but because of their favourable PK profile. On the basis of these antecedents we decided to investigate the distribution of AZT in plasma and milk after IV, IM and IMM administration in healthy and mastitic lactating Holstein cows and cows at drying-off (Turic et al., 2003a; 2003b; 2006; Errecalde et al., 2003; Lucas et al., 2009b; 2009c).

There are no AZT susceptibility studies on *S. aureus* isolated from bovine mastitis cases. However, there are data for other macrolides. Usually when running antibiograms, an erythromycin disk is used to evaluate susceptibility to the macrolide family. Variable percentages of macrolide resistant *S. aureus* ranging from 1.9% to 26.3% have been reported in several studies (Ziv., 1980a; Owens et al., 1997; Andrade et al., 2000). At present there are

highly specific methods for susceptibility testing of veterinary pathogens (CLSI, 2008). The AZT MIC₅₀ calculated for the 51 *S. aureus* isolations was 0.5 µg.mL⁻¹ and the MIC₉₀ was 1 µg.mL⁻¹ (Lucas et al, 2009c). Although it is not advisable to compare MICs of different ATM agents, it is worth stating that erythromycin MIC₉₀ for bovine isolated *S. aureus* was reported as 0.5 µg.mL⁻¹ in several publications (Giannechini et al., 2002; Russi et al., 2008).

The most prominent pharmacokinetic characteristic of AZT is the presence of high tissue concentrations which are maintained a long time after serum concentrations decline to very low levels (Fig 11 A y B). This characteristic was demonstrated after its IV and IM administration (Tables 12 and 13) (Turic et al., 2003a; 2003b), and was coincident with some authors in goats (Cárceles et al., 2005) and humans (Foulds et al., 1991). AZT T_{1/2λ} was long, an expected finding, according to the characteristics of this ATM.

Milk AZT levels resulted much higher than those found in serum (Fig. 11 A y B), after each IV and IM administration which is a logical finding according to the lipophilicity and wide distribution of the drug.

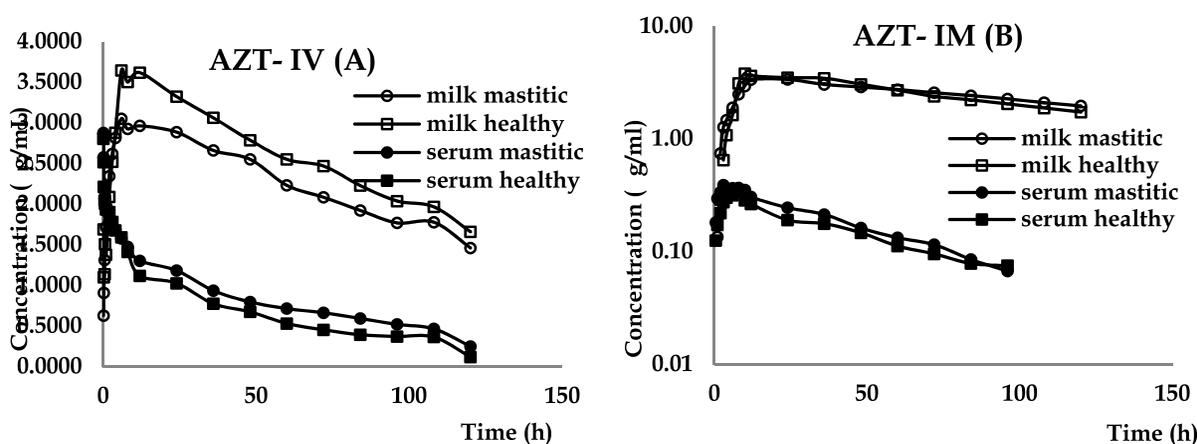


Fig. 11. Mean concentration of AZT in serum and milk of healthy and mastitic lactating cows after IV (A) and IM (B) administration (10 mg.kg⁻¹)

AZT exhibited major penetration into milk and it was cleared rather slowly. PK parameters indicated a high retention of the drug in peripheral compartments. The T_{1/2λ} in milk after each route of administration was always at least four times longer than that in plasma. AZT T_{1/2λ} clearly suggested that milk concentrations decrease more slowly than plasma ones.

Later we performed other assay with AZT, but in this case it was administered intramuscularly in two doses of 10 mg.kg⁻¹ body weight with a 48 h interval (Lucas et al., 2009c). The experimental animals were allocated by production level (high and low production levels) and the quarters were grouped by health state (Fig. 12 A and B). The T_{1/2λ} in milk after first administration was at least four times longer than that in plasma. AZT T_{1/2λ} suggested that milk concentrations exhibited a tendency to decrease more slowly than plasma ones. The same pattern was observed after a single 10 mg.kg⁻¹ IM dose of AZT to lactating Holstein cows (Turic et al., 2003a).

Parameter	Serum healthy	Serum mastitic	Milk healthy	Milk mastitic
λ_z (h ⁻¹)	0.019±0.006	0.013±0.001	0.007±0.003	0.007±0.001
$t_{1/2\lambda_z}$ (h)	38.6±9.11	53.07±3.36	113.3±39.5	111.2±21.1
V_{area} (ml.kg ⁻¹)	6325.6±1467.9	6486.3±318.6		
$AUC_{0-\infty}$ (h.µg/ml)	89.0±13.4	84.8±2.65	599.4±141.3	511.2±113.3
MRT (h)	52.1±8.94	69.5±7.61	163,6±58,6	159,6±34,7
T_{max} (h)			9.0±3.29	12.0±9.86
C_{max} (µg/ml)			3.82±0.49	3.13±0.66
AUC_M/AUC_S			389.4±69.4	232.3±52.7

Table 12. Serum and milk PK parameters obtained after one IV administration of AZT at a dose of 10 mg.kg⁻¹ in healthy and mastitic lactating Holstein cows

Parameter	Serum healthy	Serum mastitic	Milk healthy	Milk mastitic
λ_z (h ⁻¹)	0.018±0.004	0.015±0.003	0.008±0.003	0.005±0.001
$t_{1/2\lambda_z}$ (h)	39.11±7.64	47.31±9.75	105.19±40.18	135.96±39.49
$AUC_{0-\infty}$ (h.µg/ml)	36.26±8.59	26.09±1.71	561.89±60.55	713.36±249.99
MRT (h)	53.43±6.80	63.22±10.92	158.38±54.21	201.03±56.06
T_{max} (h)	3.00±1,549	2.17±0.41	17.33±10.56	13.67±5.13
C_{max} (µg.ml ⁻¹)	0.92±0.11	0.66±0.03	4.35±2.17	3.48±0.42
F (%)	41.38±13.98	18.860±2.51		
AUC_M/AUC_S			1041.57±516.7	1422.57±350.
$C_{max M}/C_{max S}$			4.85±2.67	5.26±0.73

Table 13. Serum and milk PK parameters obtained after one IM administration of AZT at a dose of 10 mg.kg⁻¹ in healthy and mastitic lactating Holstein cows

When comparing PK parameters by grouping quarters according to health status, it was observed that AZT was eliminated more slowly from and $AUC_{0-\infty}$ was substantially higher in mastitic quarters. Although this was an unexpected finding (the pKa partition hypothesis suggests the opposite), it is coincident with previously reported data (Turic et al., 2003a). Milk pH in the experimental animals ranged between 6.5 and 7.5 with the majority of values around 7.0. Average pH from all mastitic quarters was 7.13 ± 0.23 and from healthy quarters was 6.90 ± 0.21 (see Table 14). This is a normal finding for animals carrying subclinical mastitis. AZT is a weak base with a pKa value of 8.74, as a consequence, by application of the Henderson-Hasselbach equation, there would be approximately double AZT molecule dissociation in mastitic milk and more than three times in milk of healthy animals in comparison with plasma (see Table 14). Alkaline drugs (like AZT) are trapped in acidic compartments. This theoretical considerations could not, however, be confirmed by the experimental findings reported here. Azithromycin (IM) gave rise to very low plasma AUCs, which could be explained by its very high liposolubility and penetration into tissues. Although higher AUC was expected in milk of healthy animals (more acidic), which is a

common finding with the classic macrolide antibacterials, we found exactly the opposite. In our experiment, the highest concentrations were determined in the milk of mastitic animals, with an $AUC_{milk} / AUC_{plasma}$ ratio extremely high. Our explanation for this unexpected finding is the amount of somatic cells (SCC) present in mastitic milk in comparison with the normal milk. In the former case, the number of SCC was several times above those in normal milk. Mastitic milk normally exhibits very high cell counts as consequence of the inflammatory reaction. As it is known, AZT is able to reach high concentrations at infected sites, as a result of increased delivery from phagocytes (Lucas et al, 2009c). On this basis, we consider that the inflammatory reaction (and the high amount of cells) in the infected quarters is the main reason for the differences found between mastitic and healthy quarters.

	Plasma	Mastitic milk	Healthy milk
pH	7.40	7.13	6.90
Dissociated/non dissociated molecules	21.88/1	40.74/1	69.18/1
Theoretical ratio of dissociated molecules	1	2.86	3.16
Experimental Milk/plasma AUC		743.60	482.72

Table 14. Average plasma pH, milk pH (mastitic vs. healthy), dissociation as a function of AZT pKa (8.74), theoretical ratio milk/plasma and experimental AUC ratio milk/plasma

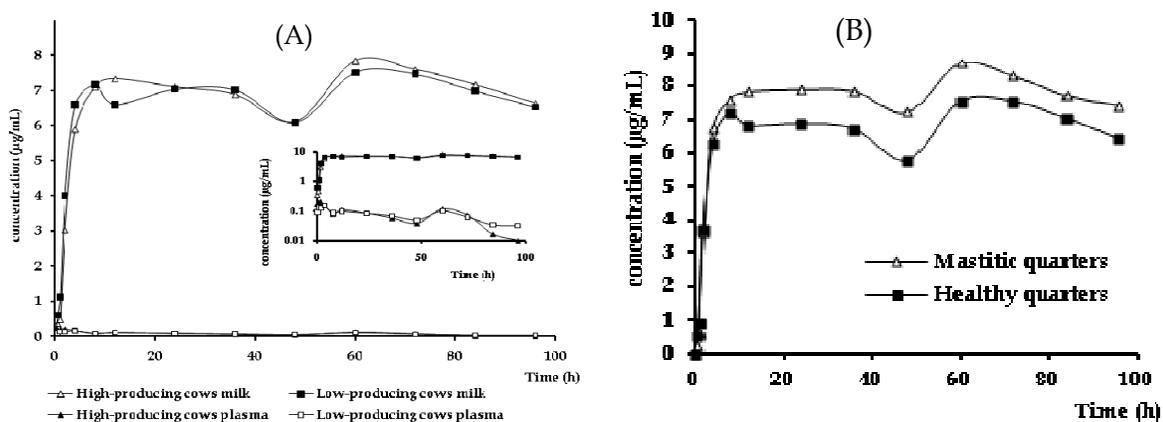


Fig. 12 (A) Mean plasma and milk concentrations of AZT in high-producing and low-producing cows after two 10 mg.kg^{-1} IM doses with a 48-h interval. (B) Mean concentrations of AZT in mastitic quarters and healthy quarters after two 10 mg.kg^{-1} IM doses with a 48-h interval

A significant AZT fraction could be trapped in the milk-cell compartment without participating of the plasma:milk equilibrium, largely dependent on the pKa - pH relationship. The $AUC_{0-\infty}$ ($P \leq 0.05$) and the MRT were higher in whole milk from mastitic quarters, which may indicate that the drug is present in higher amounts and persists during longer time in mastitic quarters than in healthy ones. At the same time, the F_{milk} of AZT was higher in the mastitic quarters indicating a different PK profile of AZT depending on the quarter status. The previous data, reported after a single 10 mg.kg^{-1} IM dose of AZT to lactating Holstein cows, support our observations (Turic et al., 2003a). The Cl_{mam}/F showed that AZT elimination was faster in healthy quarters than in mastitic quarters. Separating the

groups according to median production allowed us to identify observations that may be extrapolated to situations of major differences in productive levels (Fig. 12 A). The $AUC_{0-\infty}$ was higher in quarters of low-producing cows than in quarters of high-producing cows. It is possible that a lower milk production causes a slower antibiotic elimination, with a lower Cl_{mam}/F value and more prolonged $T_{1/2\lambda}$. On the basis of these results, we could suggest that low-producing cows have a high tendency to exceed LMR in milk while high-producing cows can eliminate (and dilute) the drug fast enough so as to diminish “contact time” and clinical efficacy possibilities.

AZT is a time dependent bacterial killing antibiotic with prolonged persistence (PAE, PASME). Thus, for this group, both $t > MIC$ and AUC_{24h}/MIC ratio play an important role in planning the dosage regimens. According to microbiological results, the MIC for *S. aureus* of bovine udder origin was $0.5\mu g.mL^{-1}$. The AUC_{24}/MIC was greater than 100 in both healthy as mastitis cows (Table 15). The times above MIC were longer than 95 h for all cases.

Six healthy and six mastitic lactating cows received one IMM AZT syringe containing 125 mg in each mammary quarter for three consecutive milkings; and six healthy and six mastitic Holstein cows at drying-off received one IMM AZT syringe containing 500 mg in each mammary quarter (Turic et al., 2003a; Errecalde et al., 2003).

For the study in lactating cows, serum profiles evolved with three peaks and a final elimination phase after the last infusion. Serum penetration from milk was fast and the three peaks were in the same order of magnitude both in healthy and mastitic animals (Fig. 13 A). AUC serum concentration was however higher in healthy animals than mastitic animals. This resulted an unexpected finding, since AZT is a basic drug. No significant differences were found either in $T_{1/2\lambda}$ or MRT between healthy and mastitic animals.

Parameters	Mastitic \pm SD	Healthy \pm SD	H-prod \pm SD	L-prod \pm SD
AUC_{0-24h}/MIC_{90}	156.53 ± 39.68	152.46 ± 33.19	152.53 ± 38.93	155.11 ± 31.51
$T > MIC_{90}$	96.09 ± 0.74	96.48 ± 1.20	95.83 ± 0.00	96.88 ± 1.80

* MIC_{90} of AZT against the 51 *S. aureus* isolated

Table 15. PK/PD parameters of AZT in mastitic and healthy quarters; and in quarters of high-producing cows and quarters of low-producing cows after two 10 mg. Kg^{-1} IM doses with a 48-h interval

4.3.2.1 AZT IMM administration

Milk profiles, on the other hand, showed interesting differences. Peak concentrations resulted much higher in mastitic animals (Fig. 13 A). $T_{1/2\lambda}$ and MRT resulted in the same order of magnitude in both groups and the differences lacked statistical significance. Areas under the curves, however, showed major differences. In the case of the healthy animals $AUC_{0-\infty}$ was in the order of 503.03 and $1615.65 \mu g.h.mL^{-1}$ in mastitic animals, the difference being statistically significant (Table 16). This difference in AUC was coincident with the differences found in serum, although these were not statistically significant.

Once again we found higher AZT concentrations in milk of mastitic animals than in milk of healthy animals after IMM administration, similarly as the situation occurred after IM

administration. As AZT is so penetrating in tissues and cells, and mastitic milk is so rich in somatic and inflammatory cells, the drug becomes included into the more acidic cellular compartment, thus hindering its participation in the milk-serum diffusion process (despite its pKa), and this retains high amounts of AZT in milk by cell trapping. All this evidence supports the fact that AZT is a penetrating azalide with concentrations several times higher in tissues and milk than in plasma. If we observe the ratios $AUC_{(milk)}/AUC_{(serum)}$ and we compare them with those obtained when the drug was administered intramuscularly we could conclude that the high availabilities in milk compared to those of serum are independent of the route of administration.

But, in the study with cows at drying-off, we found some differences: AZT milk concentrations in mastitic and healthy animals were similar (Fig. 13 B and Table 17). One explanation for this is that physiology of the mammary gland during the dry period differs markedly from that during lactation. Very few cells (less than 2% are epithelial cells) and total leukocyte concentration increase rapidly in early involution and the milk fat and casein may decrease the leukocytes phagocytic function.

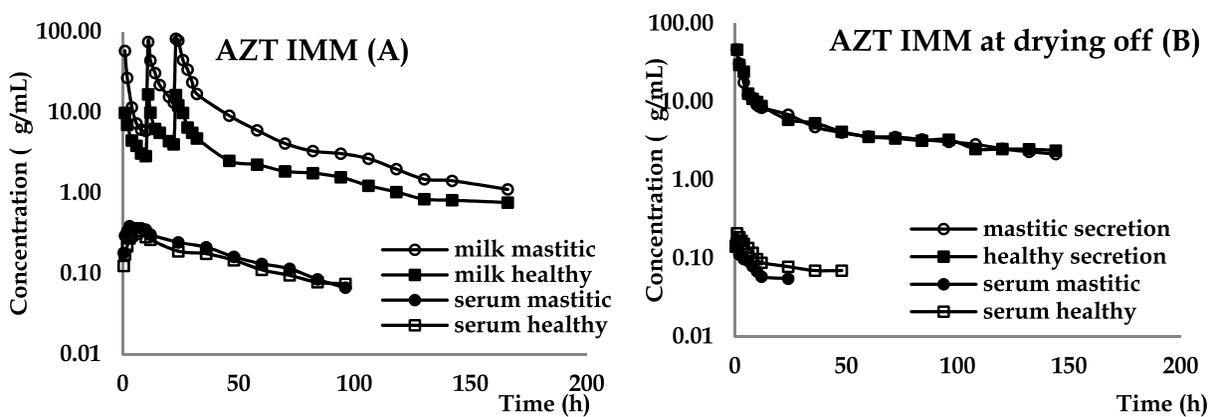


Fig. 13. Mean serum and milk AZT concentrations after one IMM AZT syringe containing 125 mg in each mammary quarter for three consecutive milkings in healthy and mastitic lactating cows (A). Mean serum and milk AZT concentrations after IMM syringe containing 500 mg in each mammary quarter of healthy and mastitic cows at drying off (B).

Finally, the excellent milk availability observed allows us to consider AZT as a potential antimastitic drug, although we have to fit the dosage through PK-PD modeling and corroborate with efficacy studies in the future. As was mentioned in previous paragraphs, the site where the pathogen is located represents one of the real challenges of ATM chemotherapy of the mammary gland. The pathogen can be in milk, or tissues. In the last case it can be in the interstitium or in cells. And in this case it can be in the cytoplasm or in fagolysosomes. As can be easily understood the deeper the location of the microorganism the more difficult will be to reach it by the ATM. Furthermore, if the ATM reaches the site of the microorganism, it has to exert its antibacterial effect and to do this it needs some special conditions, being the pH a critical one. And the pH becomes more acidic the deeper in tissues and cells it is measured. As illustrative figures we can mention the pH of plasma of 7.4, of interstitium 7.0, of cytoplasm 6.5 and of fagolysosome of 5.0. We evaluated the effect of the pH variation on the antibacterial activity of AZT against strains of *S. aureus* isolated of mastitic quarters. *S. aureus* strains isolated and *S. aureus* ATCC 25923 were tested at pH 7.4,

6.5 and 5.0, in order to simulate the conditions of acidity of plasma, tissue and subcellular structures which are commonly associated with *S. aureus* intracellular persistence. The results at pH 7.4, were consistent with those reported by CLSI 2008 (MIC: $1\mu\text{g}\cdot\text{mL}^{-1}$). However, MIC was approximately 16 times higher at pH 5.0 than at pH 7.4 (Fig. 14).

Parameter	Serum healthy	Serum mastitic	Milk healthy	Milk mastitic
λ_z (h^{-1})	0.078±0.04	0.050±0.02	0.01±0.00	0.02±0.00
$t_{1/2\lambda_z}$ (h)	11.76±7.31	16.20±7.20	59.67±12.48	46.85±20.87
$\text{AUC}_{0-\infty}$ (h.µg/ml)	3.51±2.25	2.74±1.11	503.03±49.47	1615.65±501.9
MRT (h)	17.37±10.56	24.69±11.20	76.37±18.32	52.41±28.39
T_{\max} (h)	22.75±0.27	22.67±0.26		
C_{\max} (µg/ml)	0.23±0.05	0.13±0.02		
F (%)	2.03±1.50	1.06±0.43		
$\text{AUC}_M/\text{AUC}_S$			143.31	589.65

Table 16. Serum and milk PK parameters obtained after one IMM AZT syringe containing 125 mg in each mammary quarter for three consecutive milkings in healthy and mastitic lactating Holstein cows

Parameter	Serum healthy	Serum mastitic	Milk healthy	Milk mastitic
λ_z (h^{-1})	0.02±0.01	0.08±0.03	0.01±0.00	0.01±0.00
$t_{1/2\lambda_z}$ (h)	34.73±10.96	10.18±4.77	107.60±27.72	99.09±28.46
$\text{AUC}_{0-t_{\text{last}}}$ (h. µg/ml)	3.53±1.28	1.35±0.81	760.00±196.3	747.16±223.94
$\text{AUC}_{0-\infty}$ (h.µg/ml)	6.73±2.31	2.12±1.14	1136.11±262.	1056.71±171.4
MRT (h)	15.09±3.15	6.87±1.82	131.18±35.31	122.51±46.32
T_{\max} (h)	1.25±0.61	2.08±2.18		
C_{\max} (µg/ml)	0.22±0.08	0.21±0.19		
F (%)	4.36±1.52	1.14±0.67		
$\text{AUC}_M/\text{AUC}_S$				

Table 17. Serum and milk pharmacokinetic parameters obtained after one IMM AZT syringe containing 500 mg in each mammary quarter of healthy and mastitic Holstein cows at drying off

4.3.3 Tylosin

Tylosin (TYL), other antibiotic of the macrolide group, is commonly used in food animal practice. Because it is an organic base ($\text{pK}_a = 7.1$), moderately bound by serum proteins (40%), with a high degree of lipid solubility (Lucas et al., 2007), TYL would be expected to be widely distributed in body fluids and tissues. The MIC of TYL for *S. aureus* was $<1\mu\text{g}\cdot\text{mL}^{-1}$ for most isolates studied. We determined the elimination milk profile of TYL after IM administration at multiple dose schemes.

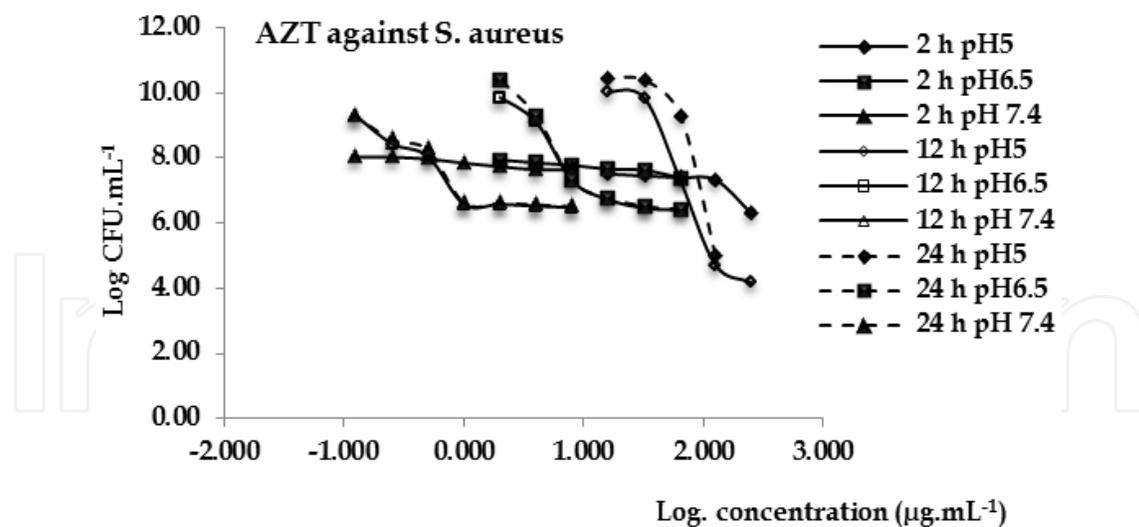


Fig. 14. Inhibition of *S. aureus* exposed to different concentrations (expressed as Log₁₀) of AZT (0.25 MIC; 0.5MIC; 1MIC; 2MIC; 4MIC and 8 x MIC) in function of the time and the medium pH (5; 6.5 and 7.4)

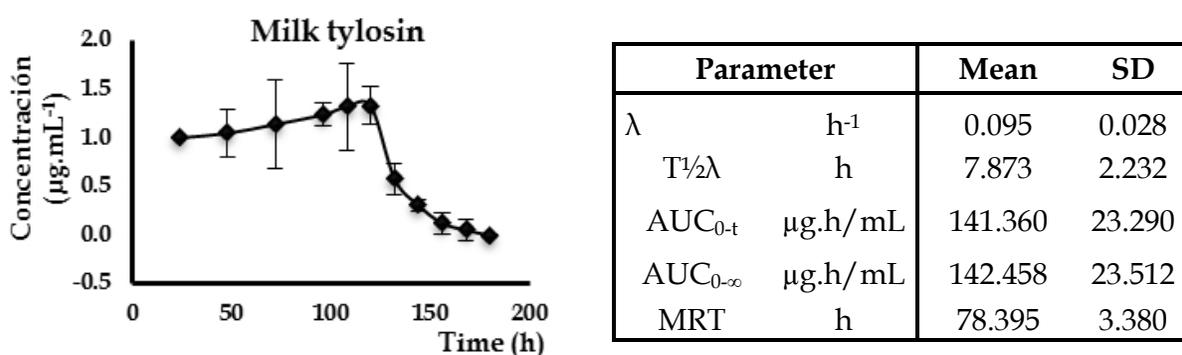


Fig. 15. Milk concentrations of TYL after its multiple (five doses every 24 hours) IM administration (10 mg.kg⁻¹)

The objective was to calculate the withdrawal time in milk necessary for TYL to reach acceptable limits for human consumption. Healthy Holstein lactating cows received 5 intramuscular doses of TYL at 10mg.kg⁻¹ every 24 hours (Figure 15). Milk samples were obtained from the four mammary quarters before the start of treatment, during and post-treatment every 12 hours until to 216 hours. The withdrawal time was calculated using the harmonized Time to Safe Concentration (TTSC) recommended by the European Union. The time required for milk to carry TYL concentration below the maximum residue limits (MRL 50 ppb) was 120 h post last TYL dose.

4.4 Tetracyclines

Oxytetracycline (OTC) is the most representative member of this class of ATMs. OTC is widely used in veterinary medicine worldwide. Its use is widespread due to its broad spectrum (not only against bacteria but also against some chlamydia, rickettsia and protozoa), and certain PK properties as its wide distribution throughout the body and prolonged therapeutic effects of some long-acting formulations. Our team has performed a

review of clinical aspects and major PK characteristics of the preparations commercially available (Mestorino & Errecalde, 1997). The PK of OTC has been studied by several authors (Schifferli et al, 1982, Errecalde, 1992; Errecalde et al, 1997). The new long-acting formulations have allowed the maintenance of high concentrations for periods much longer than the classical preparations, which represented a significant change in the practicality and in the usage habits, but also in maintaining residual levels for long periods in different tissues. Moreover, these formulations result in a significantly longer persistence of milk concentrations. The popularity of these preparations is extended to virtually all types of exploitation. Although the use of these long acting formulations is justified especially in extensive cattle exploitations, in other kind of management (i.e. more intensive), where the animals can be daily treated and controlled, is not justified. The use of tetracyclines with long elimination half-lives in dairy cows has impacted on repeated violations of maximum permitted levels of these antibiotics in milk. Problems that may emerge from the misuse of antibiotics are especially related to the presence of sub-therapeutic concentrations and the resultant emergence and dissemination of bacterial resistant strains in both animals and humans. Indisputably, the possibility of transference of portions of DNA carrying genes encoding resistance between bacteria is one of the issues of major concern to people with different responsibilities in the area. Direct toxicity must not be discarded.

Tetracyclines achieve milk concentrations that are approximately in the range of those of blood. They are second-choice parenteral antibiotics for serious infections of the udder caused by Gram positive organism and possibly by coliforms, although susceptibility among the later is controversial. In previous studies we evaluated the serum and milk PK behaviour of OTC after administration of therapeutic doses of three commercially available preparations at 5, 10 and 20 % by the IM route. In Figure 16 A and B, the plasma and milk OTC concentrations after IM administration of three different formulations is represented.

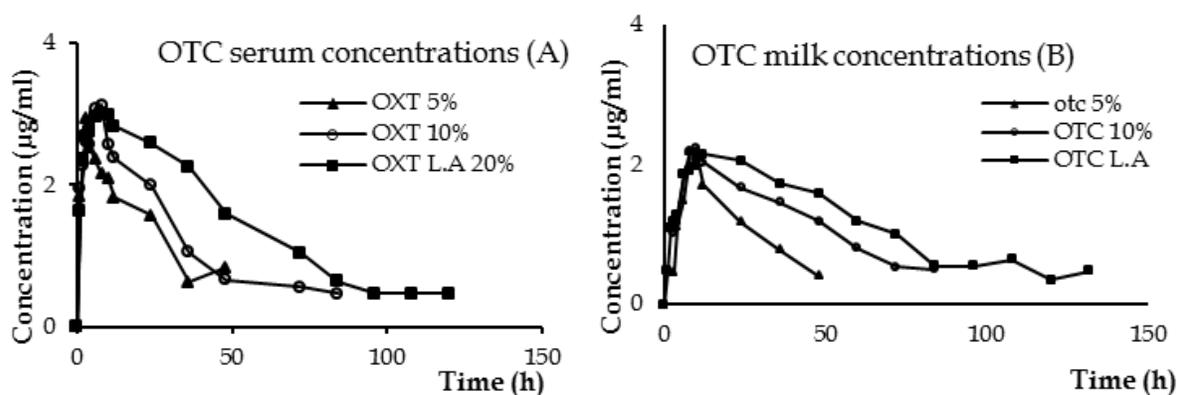


Fig. 16. Serum (A) and milk (B) OTC concentrations after IM administration of three different formulations to milk cows in production.

The 20% solution presented the higher area under the curve milk concentration versus time and the longest elimination half-life (Table 18). The withdrawal periods from milk for the 5%, 10% and 20% solutions resulted 3.5, 5 and 8 days respectively.

Addition of milk to Mueller-Hinton susceptibility test medium permitted measurement of milk effect on agar disc diffusion zone diameters obtained from *S. aureus* field isolates and

stock strains. Milk reduced the activity of different ATM agents, as novobiocin, streptomycin, gentamycin, tetracycline, and vancomycin (Owens & Watts, 1987). As was previously said, MIC is used in conjunction with PK data to determine the more appropriate surrogate PK/PD parameter. Problems arise in vivo, however, due to the physiological status of the host, site of infection, and properties of the ATM. Often, the concentration of active ATM at the infection site is quite different from that in serum or milk. Antimicrobial concentrations are lower in ischemic areas, scar tissue, and abscess contents. Also, pH, protein binding, and also normal metabolism and renal excretion mechanism reduce concentration of available ATM (Thornsberry and Sherris, 1985). Standard susceptibility tests measure ATM effectiveness in conditions extremely different from those in the udder.

Parameters	OXT 5%		OXT 10%		OXT 20%	
	Serum	Milk	Serum	Milk	Serum	Milk
	Mean±SD	Mean ±SD	Mean ± SD	Mean ±SD	Mean ±SD	Mean ±SD
C_{max} (µg.ml⁻¹)	2.94±0.74	2.46±0.49	3.37±0.54	2.35±0.27	3.39±0.43	2.30±0.33
T_{max} (h)	3±0	9.50±1.91	7.50±1.91	9.00±1.15	6.25±3.30	17.50±7.55
β (h⁻¹)	0.035 ±0.02	0.049±0.018	0.037±0.018	0.024±0.006	0.022±0.007	0.025±0.017
T^{1/2} β (h)	23.06±8.53	16.16±7.31	22.94±13.04	31.16±10.16	34.07±11.00	36.74±17.61
MRT (h)	33.68±12.01	29.97±11.96	33.35±16.07	51.88±16.19	52.21±11.85	64.94±19.57
AUC (µg.ml/h)	92.72 ±26.77	62.67±13.60	114.64 ±32.81	125.21 ±21.18	196.05 ±32.50	166.43 ±28.10
AUC₀₋₂₄ (µg.ml/h)		32.51±7.59		40.49±6.03		43.14 ± 6.88
C_{maxS}/C_{maxM}		1.19		1.43		1.47
AUC_M/AUC_S		0.68		1.09		0.85

Table 18. Serum and milk PK parameters obtained after administration of three OTC formulations in Holstein healthy lactating cows

In bovine mastitis, the unique environment of the mammary gland presents a problem for determining usefulness of chemotherapeutic agents against a given organism. Milk proteins, lipids, pH, and ionic characteristics can reduce concentration of active ATM (Ziv, 1980a). A simple in vitro method for determining the effect of milk on ATM activity could be of use when examining ATMs for possible use in mastitis therapy (Owens & Watts, 1987).

The MIC₉₀ of tetracycline (used as group representative) among *S. aureus* determined by different authors was between 0.5 - 1 µg.mL⁻¹ (Pol and Ruegg, 2007). If we consider than the MIC of tetracycline in milk could increase 4 to 32 times that in MH, the *S. aureus* mastitis treatment with OTC has not real possibilities of success, because the OTC concentrations reached in milk are insufficient for achieving the appropriate PK/PD predictors of therapeutic efficacy against *S. aureus* (AUC₂₄/MIC>100). An irreversible binding between tetracycline and large molecules of milk, which might be due to a hydrophobic interaction, was demonstrated by a dialysis test, suggesting the observed impairing effect was due to the action of milk on the tetracycline being tested. Further investigation revealed that much of the reduction of tetracycline activity in milk was attributable to the milk protein casein, while other heat-sensitive components in milk also play some roles (Kuang, et al., 2009).

4.5 Fluoroquinolones

Enrofloxacin (ENR) is a quinolone widely used for treatment of various infectious diseases in cattle caused both by Gram-positive and Gram-negative bacteria, but is not specifically recommended for bovine mastitis treatment; although high concentrations are reached and maintained in milk following parenteral administration. The MIC₉₀ value found by Russi et al., (2008) was similar than the reported for isolates from Uruguay and (Giannechini et al., 2002; San Martín et al., 2002).

4.5.1 Danofloxacin

Danofloxacin (DAF) is a fluoroquinolone (FQ) ATM drug developed for use in veterinary medicine. DAF shows a broad spectrum of activity against most Gram-negative, Gram-positive bacteria and mycoplasma, but has poor activity against anaerobes (Shojaee Aliabadi & Lees, 2003). FQ share some characteristics such as a broad spectrum of bactericidal activity, a large volume of distribution, low plasma protein binding and relatively low minimal inhibitory concentrations (MICs) against target microorganisms (Otero et al., 2001a; 2001b; Mestorino et al., 2009). Danofloxacin 18% was demonstrated to be effective in the treatment of bacterial pneumonia caused by *P. multocida*, *M. hemolytica* and *H. somnus* or bacterial enteritis (Mestorino et al., 2009) given as a single injection at a dose rate of 6 mg.kg⁻¹ of body weight, or two doses 48 hours apart, as needed. This formulation has the advantage of being safe and effective with a single dose or at maximum two doses, as handling animals many times for treatment is not practical. The concept of the high dosage in a single injection is that, after injection, the drug is available in high concentrations sufficient to kill all the sensitive bacteria during a relatively short period of time (Mestorino et al., 2009). This reduces the selection pressure for resistance. These characteristics suggest that it could be useful for the treatment of bovine mastitis caused by *S. aureus*. In order to investigate this possibility, the PK profile of DAF 18% was studied in plasma, milk and various tissues in dairy cows, when administered as a single subcutaneous injection at a dose of 6mg.kg⁻¹ (Fig. 17 A, B, C and D). DAF was rapidly absorbed and reached peak plasma concentrations of 0.53 µg.ml⁻¹ within 2 hours after injection. Distribution of DAF from plasma to all sampled tissues and milk was extensive (Table 19). Peak milk concentrations of 1.37µg.ml⁻¹ were achieved 8 hours post-injection. Maximum concentrations (9.73 µg.g⁻¹) in udder tissue (average of 4 quarters) and uterus (2.53 µg.g⁻¹) were achieved 6 and 4 hours after injection, respectively. Maximum concentrations in intestinal tract tissue and lymph nodes ranged from 3.6 µg.g⁻¹ (duodenum) to 10.22 µg.g⁻¹ (lymph nodes), and were reached between 2 h and 12 hours after injection. Plasma area under the curve (AUC_{pl}) was 9.69 µg.h/ml. Plasma elimination half life (T_{1/2β}) was 12.53 h. DAF has a very high volume of distribution (Vd = 5.79 l.kg⁻¹). AUC values for the various tissues and milk greatly exceeded AUC_{pl}. Elimination half life from milk and tissues varied between 4.57 hours to 21.91 hours and the milk withdrawal time was 73.48 h (Table 19). The reported results support the potential use of DAF in the treatment of mastitis and other infections in milk cows with three days of withdrawal.

Later we performed other study in cows with subclinical mastitis, who received 10 mg.kg⁻¹ subcutaneous (SC) 18% DAF(Fig. 18). The MIC₅₀ calculated for *S. aureus* isolated from

bovines with subclinical mastitis was $0.25 \mu\text{g.mL}^{-1}$ and the MIC_{90} was $1 \mu\text{g.mL}^{-1}$. Gram-negative bacteria have a MIC range of $0.05\text{-}2.5 \mu\text{g.mL}^{-1}$, while for Gram-positive bacteria it was $0.25\text{-}5 \mu\text{g.mL}^{-1}$ (Shem-Tov et al, 1998). Although, there are no references for DAF MIC against *S. aureus*, it has been proved that enrofloxacin MIC is $\geq 1 \mu\text{g.mL}^{-1}$ (Cester et al, 1992). The MIC_{90} was determined in order to calculate PK/PD parameters. Milk maximum concentration (C_{max}) was $2.99 \pm 0.88 \mu\text{g.mL}^{-1}$ (6.13 h post-administration) whereas plasma C_{max} was $1.45 \pm 0.26 \mu\text{g.mL}^{-1}$ (1.17 h post-administration). DAF was eliminated with mean half-lives of 7.56 ± 2.53 , and 4.43 ± 1.36 h from milk and plasma respectively. The mean area under the concentration versus time curve from 0 to 24 hours ($\text{AUC}_{0\text{-}24\text{h}}$) was $34.84 \pm 10.97 \mu\text{g.h/mL}$ in milk and $8.71 \pm 1.14 \mu\text{g.h/mL}$ in plasma.

Parameter	Plasma X	SD	Milk X	SD
β (h^{-1})	0.06	0.01	0.15	0.02
$T_{1/2} \beta$ (h)	12.53	1.47	4.57	0.46
K_{abs} (h^{-1})	1.46	0.91	0.32	0.06
$T_{1/2 \text{ abs}}$ (h)	0.64	0.39	2.27	0.48
MRT (h)	18.38	2.52	8.34	1.31
C_{max} ($\mu\text{g.mL}^{-1}$)	0.53	0.13	1.37	0.73
$C_{\text{maxM}}/C_{\text{maxPL}}$	---	---	2.68	1.35
$T_{1/2\beta\text{M}}/T_{1/2\beta\text{PL}}$	---	---	0.37	0.08
T_{max} (h)	2.17	0.98	8.67	2.07
AUC ($\mu\text{g.mL/h}$)	9.69	1.41	15.46	5.42
WT (h)			73.48	

Table 19. DAF PK parameters (mean \pm SD) obtained in plasma and after its subcutaneous administration (6 mg.kg^{-1})

When comparing PK parameters by grouping quarters by level of production, it was observed that DAF was eliminated more slowly from quarters of low-producing cows (Fig 18 B). Concomitantly, the MRT in milk from quarters of low-producing cows was higher than the MRT determined in milk from quarters of high-producing cows.

FQ are considered to have a concentration-dependent effect, although a time dependent bactericidal effect against some Gram-positive bacteria has also been described (Cester et al. 1996). DAF exhibited the same behavior against *S.aureus*, because it was strongly bactericidal at higher concentrations and when increases contact time. Importantly, the potency of DAF, evaluated by the MIC, is not affected by pH changes (Fig.19). The WT resulted longer in milk of low production than in milk of high production cows. Separating the groups according to the health status (Fig 18 A), it was observed that there were no statistically significant differences between the PK behaviour in both groups. The level of production had a significant effect ($P < 0.05$) on the milk elimination half-life ($T_{1/2\lambda}$) and on the mean residence time (MRT) of DAF in milk. The infection presence in the mammary quarters had a significant effect ($P < 0,05$) on T_{max} and MRT in milk. The incidence of infection and level of production on the PK of danofloxacin in milk cows have to be considered when designing dosage regimens for this drug.

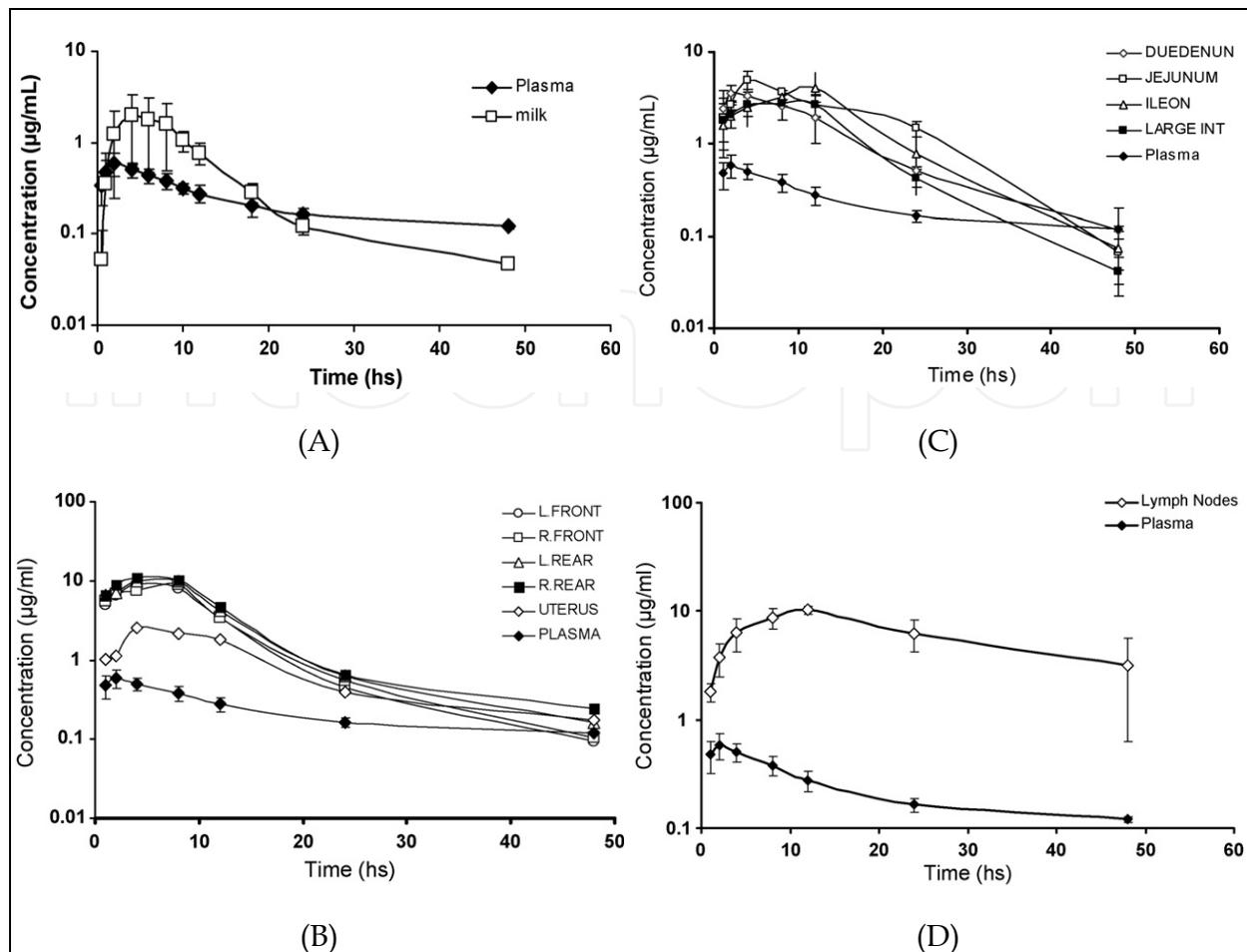


Fig. 17. Mean DAF plasma and milk concentrations (A). Mean DAF mammary, uterus and plasma concentrations (B), mean DAF duodenum, jejunum, ileum, large intestine vs. plasma concentrations (C) and mean DAF mesenteric lymph nodes vs. plasma concentrations (D), each after its SC administration in lactating cows

5. Perspectives for new therapeutic formulations

As described, *S.aureus* represents a major problem in bovine mastitis because of the poor cure rate despite the in vitro susceptibility of the acting bacteria. One possible reason for this is its intracellular location within the udder phagocytes, a place difficult to access for the majority of ATMss, which combines with reduced activity at the acidic pH of lysosomes. Other possible obstacle for antibacterial efficacy is the non-diffusion of acidic antibiotics through the lysosomal membrane due to their ionic presentation at extracellular (7.4) or cytoplasmic (6.5) pH and the very poor retention in cells of antibiotic which penetrate freely such lincomycins. Consequently, there is a clear need for more specialized dosage forms to be developed for use in the treatment of *S. aureus* bovine mastitis. Indeed, in view of the magnitude of the economic losses, every effort to develop new dosage forms should be undertaken. Such new formulations should be designed to counteract the causes of failure of antibiotic therapy for *S.aureus* and should have the following features (Gruet et al., 2001):

- Ability to penetrate phagocytes and to be retained in cells for adequate time;
- No substrate or low metabolism in the cells;

- Effective at low pH against *S. aureus*;
- Administration via local infusion through the teat canal

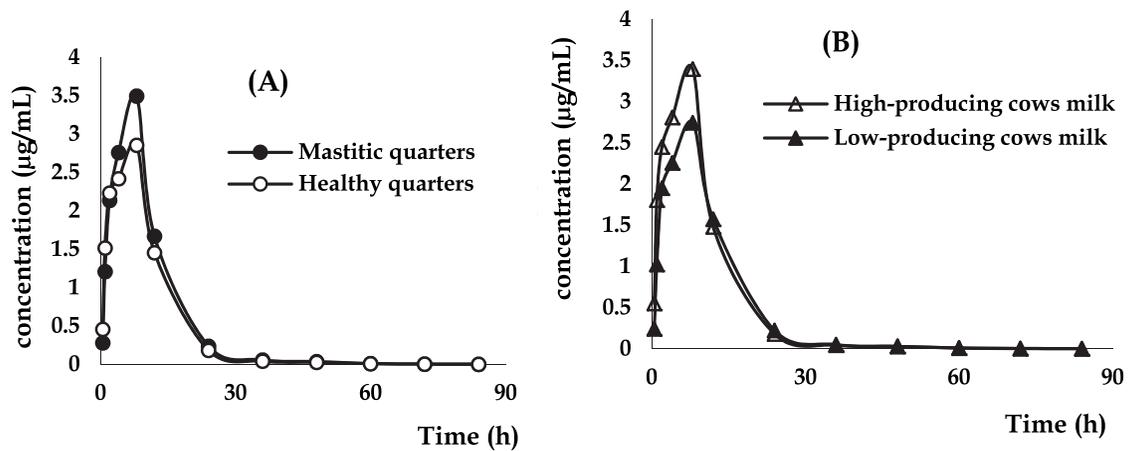


Fig. 18. (A) Mean concentrations of DAF in mastitic quarters and healthy quarters after 10 mg.kg⁻¹ IM dose (B) Mean plasma and milk concentrations of DAF in high-producing and low-producing cows after 10 mg.kg⁻¹ IM dose.

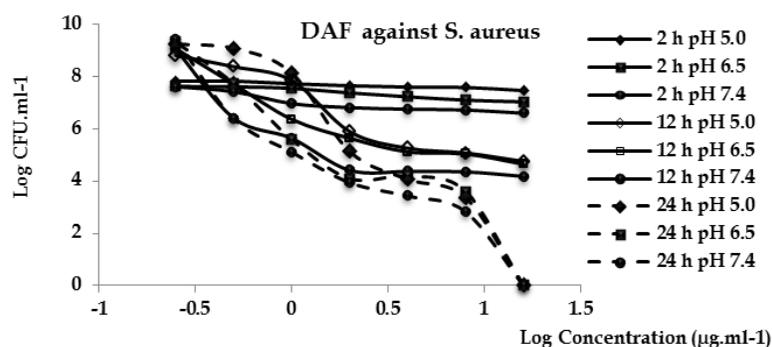


Fig. 19. Inhibition of *S. aureus* exposed to different concentrations (expressed as Log₁₀) of DAF (0.25 MIC; 0.5 MIC; 1 MIC; 2 MIC; 4 MIC and 8 x MIC) in function of the time and the medium pH (5; 6.5 and 7.4)

Delivery systems such as injectable microparticles or colloidal suspensions could fulfill some of the expectations for a new dosage form for IMM infusion. Microparticles are small, spherical particles with a diameter larger than 5µm, and can be made out of natural (gelatin or alginate) or synthetic material. Only the smallest could potentially be taken up by the phagocytes and therefore offer therapeutic value against intracellular bacteria. Colloidal suspensions include liposomes and nanoparticles. Both types are spherical particles with an average diameter of between 0.05 µm and <5µm. Liposomes are phospholipidic particles with an aqueous core. Due to their amphiphilic structure, they can incorporate either lipophilic or hydrophilic compounds. Nanoparticles are polymeric particles.

Thus liposomes, microparticles and nanoparticles may be considered potential delivery systems in the treatment of bovine mastitis by *S. aureus* since they may be taken up by the phagocytes liberating the active once inside. From the available literature, microparticles appear to be the most appropriate candidates for clinical trials in cows. Bodmeier et al. (1997) have reported the preparation of ceftiofur microparticles for the purpose of

administration to cows at drying off. The microparticles exhibited a good encapsulation ratio and were shown *in vitro* to release the drug slowly over a long period of time. This dosage form could be considered an example of possible paths to follow looking for suitable new therapeutic systems.

6. Conclusion

Milk producing cows are a very special kind of animal. An average good level of milk production is in the order of 5% of body weight daily, but everyday more “top” producers are rounding 10%. These are values that years ago were considered impossible to reach. The huge milk production increase was due to a very efficient process of selection. But these processes of increase of milk production pushed these animals to the border between physiology and pathology. In these conditions slight modifications in the diet, environment and management can result in defensive disequilibrium and disease. It has to be noted that the defensive aspects of the mammary gland did not receive the same attention than production itself, and, as a consequence, the higher the level of production, the higher the risks of mammary pathology. The mammary gland is a rather immunologically weak organ, the phagocytic activity in the mammary gland is poor. During the first few hours after milking, neutrophils coming into the gland are active, however, in a short time, they become to “engorge” with lipid molecules and their activity slows. At the end of the intermilking interval, phagocytes are almost inactive. Dilution of defensive elements in great producers is another factor. A special consideration has to be made when *S. aureus* is present. This bacterium has the ability of penetrate cells and to reproduce very slowly there. The majority of ATMs have serious difficulties to penetrate cells, and, if they reach the site where bacteria are, they fail acting against slowly growing organisms or low pH. The combination of these factors constitutes a complex problem. The knowledge of PK and PD of the different ATMs in consideration of the physiological characteristics of the mammary gland can contribute to a more rational use of ATMs. In the present paper we presented some data on the behaviour of some ATMs in milk producing cows. The consideration of the available data in a frame of prudent use surely will increase the number of therapeutically positive results with less possibilities of emergence and dissemination of resistant bacterial strains.

Antibiotic therapy is an essential component in programs of mastitis control, but does not replace preventive hygienic measures. A rational approach to ATM treatment involves knowing the different variables that influence the outcome of therapy. Accordingly, it is of fundamental importance to determine what type of etiological agents are predominant in each farm, which are their susceptibilities and what drugs can be used to combat them, having a special consideration on the penetration and tissue distribution in the mammary compartment, penetration into cells, and ATM activity once in the target place.

The increase in the knowledge of the microbiology and pathophysiology of mastitis by *S. aureus*, together with the new tools to model PK and PD of ATMs will contribute to the design of new administration systems and therapeutic plans with improvement of clinical and microbiological efficacy without the emergence and dissemination of resistant microorganisms.

7. References

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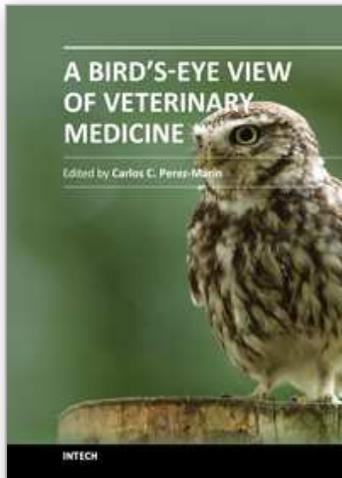
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Veterinary medicine is advancing at a very rapid pace, particularly given the breadth of the discipline. This book examines new developments covering a wide range of issues from health and welfare in livestock, pets, and wild animals to public health supervision and biomedical research. As well as containing reviews offering fresh insight into specific issues, this book includes a selection of scientific articles which help to chart the advance of this science. The book is divided into several sections. The opening chapters cover the veterinary profession and veterinary science in general, while later chapters look at specific aspects of applied veterinary medicine in pets and in livestock. Finally, research papers are grouped by specialisms with a view to exploring progress in areas such as organ transplantation, therapeutic use of natural substances, and the use of new diagnostic techniques for disease control. This book was produced during World Veterinary Year 2011, which marked the 250th anniversary of the veterinary profession. It provides a fittingly concise and enjoyable overview of the whole science of veterinary medicine.

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Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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