Serum pharmacokinetics and tissue concentrations of a new recrystallized enrofloxacin hydrochloride-dihydrate in hamsters

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Abstract: Pharmacokinetics (PK), pharmacodynamics (PD), and PK/PD ratios of a new solvate of enrofloxacin (enrofloxacin hydrochloride-dihydrate; enro-C), were studied in hamsters. Enrofloxacin from Baytril® 5% (enro$_R$) served as the reference preparation. Two groups of 60 Syrian golden hamsters were intramuscularly injected individually with 10 mg/kg of enro$_R$ or with enro-C. Tissue and serum samples were obtained for 72 h; enrofloxacin concentrations were determined by HPLC with UV detection. Ninety percent minimum inhibitory concentrations (MIC$_{90}$) were determined for strains of methicillin-resistant *Staphylococcus aureus*, *Leptospira interrogans*, and *Escherichia coli*. All PK variables were statistically different between groups (P < 0.01). C$_{MAX}$ of enrofloxacin for enro-C was 17.3 µg/mL and it was 2.6 µg/mL for enro$_R$. AUC was considerably higher for enro-C (459.2 µg/mL h vs. 19.9 µg/mL h). There were no statistically significant differences in MIC$_{90}$ values between enro$_R$ and enro-C. Tissue concentrations of enro-C in all cases were higher and remained above the MIC for longer periods than those of enro$_R$. Relevant PK/PD ratios for enrofloxacin (AUC/MIC ≥ 125 and C$_{MAX}$ > MIC = 10–12) are consequently superior for enro-C. Given the outstanding PK/PD ratios of enro-C, this new moiety is proposed as a possible solution when high tissue concentrations of enrofloxacin are necessary.

Key words: Enrofloxacin, enro-C, hamster, pharmacokinetics/pharmacodynamics, tissue concentrations, *Staphylococcus aureus*, *Leptospira interrogans*, *Escherichia coli*

1. Introduction

Enrofloxacin (1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3 quinolone carboxylic acid) is an antimicrobial agent that belongs to the group of improved synthetic 6-fluoroquinolones (1,2). It has a broad spectrum of antibacterial activity and is one of the most used antibacterials in veterinary medicine. The interest of the pharmaceutical and medical communities in fluoroquinolones has not decreased during the last 10 years, and many new derivatives have been developed or are under investigation. Traditionally, it has been advanced that chemical substitutions in positions 6, 7, and 8 of the quinolone core group result in only subtle differences in antimicrobial activity (2,3). Hence, clear advances among modern fluoroquinolones are inconspicuous at best. As far as enrofloxacin in veterinary medicine is concerned, important differences in bioequivalence in various domestic species (4–7) may contribute to the appearance of bacterial-resistant strains, given that many pharmaceutical preparations fail to reach the optimal peak concentrations required for a proper pharmacokinetic/pharmacodynamics (PK/PD) ratio.

Enrofloxacin is a faintly yellowish to light yellow crystalline substance soluble in KOH and glacial acetic acid and only slightly soluble in water at pH 7 (0.6 mg/mL) (8). In solution and depending on the pH of the media, enrofloxacin can exist in 4 possible forms: as an acidic cation, a neutral unionized species, an intermediate zwitterion, and a basic ion. At physiological pH, this drug is lipid-soluble and therefore shows good penetration to tissues (3,9). Optimal clinical efficacy of enrofloxacin has been linked to specific PK/PD ratios, i.e. maximum serum concentration (C$_{MAX}$) should reach at least 10–12 times the value of the minimal inhibitory concentration (MIC) (C$_{MAX}$ > 10–12 MIC), and the area under the serum concentration vs. time curve (AUC$_{0-24}$)/MIC should be equal to or higher than 125 (AUC/MIC ≥ 125) (10). These ratios are not always accomplished for enrofloxacin due to many factors, such as defective maneuvers to deliver the correct dose (11,12), the lack of bioequivalence of many pharmaceutical preparations (4–7), and the presence of resistant bacteria to this antimicrobial agent.

The development of solvates (also known as polymorphs) or pharmaceutical derivatives of active

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principles such as salts, molecular complexes, and cocrystals represents extensions of chemical space wherein enhanced molecules with new chemical and physical properties may lead to extended use of a given active principle (13). A new solvate of enrofloxacin is already available for research (enrofloxacin hydrochloride-dihydrate, enro-C) (14), and it has shown improved PK values in broilers (15). Hence, the aim of this trial was to determine the PK and the PK/PD ratios of enrofloxacin from enro$_R$ and from enro-C in Syrian golden hamsters while assessing as pharmacodynamic markers the MIC values for methicillin-resistant *Staphylococcus aureus*, *Leptospira interrogans*, and *Escherichia coli*.

2. Materials and methods

Study design and animal handling complied with Mexican regulations for use of experimental animals based on international normativity and Mexican prescripts in NOM-062-ZOO-2001 (http://www.senasica.gob.mx/?doc=743). Hence, it was reviewed and approved in November 2012 by the bioethics committee of the National Autonomous University of Mexico (UNAM), named the Institutional Committee for the Care and Use of Experimental Animals (CICUAE), at the Faculty of Veterinary Medicine (FMVZ). Enrofloxacin as the parent compound was obtained from Globe Chemicals S.A. de C.V. (Mexico City) with 99.97% purity, and the reference enrofloxacin preparation was Baytril® 5% (Bayer, Mexico City). Enrofloxacin hydrochloride-dihydrate (enro-C) was synthesized in our laboratory (Patent Submission Number 472715; Instituto Mexicano de la Protección Industrial, Mexico City).

The trials were carried out at the experimental unit of the Veterinary College, UNAM, in Mexico City. Each preparation was assessed with 60 female Syrian golden hamsters (*Mesocricetus auratus*), 7 weeks old and weighing 191 ± 6.3 g. Animals were individually housed in cages with a controlled environment (lights on 0700–1900 hours, temperature 22 °C). Food and water were provided freely. Supported by technical assistance, each animal received, based on its individual weight, a dose of 10 mg/kg intramuscularly of either drug (enro$_R$ or enro-C) in a volume of 0.05 mL using 1-mL syringes and 22-gauge hypodermic needles. The injection site was in the caudal thigh; pH of enro$_R$ from Baytril 5% was 10.4, while pH of enro-C 5% suspension was 6.8. After individual drug administration, 6 hamsters were sacrificed each time by carbon dioxide asphyxiation; a blood sample was obtained when the hamsters were unconscious by intracardiac insertion of the needle and prior to respiratory arrest. Unconsciousness occurred with no apparent stress and death occurred at a sufficient time-lapse so as to manipulate the animals. Samples were obtained from the lungs, heart, kidneys, liver, muscle, skin, fat, duodenum, and cecum at

0.5, 1, 2, 4, 8, 12, 24, 48, 60, and 72 h for blood and at 4, 12, 24, 48, and 72 h for tissue. Blood samples were immediately centrifuged at 3000 × *g* for 15 min and approximately 1–1.5 mL of serum was recovered, identified, and frozen (–4 °C) in Eppendorf tubes until analyzed. The tissue samples were also frozen (–20 °C) until analyzed.

Serum and tissue enrofloxacin concentrations were determined by high-performance liquid chromatography (HPLC) as described by Elmas et al. (16), using a Jasco XLC HPLC system (JASCO X-LC 3159, Mexico City) with a quaternary pump (PU-2089 Plus JASCO Serial No. C160960865, Mexico City), a Symmetry C18 column (4.6 mm × 100 mm, 3.5 µm; Waters, Mexico City), a C18 pre-column (4.6 mm × 20 mm, Waters, Mexico City), and a UV detector (UV-2075 Plus JASCO Serial No. 0262060866, Mexico City) set at 278 nm.

Extraction from serum samples (0.5 mL) was carried out using dichloromethane (1.5 mL, Merck, Mexico City). Extraction from tissue samples (1 g) was achieved by KOH at pH 12. Enrofloxacin was analyzed by reverse-phase chromatography for tissues and serum. The mobile phase was a mixture (pH 2.2) of potassium dihydrogen phosphate (KH_2PO_4) and acetonitrile (80:20, v/v). Heptane sulfonic acid-Na (1.1 g/L) was added as an ion-pairing reagent. For both serum and tissue samples, the flow rate of the mobile phase was 2.0 mL/min and the run time was 10 min.

For serum samples, the method used was linear from 0.05 µg/mL to 50 µg/mL, while linearity was shown for all tissues from 0.1 µg/g to 500 µg/g. Limit of detection and quantification in serum samples was 0.01 to 50 µg/mL and it was 0.05 to 500 µg/g for tissue samples. Recovery values were 94% for serum and 85%, 86%, 90%, 93%, 93%, 91%, and 94% for fat, skin, muscle, heart, lung, kidney, and liver, respectively. Coefficient of variance was 6% for serum and <8% for tissues; interassay values were 6% for serum and <9% for tissues.

A computerized curve stripping program, PKAnalyst (MicroMath, St. Louis, MO, USA), was used to fit and analyze the concentration-versus-time patterns for each group. Models of best fit (r³ 0.99) were chosen after analysis by use of the residual sum of squares and the minimal Akaike information criterion. Best fit for the enro-C route was obtained by use of model 3, whose general formula is:

$$Concentration\ (Time) = \frac{Dose \bullet K_{AB}}{Volume\ K_{AB} - K_{elim}} e^{-Kelim.Time} - e^{-KAB.Time}$$

Variables obtained were: AUC = area under the curve; $AUMC_{0-\infty}$ = area under the first moment curve from 0 to ∞ with extrapolation of the terminal phase; MRT = mean residence time; $T_{1/2\beta}$ = elimination half-life; C_{MAX} = serum peak concentration, T_{MAX} = peak time.

For enro$_R$, model 13 was chosen, and its general formula is:

$$Concentration\ (Time) = Ae^{-a \cdot Time} + Be^{-b \cdot Time} + Ce^{-KAB \cdot Time}$$

Variables obtained were as above. In addition, relative bioavailability was calculated as follows:

$$\text{Concentration (Time)} = \frac{AUC_{enroR}}{AUC_{enro\text{-}C}} \times 100$$

Statistical differences in serum and tissue concentrations between $enro_R$ and enro-C were carried out through the Mann–Whitney U test. Probability values of <0.05 were considered to be significant. All statistical analyses were performed using SPSS 14.0 for Windows.

A total of 94 methicillin-resistant *Staphylococcus aureus*, 125 *Escherichia coli*, and 45 *Leptospira interrogans* isolates were collected from clinical veterinary cases at the Department of Microbiology, School of Veterinary Medicine, UNAM. All organisms had undergone no more than 5 passages. The strains were subcultured twice on 5% sheep blood Colombia agar (35 °C for 16 to 18 h) prior to MIC testing.

Minimum inhibitory concentrations were determined for both enrofloxacins ($enro_R$ and enro-C) using the microdilution method as described by the CLSI (17). Briefly, 3 to 5 colonies of an 18-h culture were removed, suspended in 5 mL of sterile 0.85% saline, and adjusted to yield approximately 5×10^5 CFU per mL. A 10-µL aliquot of this suspension was then transferred to 10 mL of Mueller Hinton agar supplemented with yeast extracts, complement, and 5% sheep blood. A 50-µL aliquot of this suspension was then dispensed into each well of a 96-well microdilution plate containing a serial prediluted antimicrobial agent in the same culture broth. The microdilution plates were then incubated at 35 °C for 18 to 24 h.

Leptospira interrogans microorganisms were maintained by continuous culture in Ellinghausen-McCullough-Johnson-Harris medium (Becton Dickinson de Mexico S.A de C.V., Mexico City) and were evaluated by the broth microdilution and macrodilution susceptibility tests described by Murray and Hospenthal (18). Two parallel runs were performed at different times to determine the reproducibility of results. For each isolate and antibacterial drug, independent experiments were performed on different days to obtain the MIC. A theoretical minimal bactericidal concentration (MBC) was calculated, multiplying by 16 the value of MIC. The following quality control strains recommended by NCCLS were included with each batch of tested bacteria: *S. aureus* ATCC 29213, *E. coli* ATCC 25922, and *L. interrogans* ATCC 56601. To investigate possible differences in the susceptibility patterns of the bacterial species tested, the Kruskal–Wallis test was performed for all MICs and MBCs. The level of significance was P < 0.05.

3. Results

Hamsters did not show distinguishable signs of pain or discomfort after injection of the 5% suspension of enro-C, and no inflammatory response became evident at the injection site after administration. A small, apparently painless, faint bulge was detectable in hamsters at the site of the injection with $enro_R$. No other side effect was observed during the short time period that this trial lasted.

Table 1 summarizes mean ± 1 SD values for all PK variables obtained. All parameters were statistically

Table 1. Pharmacokinetic variables for the 2 groups of Syrian golden hamsters after a single intramuscular injection of 10 mg/kg of either the reference enrofloxacin from Baytril 5% ($enro_R$) or a 5% suspension of enrofloxacin hydrochloride-dihydrate (enro-C).

Parameter	enro-C	$enro_R$
C_{MAX1} (µg/mL)	17.3 ± 4.5 [a]	2.6 ± 0.7 [b]
C_{MAX2} (µg/mL)	6.5 ± 1.1	-
T_{MAX} (h)	8.2 ± 0.7 [a]	2.8 ± 0.2 [b]
T_{MAX2} (h)	48 ± 1.8	-
$T\frac{1}{2}_{\beta1}$ (h)	7.0 ± 0.2 [a]	1.9 [b] ± 0.1
$T\frac{1}{2}_{\beta2}$ (h)	6.6 ± 0.2	-
$AUC_{0-\infty}$ (µg/mL h)	459.2 ± 44.7 [a]	19.9 ± 1.7 [b]
$AUMC_{0-\infty}$ (µg/mL h²)	17123.1 ± 403.5 [a]	111.0 ± 10.3 [b]
MRT (h)	37.3 ± 2.2 [a]	5.6 ± 0.8 [b]
F_R (%)	2307	-

[a,b]: The values within a row with no common superscript differ significantly (P < 0.05).

η = 6; C_{MAX} = serum peak concentration, T_{MAX} = peak time; $T_{\frac{1}{2}\beta}$ = elimination half-life; $AUC_{0-\infty}$ = area under the curve from 0 to ∞; $AUMC_{0-\infty}$ = area under the first moment curve from 0 to ∞ with extrapolation to the terminal phase; MRT = mean residence time; F_R = relative bioavailability.

significant between groups (P < 0.01). C_{MAX} values of enrofloxacin after administration of enro-C were 6.7 times higher than the corresponding values obtained for $enro_R$ (17.3 ± 4.5 µg/mL vs. 2.6 ± 0.7 µg/mL, respectively). $AUC_{0-¥}$ values for enro-C were 23 times higher than the value for $enro_R$ (459.2 ± 2.87 µg/mL h vs. 19.9 ± 1.08 µg/mL h). This resulted in a much higher relative bioavailability of enro-C (2307%) as compared to $enro_R$. MRT was also increased from 5.6 ± 0.8 h for $enro_R$ to 37.3 ± 2.2 h for enro-C. Figure 1 shows the referred serum concentrations vs. time profiles of enrofloxacin for both forms of this antibacterial drug; the MIC values for methicillin-resistant *S. aureus*, *E. coli*, and *L. interrogans* are highlighted. When the obtained PK data are integrated to PK/PD ratios, enro-C showed statistically significant higher values (P < 0.05 or less).

Comparative tissue concentrations of enrofloxacin from $enro_R$ and enro-C in hamsters revealed prominent improvements in concentrations with enro-C in all tissues analyzed, as shown in Figures 2–4. Additionally, the period
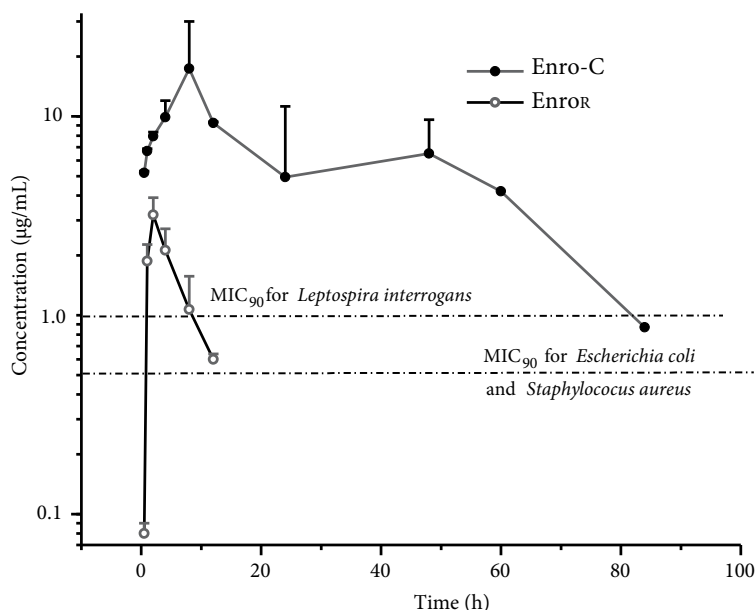

Figure 1. Serum concentrations of enrofloxacin in Syrian golden hamsters after a single intramuscular injection of 10 mg/kg of either the reference enrofloxacin from Baytril 5% ($enro_R$) or a 5% suspension of enrofloxacin hydrochloride-dihydrate (enro-C).
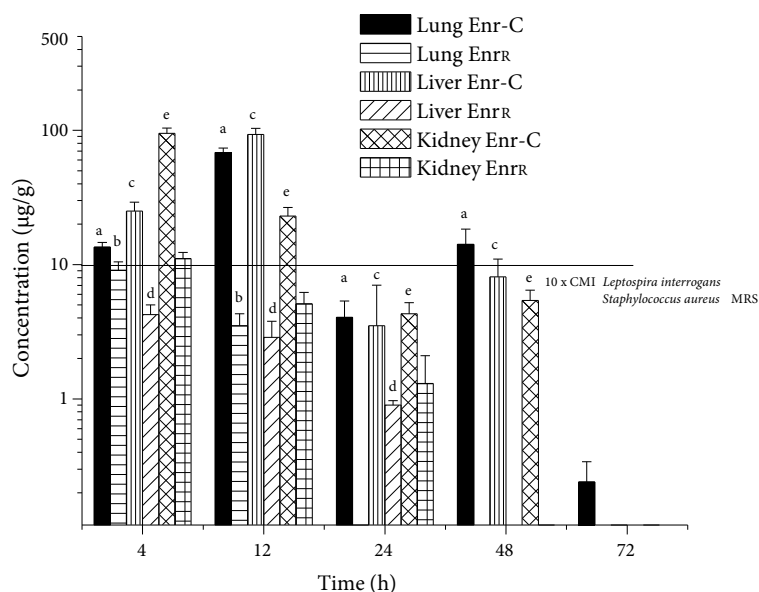

Figure 2. Lung, liver, and kidney concentrations of enrofloxacin after a single intramuscular injection of 10 mg/kg of either the reference enrofloxacin from Baytril 5% ($enro_R$) or a 5% suspension of enrofloxacin hydrochloride-dihydrate (enro-C).
[a,b,c,d,e,f]: The values within a column in each time with no common letter differ significantly (P < 0.05).
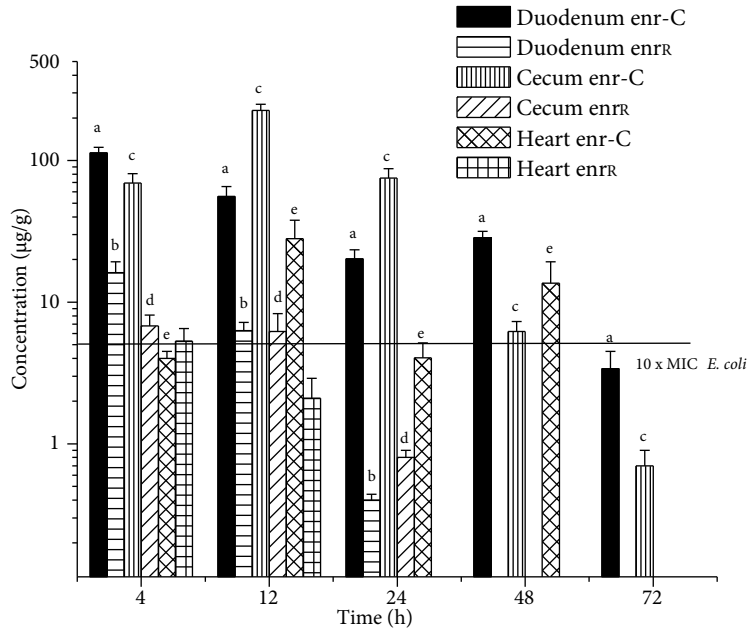
Figure 3. Duodenum, cecum, and heart concentrations of enrofloxacin after a single intramuscular injection of 10 mg/kg of either the reference enrofloxacin from Baytril 5% (enro$_R$) or a 5% suspension of enrofloxacin hydrochloride-dihydrate (enro-C).
[a,b,c,d,e,f]: The values within a column in each time with no common letter differ significantly (P < 0.05).
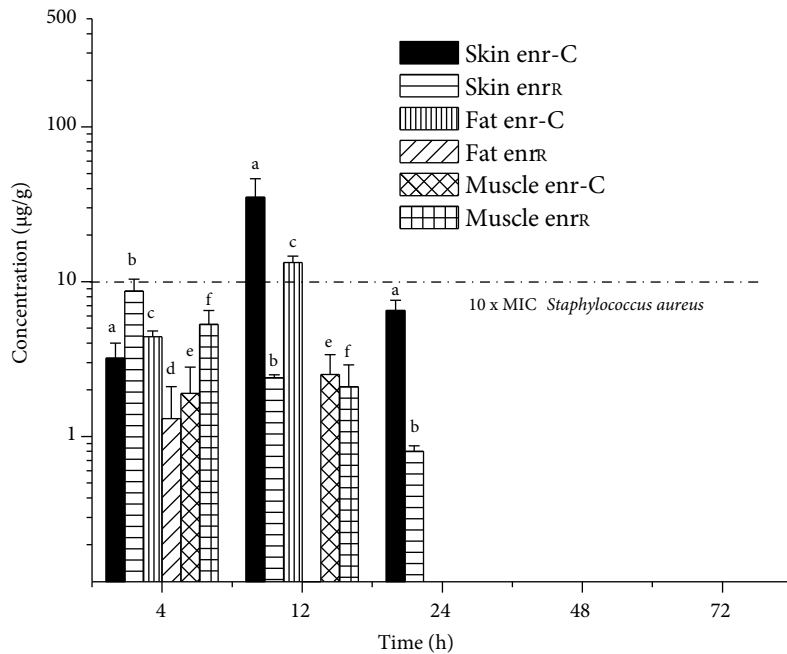

Figure 4. Skin, fat, and muscle concentrations of enrofloxacin after a single intramuscular injection of 10 mg/kg of either the reference enrofloxacin from Baytril 5% (enro$_R$) or a 5% suspension of enrofloxacin hydrochloride-dihydrate (enro-C).
[a,b,c,d,e,f]: The values within a column in each time with no common letter differ significantly (P < 0.05).

of time for which enrofloxacin was at or above MIC levels was extended in all tissues analyzed in the enro-C group.

Table 2 shows the MIC and the MBC for the 3 pathogens cultured with either enro$_R$ or enro-C. A slight tendency in favor of enro-C can be noticed. However, no statistically significant (P < 0.05) difference was obtained for any of the 3 bacteria tested as far as this value is concerned.

Table 2. Minimum inhibitory concentration (MIC$_{90}$) and theoretical mutant bactericidal concentration (MBC) for enrofloxacin hydrochloride-dihydrate (enro-C) and enrofloxacin.

| Dilution (µg/mL) | Staphylococcus aureus | | Escherichia coli | | Leptospira interrogans | | | |
| | Micro | | Macro | | Micro | | Macro | |
	enro-C	enro$_R$	enro-C	enro$_R$	enro-C	enro$_R$	enro-C	enro$_R$
4	1	1	-		4	6	4	6
2	1	3	1	2	2	5	2	5
1	4	6	6	8	38	31	38	31
0.5	85	82	112	108	1	3	1	3
0.25	3	2	4	4	-	1	-	1
0.125	-	-	1	2	-	-	-	-
MIC$_{90}$	0.5	0.5	0.5	0.5	1	1	1	1
MBC C$_{MAX}$/MIC > 16	34.6	5.2	34.6	5.2	17.3	2.6	17.3	2.6

4. Discussion

Enrofloxacin is one of the most extensively used antibacterial drugs in Latin America (19). It possesses low toxicity; i.e. it has been described by manufacturers that enrofloxacin at doses of up to 100 mg/kg body weight (20 times the recommended dose) caused no significant adverse effect in dogs (http://www.baytril.es/scripts/pages/es/productos/seguridad-y-toxicologia/index.php). In areas of the world where enrofloxacin is still a choice for treating domestic and production species, lack of bioequivalence in generic preparations seems to be the constant (4–7). Hence, PK/PD ratio markers for the specific pathogen are often not achieved, i.e. C$_{MAX}$ > 10–12 MIC and AUC$_{0-24}$/MIC ≥ 125. Failure to attain these ratios has been linked to an increase in bacterial resistance and lack of clinical efficacy (20). Synthesis of a solvate of enrofloxacin (enrofloxacin hydrochloride-dihydrate; enro-C) (13) with improved pharmacokinetics has been advanced as means to obtain improved PK/PD ratios (14,15) and, ideally, to achieve theoretical mutant-preventive concentrations (C$_{MAX}$ ≥ 16 MIC) (21). Enro-C exhibits different physicochemical properties from the parent compound, mainly water solubility (13) and pharmacokinetics, as has been so far shown in broilers (15). In this latter case, enro-C somehow improved key PK features, namely AUC$_{0-\infty}$, T½b, and C$_{MAX}$, and consequently PK/PD ratios, that allow the prediction of better clinical outcomes with enro-C as compared to enro$_R$. For example, if MIC values for a partly resistant wild-type E. coli are incorporated (0.5 µg/mL in this study), the referred ratios will only be achieved by enro-C

(C$_{MAX}$/MIC = 35 and AUC/MIC = 918.4), while enro$_R$ ratios will be unsatisfactory (C$_{MAX}$/MIC = 5.2 and AUC/MIC = 39.8). For Leptospira interrogans, MIC values of 1 µg/mL will result in C$_{MAX}$/MIC = 17.5 and AUC/MIC = 459.2 for enro-C, while the same ratios will render enro$_R$ an inadequate choice to treat leptospirosis in this species (C$_{MAX}$/MIC = 2.6 and AUC/MIC = 19.9).

It is worth noting that enrofloxacin concentrations in all tissues obtained after the intramuscular administration of 10 mg/kg of enro-C exceeded by 10 the MIC$_{90}$ for the 3 pathogens tested for 24 h. Furthermore, enro-C may provide, in many cases, mutant-preventive concentrations (C$_{MAX}$/MIC ≥ 16) (21). That is, C$_{MAX}$/MIC = 35 for E. coli and S. aureus methicillin-resistant, and C$_{MAX}$/MIC = 17.5 for L. interrogans in this study.

It is known that commercially available enrofloxacin is not a therapeutic option to treat leptospirosis in domestic species (4–6). However, the PK/PD ratios obtained for enro-C merit further research as far as treating clinical cases of leptospirosis is concerned. That is, the MIC$_{90}$ value for Leptospira sp. was 1 µg/mL; tissue concentrations in target tissues were well above this value and in some instances >10 times higher for 12 h, such as in the lungs, liver, kidney, duodenum, and cecum. In such a context, it is clear that enro-C is a paradox, because it behaves as a new antibacterial drug while being the same enrofloxacin used in veterinary medicine. The difference between enro$_R$ and enro-C is apparently linked to the improved PK/PD ratios here described for enro-C. Nevertheless, for enro-C, further work is needed to analyze its rate of biotransformation into ciprofloxacin and other

metabolites. Additionally, field studies should be performed with different doses and administration routes in target species, as well as to review if adverse drug reactions are not different from the parent moiety.

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