

Serum and milk concentrations of enrofloxacin in cows intramammarily treated with a new enrofloxacin-polymorph¹⁾

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Summary

The success rate in treatment against *Staphylococcus aureus* mastitis with antibacterial drugs is marginal. Low antibacterial drug concentrations in the mammary tissue is part of the reason for this failure. Enrofloxacin has only been administered on few occasions through the intramammary route i.e., combined with gentamicin in an undisclosed vehicle or dose. A new solvate form of enrofloxacin (enrofloxacin hydrochloride-dihydrate (enro-C)) prepared as 1.5% suspension pH 5.5 was tested in a field mastitis outbreak due to coagulase-negative *S. aureus* (CNS) in F1 (Holstein/Zebu) cows aged 5-8 years destined to be culled. Enro-C was administered intramammarily daily (300 mg/infected quarter) for 8 days both to healthy and mastitic cows; milk and serum enro-C concentrations were determined on days 1 and 8. Maximum serum concentration (C_{MAX}) values were similar in both groups (9.4 to 10.7 µg/mL). The area under the moment curve (AUMC) for enrofloxacin increased only by 11% in healthy cows and by 10% in infected cows from day 1 to day 8, a fact that suggests little accumulation with this dose regime. Peak concentrations in milk ranged from 18.5 to 19.8 µg/mL on day 1 and from 20.2 to 22 µg/mL on day 8. Cure rates on day 21 after treatment were 75% (12 out of 16 cows) or 69.4% (26 out of 36 glands). Somatic cell counts and California mastitis test showed a positive trend in cured animals. Uniquely high milk enrofloxacin and ciprofloxacin concentrations were obtained after intramammary administration of enro-C. These concentrations seem effective for treating CNS mastitis. The feasibility of incorporating this experimental pharmaceutical preparation of enrofloxacin is discussed.

Keywords: intramammary-administration, cow, enrofloxacin, enro-C, mastitis, *Staphylococcus aureus*

Therapeutic success rates in treating subclinical and clinical mastitis by *Staphylococcus aureus* are marginal. Sub-effective concentrations in milk and/or insufficient contact time with the affected mammary tissue are common causes for antibiotic failure (26). While parenteral enrofloxacin preparations are not usually recommended to treat bovine mastitis due to *Staphylococcus aureus*, high milk concentrations of enrofloxacin and its main metabolite (ciprofloxacin) are reached and maintained after parenteral administration (14). However, it must be considered that actual antibacterial activity can be partly impaired by the presence of milk and inflammatory secre-

tions (10). It is then likely to consider that higher concentrations of enrofloxacin and ciprofloxacin can improve clinical efficacy. This has been suggested when treating mastitis due to *Staphylococcus aureus* in sheep by means of parenteral administration of enrofloxacin (2). Intramammary administration may overcome the problem of insufficient concentrations of enrofloxacin in mammary tissue. However, this administration route is not recommended for standard preparations of enrofloxacin. A thorough search of the available literature revealed that enrofloxacin has been administered through the intramammary route on only few occasions i.e., combined with gentamicin in an undisclosed vehicle or dose (15). However, to the best of our knowledge, no commercial preparations

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for this route exist in most countries, and the available products for parenteral administration have a pH at or above 10.4; a fact that makes intramammary administration of the drug rather unadvisable (28). However, a new recrystallized enrofloxacin polymorphic form, enrofloxacin hydrochloride-dihydrate (enro-C) (19), has shown higher water solubility than the parent compound and a superior bioavailability than reference enrofloxacin in broiler chicken (12). As a 1.5% suspension, enro-C exhibits a pH of 5.5, and it does not irritate the mammary gland when intramammarily administered (17). Thus, a study to quantify milk and serum enrofloxacin and ciprofloxacin concentrations after intramammary administration of 300 mg of 1.5% enro-C aqueous suspension to cows was conducted. The efficacy against chronic *Staphylococcus aureus* mastitis was also assessed in a field outbreak.

Material and methods

Animals. All procedures were approved by the Institutional Committee for Research, Care and Use of Experimental Animals of the National Autonomous University of Mexico (UNAM), and conducted according to the Mexican Official Regulation NOM-062-ZOO-20 (http://dof.gob.mx/nota_detalle.php?codigo=762506&fecha=22/08/2001). Accordingly, all cows were slaughtered once the experiment was finished. This trial was carried out in Martínez de la Torre, Veracruz, a town in the Mexican tropic (20.0667 longitude; 97.05 latitude), in a dual-purpose dairy farm with F1 (50% Holstein-50% Zebu) cows in milk (mean production, 13.8 L/day) and mean weight of 625 ± 23.5 kg. Thirty-one cows from the herd were found infected with coagulase-negative *Staphylococcus aureus* (CNS). Intramammary infection (IMI) due to *Staphylococcus* sp. was regarded as chronic in these animals after repeated pathogen identification, as shown in Table 1, where other relevant features are also presented. None of the cows included in this trial were treated with an antibacterial drug for the current infection and had had their last antibacterial treatment at least a month ago. Chosen cows were divided by simple randomization through computer-generated random numbers, into two groups: 16 cows (16 glands infected) were treated with enro-C (Mi-Tx), and 15 cows (15 infected glands) were left untreated as controls (Mi-C). A third group of 12 cows was also treated with enro-C; these animals had no previous

history of *Staphylococcus aureus* IMI (Ni-Tx). Each group was relocated in different grounds, at least 1 km apart from each other, and were handled and milked in different settings to avoid cross-contamination.

Treatments. A batch of re-crystallized enrofloxacin was prepared as reported in Patent 472715 (Instituto Mexicano de la Protección Industrial, Mexico). This process yields enrofloxacin hydrochloride-dihydrate. Enrofloxacin was purchased from Globe Chemicals (Mexico City) with 99.97% purity. Enrofloxacin hydrochloride suspension (1.5% base) was prepared by slowly adding weighed amounts of polyvinylpyrrolidone K-30 and sodium chloride to a mixture of polypropylene glycol and cold injectable water with continuous stirring until complete dissolution. Then, enrofloxacin hydrochloride-dihydrate was added to the suspension, with stirring until complete dispersion; pH was adjusted to 5.5 with diluted hydrochloric acid, adding water to the final volume. The suspension was filtered through a 0.22- μ m pore membrane to yield a sterile, yellowish, transparent, particle-free liquid.

After the cows had been milked under aseptic conditions, the udder was washed, and each teat was cleaned thoroughly with a warm chlorhexidine-based antiseptic solution (Tetramast® Brovel, Mexico); teats were dried and the meatus was wiped with an alcohol-infused pad. Then, 300 mg of enro-C per affected quarter were deposited midway into the teat with a 20-mL intramammary, soft-plastic tip syringe, without massaging the gland. This procedure was repeated daily for 8 days in all treated cows regardless of age, days in milking period, milk production, and individual mastitis history.

Sampling. Pharmacokinetic data with a single dose of 300 mg/cow was obtained from 8 cows in the Mi-Tx group and 8 cows in the Ni-Tx group. Serial blood samples were obtained by direct jugular vein puncture with a Vacutainer kit and 20-gauge needles (10 mL/sample), at 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, and 24 h on day 1 and on day 8 of treatment (168.25, 168.5, 169, 170, 174, 176, 178, 180, 182 and 192 h). Serum was recovered by centrifugation at 3000 rpm for 15 min, labeled, and frozen at -20°C until analyzed. Milk samples were also obtained 2, 8, and 24 h after the first treatment, the last two sampling times coinciding with established milking times. Samplings were repeated on day 8 of enro-C intramammary treatment.

Bacteriological diagnosis. Additional milk samples (10 mL) were collected in sterile test tubes and immediately transported to the laboratory for microbiological analysis at $3-4^{\circ}\text{C}$. Each sample was vortexed for 5 min; then, 30 μ L of inoculant was spread in blood and on MacConkey agar plates and incubated under aerobic conditions at 37°C for 24-48 h (1). Colonies were classified according to morphological and biochemical characteristics, including Gram stain, catalase and coagulase activity, thermonuclease production, lysostaphin sensitivity, and anaerobic fermentation (glucose and mannitol) in accordance

Tab. 1. Characteristics (mean \pm SD and ranges) of the F1 (50% Holstein – 50% Zebu) dual-purpose cows included in this trial

Group	Age (years)	Lactation number	Days in milk	Individual milk production (kg)	Previous mastitis cases*
Mi-Tx (16 cows)	6.3 ± 1.0 (5-8)	4.3 ± 1.0	174 ± 16	2088 ± 152	4.2 ± 1.4 (3-6)
Mi-C (15 cows)	6.3 ± 1.3 (5-8)	4.3 ± 1.3	168 ± 19	2010 ± 187	4.6 ± 1.3 (2-7)
Ni-Tx (12 cows)	6.6 ± 1.1 (5-8)	4.6 ± 1.3	178 ± 20	2848 ± 210	0.5 ± 0.5 (0-1)

Explanations: * Diagnosed as *Staphylococcus aureus* IMI during the last lactation period; Mi-Tx – *S. aureus*-infected, enro-C-treated; Mi-C – *S. aureus*-infected, untreated; Ni-Tx – non-infected, enro-C-treated.

with established protocols (4). Samples were processed in triplicate.

Two additional samples were collected for the California mastitis test (CMT) and for somatic cell count (SCC) by an electronic cell counter (DeLaval DCC®, Sweden). Bacteriological diagnosis, CMT, and SCC were repeated 14 and 21 days after the eight-day treatment scheme with enro-C, to assess whether or not a bacteriological cure was attained.

Cows included in this trial had at least four previous diagnoses of IMI due to *Staphylococcus* sp. in the current lactation cycle. Intramammary infection was defined as positive when *Staphylococcus* spp. was isolated and quantified as ≥ 30 CFU/30 μ L of inoculum in at least 2 out of 3 replicates (National Mastitis Council. Research Committee Report. <http://nmconline.org/docs/InterpretBactResults.pdf> (1987)). Cure criterion was the absence of the pathogen in two consecutive milk samplings, 14 and 21 days after the last treatment. Table 2 shows the biochemical tests identifying the mastitis causative microorganism as coagulase-negative *Staphylococcus aureus*.

Chromatographic enrofloxacin and ciprofloxacin determination. Enrofloxacin and ciprofloxacin determination in milk and serum samples was based on an HPLC method (13). Briefly, each sample was added with 1 mL of methylene chloride and centrifuged for 5 min at 10,000 rpm. Aqueous phase was discarded and organic phase was evaporated. Residues were reconstituted in the acetonitrile: methanol: water (17: 3: 80) mobile phase with phosphoric acid (4% v/v) and trimethylamine (4% v/v). The aqueous extract was analyzed by high-performance liquid chromatography (HPLC) with fluorometric detection at $\lambda_{ex} = 278$ nm and $\lambda_{em} = 460$ nm. A Jasco XLC (LC-2000Plus® JASCO Benelux B. V., de Meern, Netherlands) HPLC system with a Symmetry-C₁₈ column (4.6 mm \times 100 mm, 3.5 μ m; Waters) was used. Injection volume was 50 μ L and flow was 0.6 mL/min. Data were analyzed with the EASYCHROME 2.0 software. Sarafloxacin hydrochloride was included as internal standard. The standard calibration curve was obtained by injecting five-level concentrations ranging from 0.05 to 20 μ g/mL and measuring the peak areas of the chromatograms. The recovery percentages ranged from 95 to 104%, with a maximum of 9% standard deviation. The lowest possible standard on the calibration curve was accepted as the limit of quantitation (LOQ). LOQ for enrofloxacin and ciprofloxacin in plasma was found to be 0.1 μ g/mL. LOQ for enrofloxacin and ciprofloxacin in milk was found to be 0.2 μ g/mL.

The following pharmacokinetic variables were calculated with the Microsoft Excel (© 2013 Microsoft, USA) and WinNonlin (WinNonlin® Princeton, NJ, USA) software: absorption half-life ($T_{1/2ab}$); elimination half-life ($T_{1/2\beta}$); maximum serum concentration (C_{MAX}); time to achieve C_{MAX} (T_{MAX}); area under the curve by the trapezoidal method (AUC_t); area under the moment curve from zero to ∞ with extrapolation to the terminal phase (AUMC); mean residence time (MRT).

Statistical methods. Milk data are reported as mean and SD. Enrofloxacin and ciprofloxacin kinetic profiles were analyzed by ANOVA of repeated measurements. Post-treatment data for the presence of *S. aureus* in Mi-Tx and

Tab. 2. Biochemical features used to identify coagulase-negative *Staphylococcus aureus* in this study trial

Test	Response
Catalase activity	4+
Coagulase production	–
Thermonuclease production	+
Lysostaphin sensitivity	+
Anaerobic utilization of glucose	+
Mannitol	+

Mi-C cows, California scores, and SCC on days 14 and 21 were also recorded. CMT and SCC data were analyzed by ANOVA for repeated measurements to determine differences between baseline and post-treatment values due to treatment.

Serum pharmacokinetic parameters for enrofloxacin and ciprofloxacin in Mi-Tx and Ni-Tx cows, and on days 1 and 8 of treatment, were compared using TTOST (two *t*-tests of single tail) with δ -values not higher than 10%; $P < 0.05$ was considered as significant. Post-treatment response in Mi-Tx and Mi-C cows, with two possible responses (positive or negative), was assessed with the generalized linear model:

$$g(E(Y_i)) = g(\mu_i) = x_i'\beta$$

Where

$\mu_i = (E(Y_i))$ is a linear function relating the response means of linear predictor $x_i'\beta$

x_i' is the vector of independent variables for the *i*-th observation, and

β is the estimable regression parameters vector

Y_i component is independent and has a binomial probability distribution, since only two possible responses exist (*i* = either positive or negative).

Test potency was determined with the GPower software (Buchner, A.; Erdfelder, E. and Faul, F. (1997) How to Use G*Power. URL:http://www.psych.uniduesseldorf.de/aap/projects/-gpower/how_to_use_gpower.html (Last consulted July, 2015)). All other statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS) for Windows, Version 20 (IBM, Mexico City) (Insurgentes Sur 933, piso 1. México, 03810. mexico@pass.mx).

Results and discussion

Table 3 shows the characteristics of the cows in the three groups, the number of glands and cows treated, CMT and SCC values before and after treatment, and the rate of bacteriological cure on days 14 and 21 after treatment for Mi-Tx and Mi-C cows. Cure rate, SCC, and CMT mean values show significant differences due to treatment ($P = 0.001$). A 75% cure rate was attained (12 out of 16 cows), which represent 69.4% cured glands (25 out of 36). A G*Power analysis of these results showed a potency of 0.99. The mastitis cure rates, the values of CMT and the SCC were all noticeably reduced after treatment with enro-C.

Figures 1 and 2 show mean \pm SD for ciprofloxacin and enrofloxacin serum concentrations on days 1 and 8 in Mi-Tx and Ni-Tx cows, respectively. Serum pharmacokinetics for enrofloxacin and ciprofloxacin in

Tab. 3. Groups formed: Mi-Tx – *Staphylococcus aureus*-infected, enro-C-treated; Mi-C – *S. aureus*-infected, untreated; Ni-Tx – non-infected, enro-C-treated. California mastitis test (CMT) and somatic cell count (SCC) before and after treatment are shown, as well as the percent rate of bacteriological cure on days 14 and 21 in Mi-Tx and Mi-C cows

Group	No of cows (glands) infected	Before treatment*		After treatment*		Bacteriological cure (%)	
		Mean CMT score	Mean SCC (× 1000 cells/mL)	Mean CMT score	Mean SCC (× 1000 cells/mL)	Day 14	Day 21
Mi-Tx	16	2.75 ± 0.44	2212.5 ± 650	1.31 ± 0.47	209.8 ± 75	75 (12/16)	75 (12/16)
Mi-C	15	2.8 ± 0.4	2096 ± 695	2.9 ± 0.6	2950 ± 560	0	0
Ni-Tx	12	0.4 ± 0.5	178.5 ± 67	0.75 ± 0.62	198 ± 69	NA	

Explanations: NA – Not applicable. * Cows were treated with a single intramammary aqueous suspension of 300 mg of enrofloxacin hydrochloride-dihydrate (pH 5.5) per day, for 8 days.

these groups are presented in Table 4. No statistically significant differences in pharmacokinetic parameters were found between Mi-Tx and Ni-Tx cows. C_{MAX}

mL·h²), being the difference statistically significant ($P < 0.05$). Levels in milk did not show significant differences between healthy and mastitis-infected cows,

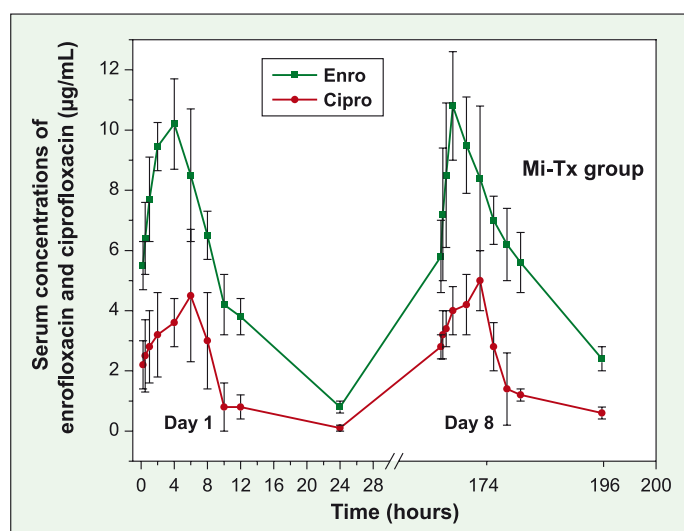


Fig. 1. Serum concentrations of enrofloxacin (enro) and ciprofloxacin (cipro) on day 1 and day 8 after intramammary administration of 300 mg of enrofloxacin hydrochloride-dihydrate (pH 5.5) in cows with mastitis caused by coagulase-negative *Staphylococcus aureus* (Mi-Tx group)

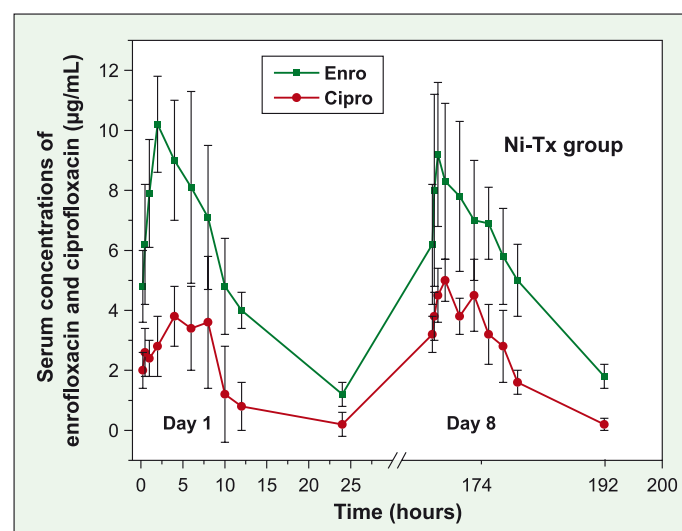


Fig. 2. Serum concentrations of enrofloxacin (enro) and ciprofloxacin (cipro) on day 1 and day 8 after intramammary administration of 300 mg of enrofloxacin hydrochloride-dihydrate (pH 5.5) in healthy cows (Ni-Tx group)

Tab. 4. Serum pharmacokinetic parameters for enrofloxacin and ciprofloxacin in cows treated with a single intramammary aqueous suspension of 300 mg of enrofloxacin hydrochloride-dihydrate. Cows were grouped as non-infected, enro-C-treated (Ni-Tx) and *Staphylococcus aureus*-infected, enro-C-treated (Mi-Tx group)

	Ni-Tx group				Mi-Tx group			
	Enro day 1	Enro day 8	Cipro day 1	Cipro day 8	Enro day 1	Enro day 8	Cipro day 1	Cipro day 8
$T_{1/2\beta}$ (h)	2.8	2.6	3.1	2.2	2.7	2.4	2.4	3.1
$T_{1/2ab}$ (h)	0.5	0.6	–	–	0.6	0.5	–	–
T_{MAX} (h)	3.4	3.1	3.1	1.4	3.3	3.3	1.8	1.0
C_{MAX} (µg/mL)	10.2	10.7	4.0	5.1	9.4	10.3	1.6	19.1
AUCt (µg/mL·h)	42.5	45.8	24.0	26.7	41.7	43.0	22.6	23.1
AUMC (µg/mL·h ²)	55.5	58.7	27.6	28.2	56.3	59.1	24.1	26.1
MRT (h)	6.8	8.4	8.8	12.2	6.7	9.5	15.6	17.7

Explanations: n = 8 cows/group; $T_{1/2ab}$ – absorption half-life; $T_{1/2\beta}$ – elimination half-life; C_{MAX} – maximum serum concentration; T_{MAX} – time to achieve C_{MAX} ; AUC – area under the curve by the trapezoidal method; AUMC – under the moment curve from zero to ∞ with extrapolation of the terminal phase; MRT – mean residence time. Considering TTOST analysis, the null hypothesis of differences between day 1 and 8 was rejected for the same analyte.

Tab. 5. Mean \pm SD milk concentrations of enrofloxacin and ciprofloxacin ($\mu\text{g/mL}$) after daily intramammary treatment of enrofloxacin hydrochloride-dihydrate for 8 days, at a dose of 300 mg/quarter (1.5% suspension, pH 5.5). Concentrations were determined in milk from the treated quarter only. Cows were grouped as non-infected, enro-C-treated (Ni-Tx), and *Staphylococcus aureus*-infected, enro-C-treated (Mi-Tx group)

Day	Hours after treatment	Enro Mi-Tx group		Cipro Mi-Tx group		Enro Ni-Tx group		Cipro Ni-Tx group	
		\bar{x}	\pm SD	\bar{x}	\pm SD	\bar{x}	\pm SD	\bar{x}	\pm SD
1	2	10.4	2.5	2.3	0.8	11.0	3.2	3.2	1.2
	8	19.8	2.8	4.9	1.2	18.5	2.5	5.2	2.2
	24	3.1	1.2	0.8	1.0	4.5	3.4	1.4	2.0
8	2	11.4	3.0	3.5	1.4	14.0	2.8	3.8	1.6
	8	20.2	3.4	5.6	0.8	22.0	1.2	5.9	1.8
	24	4.2	1.2	1.6	1.6	5.2	0.8	2.0	0.8

Explanations: n = 8 cows/group. No statistically significant differences were observed, comparing either enrofloxacin columns or ciprofloxacin columns ($1 - \beta = 0.83$, GPower 3.1.5).

with peak concentrations of 18.5-19.8 $\mu\text{g/mL}$ on day 1 to 20.2-22 $\mu\text{g/mL}$ on day 8. Figure 3 and Table 5 show mean \pm SD enrofloxacin and ciprofloxacin milk concentrations on days 1 and 8 after treatment.

The physicochemical characteristics of enro-C include higher water solubility than that of the parent molecule (19). Yet, this feature was not sufficient to make it possible to prepare a solution rather than a suspension of 300 mg of enro-C in 20 mL. Hence, the pharmaceutical preparation was conceived as a stable suspension with a particle size smaller than 0.22 μm . Nevertheless, the higher water solubility of enro-C and particle size achieved in the experimental suspension, may have accounted for the rapid absorption phase observed ($T_{1/2ab} = 0.5\text{-}0.6$ h), which then resulted in a distinctly high C_{MAX} . The total dose administered was 300 mg, and this is only a fraction of the standard dose used in pharmacokinetic studies (5 mg/kg i.e., approximately 3,000 mg/cow weighing 600 kg). As no previous reference to a dose through the mammary route is available in the literature, a relatively high intramammary dose was chosen in an attempt to comply with high concentrations of enrofloxacin at this target site. In agreement with absorption, elimination half life ($T_{1/2\beta}$) ranged from 2.4 to 2.8 h, a shorter value than previously reported for the standard preparation of enrofloxacin (approximately 6 h) (14).

It is interesting that AUMC was not noticeably increased at day 8 of medication compared to day 1 (approximately 10-11%). This parameter is in agreement with MRT values found in this study (from 6.8 to 9.5 for enrofloxacin and from 8.8 to 17.7 for ciprofloxacin), which are slightly faster than previously reported ones (14, 16). Intramuscular administration of a standard preparation of enrofloxacin at a dose of 5 mg/kg results in a MRT of 7.8 h (31). Additionally, considering that AUMC is used for assessing the extent of the distribution of a drug at steady state, the referred lack of substantial increment from day 1 to day 8 suggests that a good distribution outside the central compartment occurs on days both 1 and 8. In fact, a certain amount of reentrance into the mammary gland may be

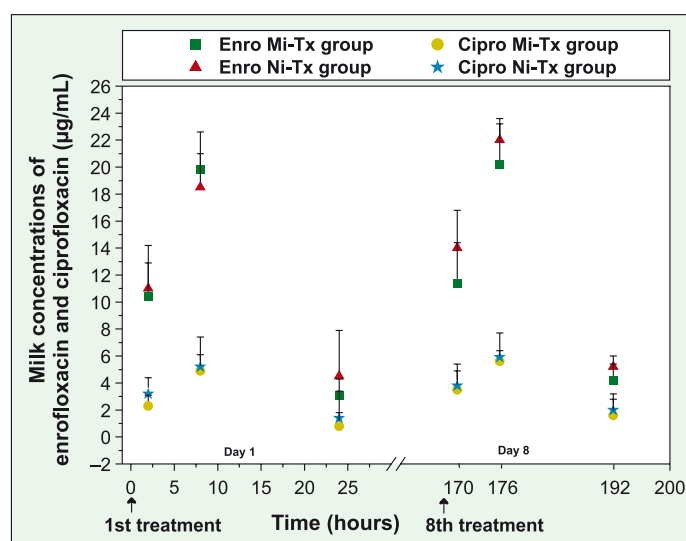


Fig. 3. Mean \pm SD milk concentrations of enrofloxacin (enro) and ciprofloxacin (cipro) on days 1 and 8 after daily intramammary treatment with 300 mg of enrofloxacin hydrochloride-dihydrate (pH 5.5) for 8 days. Treatments were applied to either *Staphylococcus aureus*-infected quarters (Mi-Tx group) or healthy ones (Ni-Tx group)

occurring, considering that the peak level in milk was augmented from 19.8 $\mu\text{g/mL}$ on day 1, to 22 $\mu\text{g/mL}$ on day 8. However this difference is not statistically significant, suggesting a first-order elimination kinetics of enrofloxacin through milk. In Figure 3 it is possible to observe that concentrations of enrofloxacin in milk after 24 h are approximately 3 $\mu\text{g/mL}$ in Mi-Tx cows and 4.5 $\mu\text{g/mL}$ in Ni-Tx cows. In spite of enrofloxacin being a switterion molecule (30), the slight changes that occur in mammary tissue and fluids may be part of the explanation for this difference, as healthy tissue is more acidic than infected one. The same seems to apply to ciprofloxacin. Again, statistically significant differences were not obtained, which is consistent with the proposal of a first-order elimination kinetics. This observation requires confirmation with formal drug-residue depletion studies. For example, this study was carried out in F1 (50% Holstein-50% Zebu) cattle, and these animals behave in a more unpredictable and wayward manner than Holstein cows, making

it difficult to have a more constant milk production. Also, the cows in this study lived in a tropical environment with outstanding variation in weather conditions and consequently in their drinking and eating habits. Nevertheless, a relevant issue that can be stressed in these data is that very large concentrations of enrofloxacin and ciprofloxacin are obtained, even 24 h after the intramammary administration of enro-C. Furthermore, the added antibacterial contributions of enrofloxacin and ciprofloxacin should result in a considerably higher antibacterial activity than the sum of their independent antibacterial effects (18, 26). This is particularly important, considering that only 2 to 3 µg/mL of enrofloxacin are found in milk after a standard dose of enrofloxacin, injected IM (13, 14), and that cow's polymorphism can be a clinically relevant factor affecting the concentrations of enrofloxacin and ciprofloxacin in milk after parenteral administration of the drug (23).

It has been stated that the use of enrofloxacin requires specific plasma pharmacokinetic/pharmacodynamic (PK/PD) ratios to optimize clinical efficacy i.e., C_{MAX} should be 10-12 times the value of the minimal inhibitory concentration (MIC), and the ratio AUCt/MIC should be equal to or higher than 125 (31). Considering that the *S. aureus* breakpoint for enrofloxacin is 0.25 µg/mL (8, 21, 26), the usually obtained concentrations of enrofloxacin and ciprofloxacin after the IM administration of enrofloxacin (5 mg/kg) would seem sufficient for clinical efficacy (18, 26). However, if higher milk concentrations are sought, such as in the case of enrofloxacin, a concentration-dependent antibacterial drug (2), intramammary administration of enro-C can prove useful. Taking only serum concentrations of enrofloxacin on day 1 in this study and 0.25 µg/mL as the breakpoint for *S. aureus*, PK/PD ratios would be: AUCt/MIC = 170 and $C_{MAX}/MIC > 40$. If the maximum concentration of enrofloxacin obtained for milk 8 h after a single 300 mg/gland dose is considered (19.8 µg/mL), the $C_{MAX \text{ in milk}}/MIC$ ratio would be 79. This latter ratio points to a clear advantage of intramammary administration of enro-C over the IM administration of enrofloxacin. Furthermore, the direct $C_{MAX \text{ in milk}}/MIC$ ratio of enrofloxacin and ciprofloxacin based on concentrations as obtained by HPLC may underestimate their combined antibacterial activity *in vivo*. In this study, the mean production of ciprofloxacin was 30% of the actual concentration of enrofloxacin in both mastitis-affected and healthy cows, increasing to 38% in the last sample taken 24 h after administration of enro-C. These values are lower than previously reported data for cows (59%) (26). Different routes of administration and doses used in these studies, as well as cow breeds used, and a shorter elimination half-life found for enro-C in this study, may account for these discrepancies.

There is no clear explanation why C_{MAX} obtained in this study was much higher than the corresponding values found in other studies after the administration

of enrofloxacin through the IM route. A rapid absorption of enro-C through the mammary tissue can be advanced. This has been shown for florfenicol in lactating cows, with a three-fold increment in C_{MAX} values (from 2.3 to 6.9 µg/mL) (27). Also, higher C_{MAX} values for enro-C have been shown after oral administration in broiler chickens (12) and after IM administration in hamsters (6). Enro-C can be described as a polymorphic enrofloxacin, and it is not rare to obtain better pharmacokinetics with a re-crystallized polymorph of an active principle (7, 26); for example: rifaximin- α , a crystal polymorph, is much more bioavailable than the parent molecule (5). The higher water solubility of enro-C compared to standard enrofloxacin, and the manner in which nanoparticles are obtained in the referred preparation, may also be part of the explanation.

The most insidious and challenging etiology causing bovine mastitis is *Staphylococcus aureus* (3, 8, 11, 26). The more than 50 species and subspecies of this pathogen are usually divided into coagulase-positive and coagulase-negative *S. aureus*, the former being more virulent and prone to develop bacterial resistance (3, 8). These bacteria colonize many environments and make part of the cutaneous and mucosal flora in domestic animals and humans. *S. aureus* adheres to mammary epithelial cells, endothelial cells, and fibroblasts. These bacteria often enter the cytoplasm, escaping phagosomes and remaining in vacuoles, a process that facilitates apoptosis (11). Attempts to cure herds with this type of mastitis are a high economic investment with a low success rate (22, 29, 30). Thus, although concentrations of enrofloxacin obtained in this study seem effective for treating mastitis caused by coagulase-negative *Staphylococcus aureus*, further evaluation is needed to determine whether such a long treatment protocol is economically viable and whether treatment with parenteral enrofloxacin has a comparable clinical efficacy. Maintaining the sample size for pharmacokinetics analysis, impeded this latter comparison.

Novel approaches have been proposed to improve the prognosis of *S. aureus* mastitis i.e., the extended therapy with multiple infusions of pirlimycin (9) ceftiofur (22) or cefquinome (29). However, to the best of our knowledge there has been no attempt to treat *S. aureus* mastitis with enrofloxacin, either by the parenteral route or by the intramammary one. Among the reasons to avoid this latter route is the high pH of pharmaceutical preparations (10.4-11), usually achieved by adding KOH as vehicle in most available preparations (28). Clearly, this makes intramammary administration unwise. In contrast, the pH of the 1.5% enro-C suspension used in this trial was 5.5, and it proved to be non-irritant to the mammary gland as assessed by direct clinical inspection, herein and in a previous study (17). Nevertheless, additional studies are required to validate this observation. The 75%

efficacy with a daily use of intramammary enro-C (300 mg/quarter/day) for 8 days ranks among the best results found in the formal literature, particularly considering that cows included in this trial were old (5-7 years), and tissue damage must have already been well established (3). While the main objective of this study was to assess serum and milk enro-C concentrations after intramammary administration, a natural outbreak of mastitis due to coagulase-negative *S. aureus* allowed us to quantify treatment efficacy. Hence, any comparison with other studies in larger herds and different clinical settings should be treated with caution. For example, this trial was conducted in a tropical area with dual-purpose cattle. Nevertheless, it is worth emphasizing that these infections are in most cases refractory to routine intramammary antimicrobial therapy and tend to spread quickly, particularly in dairies with poor milking protocols (3).

Further studies are required before this antibacterial drug can be used in clinical practice, such as studies of potential toxicity of the chosen dose of intramammary enro-C to mammary cells and epidemiological pertinence of incorporating enrofloxacin as intramammary medication in terms of bacterial resistance. Regulations on the use of antibacterial drugs vary with each country. Thus, at this stage, a universal, epidemiological point of view on the use of enro-C is not possible. However, it is worth pointing out that the high concentrations of enrofloxacin and ciprofloxacin obtained in milk with enro-C are likely to reach the so-called mutant prevention concentrations (20, 23), thus reducing drastically the emergence of resistant strains. Also, if a pharmaceutical preparation of enro-C becomes available, time to reach the maximum residue limit in milk (MRL) must be established.

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