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Withdrawal time of four pharmaceutical formulations of enrofloxacin in poultry according to different maximum residues limits

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To ensure delivery of safe animal products to consumers, the withdrawal time (WDT) of drugs must be respected. Property differences among pharmaceutical formulations, for the same drugs, can lead to differences in the WDTs estimation. The WDTs of four commercial formulations of enrofloxacin (ENRO) in broiler chickens, considering MRLs established by different countries, were studied. Two hundred-thirty-four broiler chicks were allotted among four groups; the formulations were orally administered daily with 10 mg/kg bw. After treatment, six chickens of each group and two controls were slaughtered daily until day 9 post-treatment. Samples of muscle and liver were collected, and analyzed using HPLC-MS-MS. The WDTs among formulations of ENRO showed differences of 24 and 48 h. Based on the European Community and Chile MRLs of 100 μg/kg (muscle) and 200 μg/kg (liver), the WDTs did not exceed 5 days. When Japan MRL was considered (10 μg/kg), the WDTs increased up to 8 days. These results indicate that for WDTs determination, the differences among pharmaceutical formulations of a drug must be considered as well as the MRLs.

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INTRODUCTION

The fluoroquinolones are a class of compound that comprises a large and expanding group of synthetic antimicrobial agents. These drugs are used widely in the treatment of infections in human medicine and in preventive and therapeutic treatment of farm animals (ruminants, pigs and birds). In veterinary medicine, they are used for the control of early mortality and for the prophylaxis and treatment of respiratory, renal and digestive infections of poultry (Martinez *et al.*, 2006).

The administration of fluoroquinolones to food-producing animals without an adequate withdrawal time (WDT) may lead to violative concentrations of residues in foods destined for human consumption. These residues represent a risk to public health, including stimulation of bacterial resistance, alterations on intestinal microflora and hypersensivity reactions (Fàbrega *et al.*, 2008).

Enrofloxacin (ENRO) is a fluoroquinolone that was developed exclusively for veterinary use in cattle, pigs, dogs, cats and for the treatment of respiratory and intestinal diseases in chicken and turkeys (Anderson *et al.*, 2003). After administration ENRO is partly de-ethylated to ciprofloxacin (CIPRO) *in vivo*. This pharmacologically active metabolite is employed in human medicine therapeutics (Dichiara *et al.*, 2008; Taccetti *et al.*, 2008).

With the aim of minimizing the risk for human health represented by residues in the food products, Maximum Residue Limits (MRLs) for quinolones have been established by regulatory agencies of different countries. The European Commission has established for chicken tissues the following MRLs for the sum of ENRO and CIPRO: 100 μg/kg (muscle), 100 μg/kg (skin), 200 μg/kg (liver) and 300 μg/kg (kidney) (European Council Regulation, 1999). The Food Safety Regulation in Chile has established the same values (Ministry of Health, 1999). On the other hand, Japan has defined in all chicken tissues MRLs of 10 μg/kg. (Ministry of Health and Welfare, 2005).

To deliver safe food for human consumption, withdrawal times (WDTs) of pharmaceutical formulations of a drug must be fulfilled. In general terms, the WDT is the period of time required after completion of treatment needed for tissue

concentrations of the drug and/or its metabolites to deplete to less than the established MRLs (Riviere *et al.*, 1998; KuKanich *et al.*, 2005).

Worldwide, there are different pharmaceutical formulations of ENRO for oral administration in chickens. Consequently, the administration of one or another formulation of the same drug leads to violative concentrations of residues if individual WDTs are not considered. The final elimination phase depends on drug pharmaceutical formulation, dose, length of treatment, route and site of administration. Differences in formulation properties, despite being the same drug, may result in violative concentrations. In the extra vascular administration of a drug, the pharmaceutical formulation can condition the rate of absorption and consequently the final elimination phase. According to this, a formulation may require a longer WDT when the drug is slowly depleted from tissues. Otherwise, a shorter WDT can be used when faster depletion is adequately proven (KuKanich *et al.*, 2005).

This study was directed to review the WDTs of four oral pharmaceutical formulations of ENRO administered in broiler chickens, considering the different MRLs established by regulatory agencies of different countries.

MATERIALS AND METHODS

This work was performed in the Laboratory of Veterinary Pharmacology of the Faculty of Veterinary Sciences, University of Chile, and accredited under ISO 17025 Of.2005 standards.

Pharmaceutical formulations of enrofloxacin

Four commercial formulations of ENRO were used: 16%, 20% and 80% premix powder and 10% solution. The labels do not contain information about the vehicles of the different formulations. Prior to the study, the content of enrofloxacin was verified in the premixes and the solution. For this purpose, standard calibration curves of a certificate ENRO standard were performed.

Animals

Two hundred-thirty-four male broiler chickens (Ross 308 genetic) were used for this study. Animals were maintained from 1-day old in batteries (25 ± 5 °C of temperature, 50–60% relative humidity) with *ad libitum* access to water and no medicated feed. The ration was formulated according to breed standard requirements.

Experimental animals were kept in conditions in agreement with the animal welfare guidelines approved by the Bioethics Committee of the Veterinary Sciences Faculty, University of Chile, and the recommendations of the European Council Directive 2007/43. Throughout the experiment, the animals were monitored by an avian medicine veterinarian. The birds were sacrificed under the animal welfare rules of the European Council Directive 93/119/CE 1993.

On day 19, chickens were weighed and randomly allocated into five experimental groups: four groups of 54 chickens (A, B, C, D) and one group of 18 chickens (E). Groups A, B, C and D were treated with ENRO 16%, 20% and 80%, premix powder and 10% solution, respectively. Animals were treated individually with 10 mg/kg bw orally administrated once daily for five consecutive days. Drug was administrated using a gastric catheter to assure the complete ingestion of the dose. Group E remained as untreated control chickens.

From day one until day nine after treatment, six birds of each treated group, and two of the control group were sacrificed. Muscle (breast and leg) and liver samples were collected and individually stored at −70 ± 2 °C until sample preparation and chromatographic analysis.

Reagents and chemicals standards

Standards of ENRO and CIPRO were supplied by USP Reference Standard (Rockville, MD, USA) and Dr Ehrenstorfer Gmbh (Ausgsburg, Germany), respectively. The LC-grade acetonitrile, diclorometane and water were from Fisher Chemicals (Fair Lawn, NJ, USA). LC-grade methanol was supplied by Merck (Darmstadt, Germany). All other reagents were of analytical reagent grade. A 25% ammonia solution and trifluoroacetic acid were purchased from Merck.

Standard and working solutions

Standard solutions of ENRO and CIPRO were prepared in aqueous 0.03 M NaOH at 1000 μg/mL and stored at 4 ± 2 °C in the dark for no longer than 3 months. Matrix-matched calibration curves were spiked with the standard solution immediately prior to extraction.

Sample preparation

The determination of ENRO and CIPRO in chicken tissues was based on the sample preparation published by Hassouan *et al.* (2007). Briefly, four grams of homogenized tissue (muscle or liver) were placed into a tube, 500 μL of a 25% ammonia solution and 4 mL of acetonitrile were added. Samples were centrifuged 10 min at 3130 ***g***. The upper layer was transferred to other tube and extracted with 4 mL of diclorometane. The sample was centrifuged 10 min at 3130 ***g***. The upper layer was transferred to a 2 mL Eppendorf tube, and centrifuged at 9300 ***g*** for 10 min. The upper layer was transferred to a glass tube and evaporated under nitrogen stream at 40 ± 2 °C. The residue was dissolved with 150 μL of mobile phase, filtered though a 0.22-μm-pore-size polyvinylidene fluoride Millex-GV membrane. Twenty microliters were injected into the HPLC system. Residues were separated by liquid chromatography and identified by mass detection (HPLC MS/MS), based on the method published by Van Hoof *et al.* (2005). The limit of detection (LOD) and the limit of quantification (LOQ) for the analytical method were 1 μg/kg and 2 μg/kg, respectively, for ENRO and CIPRO in both matrices. These reported values are based in quantifier product ion.

Instrumentation

Liquid chromatography

The HPLC system (series 200, Perkin Elmer, Norwalk, CT, USA) consisted of two micropumps, an auto sampler, a column oven, and a vacuum degasser. Chromatographic separation was achieved on a C-18 Symmetry column of 3.5 μm (150 mm by 2.1 mm inside diameter; Waters, Milford, MA, USA). Mobile phase A was 0.1% (vol/vol) trifluoroacetic acid in methanol, and mobile phase B was 0.1% (vol/vol) aqueous trifluoroacetic acid. The gradient program consisted of a constant 20% mobile phase A with 80% mobile phase B for 5 min, increasing to 100% phase A by 5 min, returning to 20% phase A by 2 min, and holding at 20% phase A for 20 min. Chromatography was performed at 30 °C at a mobile phase flow rate of 0.2 mL/min.

Mass spectrometry

A Sciex API 4000 mass spectrometer (Applied Biosystems Sciex, Concord, ON, Canada) was used for mass detection. ENRO and CIPRO ionization was achieved using the Turbospray ionization source operated in positive mode. Conditions and settings were those described by San Martín *et al.* (2007): curtain gas (N_2) at 10 psi, ion source gases 1 and 2 at 40 psi, source temperature at 450 °C, ion spray at 5000 V, and collision gas pressure at 4 psi. MS data on precursor and product ions were collected in multiple reactions monitoring mode. Precursor and product ions, declustering potential, entrance potential, collision energy, and cell exit potential were optimized for each drug (Table 1).

Validation of analytical method

The validation of the analytical method was preformed according to the European Commission Decision 2002/657/EC (2002) guidelines using the control chicken tissues (muscle and liver) as matrix. Essential parameters in validating an analytical procedure such as specificity, recovery, repeatability, within-laboratory reproducibility, decision limit (CCα), detection capability (CCβ) and linearity. The ruggedness was evaluated to determinate if the method fulfills the performance requirements at concentration of 10 and 100 μg/kg.

Drug quantification in experimental samples

Enrofloxacin and CIPRO concentrations were calculated using the equation from the regression analysis of the matrix-matched calibration curves ($r > 0.99$) at different concentrations, to avoid extrapolations. The range of concentrations of the matrix-matched calibration curves were: 5–10–15–20–25 μg/kg and 50–100–150–200–250 μg/kg. The range of the curve used for the quantification of day one post-treatment samples was 200–400–800–1200–1400 μg/kg.

Table 1. Multiple reaction monitoring analysis of two quinolones

Analyte	Precursor ions	Product ions	DP	EP	CE	CXP
CIP	332.2	231.2*	51	10	51	16
		314.2†			29	26
ENR	360.2	316.1*	51	10	29	24
		342.1†			29	22

CIP, ciprofloxacin; ENR, enrofloxacin; DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential. *Confirmation ion, †Quantitation ion.

Determination of withdrawal times

The sum of CIPRO and ENRO at each sampling time was considered to determine WDTs in muscle and liver. For this purpose, the recommendations of the European Agency for the Evaluation of Medicinal Products (EMEA, 1995) were adopted. The WDTs were estimated from the linear regression analysis of log-transformed tissue concentration and was determined at the time when the 95% upper one-sided tolerance limit was below the MRL with 95% confidence.

RESULTS

Analytical performance

A prior WDT calculation for each formulations of ENRO, the validation of the analytical method in liver and in muscle was performed by HPLC-MS-MS. The precursor ion and two product ions were identified for ENRO and CIPRO (Table 1). Validation parameters such as recovery, repeatability, within-laboratory reproducibility, decision limit (CCα), detection capability (CCβ) and the matrix-matched calibration curve are shown in Tables 2 and 3. The analytical modifications studied in the ruggedness test were performed at the concentration of 10 and 100 ppb, and these showed no significant effect on the performance of the assay.

Estimation of withdrawal time (WDT)

The matrix-matched calibration curves of liver and muscle were linear ($r > 0.99$) and were used for drug quantification in experimental samples. The linear regression analyses of the tissue depletion of ENRO+CIPRO for each commercial formulation are shown in Figures 1 and 2. In each figure, the line for the

Table 2. The validation parameters recovery, coefficient of variation for repeatability within-laboratory reproducibility, CCα and CCβ for ENR and CIP obtained for muscle samples

	MRL 10 μg/kg		MRL 100 μg/kg	
Validation parameters	ENR	CIP	ENR	CIP
Recovery (%)	98	96	107	111
Repeatability (CV %)	14	17	13	15
Within-laboratory reproducibility (CV%)	21	24	19	17
CCα μg/kg	11.35	11.9	102.9	106.7
CCβ μg/kg	13.1	13.8	106.7	105.9
Matrix-matched calibration curves	5–10–15	5–10–15	5–10–15	5–10–15

Table 3. The validation parameters recovery, coefficient of variation for repeatability within-laboratory reproducibility, CCα and CCβ for ENR and CIP obtained for liver samples

	MRL 10 μg/kg		MRL 200 μg/kg	
Validation parameters	ENR	CIP	ENR	CIP
Recovery (%)	110	112	111	114
Repeatability (CV%)	15	19	14	17
Within-laboratory reproducibility (CV%)	24	25	21	23
CCα μg/kg	11.8	12.1	203.5	205.6
CCβ μg/kg	13.9	14.1	207.8	208.2
Matrix-matched calibration curves	5–10–15	5–10–15	5–10–15	5–10–15

upper tolerance limit with a confidence of 95% is shown. In Table 4, the duration of the WDTs for each pharmaceutical formulation considering the different MRLs are shown.

DISCUSSION

In drug tissue depletion studies for determination of WDTs, the selection and validation of suitable analytical methods must be previously accomplished. The HPLC-MS-MS method was selected because of its high specificity and accuracy, which were considered critical factors for this study. HPLC-MS-MS has been used for similar purpose by other authors (Clemente *et al.*, 2006; Hermo *et al.*, 2006; Tang *et al.*, 2006; San Martín *et al.*, 2007; López *et al.*, 2008).

As CIPRO is an ENRO metabolite, with microbiological activity and it is part of the MRL, it was also included in the analytical method validation. This metabolite is also currently used in human medicine for the treatment of bacterial infection (Aydemir *et al.*, 2006; Hickerson & Carson, 2006). The analytical method validation results indicate that the proposed method is suitable for the evaluation of the ENRO+CIPRO tissue depletion in chickens.

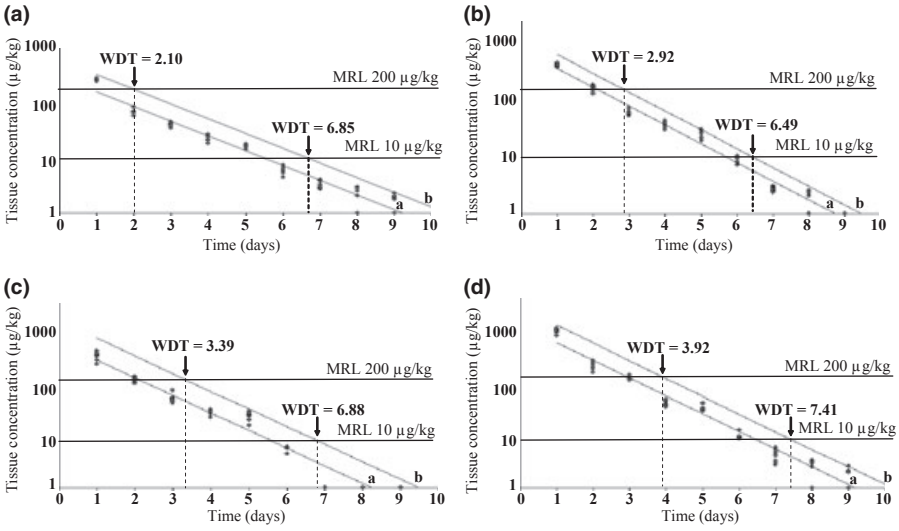

Fig. 1. Plot of liver depletion of enrofloxacin plus ciprofloxacin following the oral dose of different formulations of enrofloxacin. A: 10% enrofloxacin (r = −0.969); B: 20% enrofloxacin (r = −0.987); C: 80% enrofloxacin (r = −0.972); D: 16% enrofloxacin (r = −0.977). a: linear regression line; b: 95% tolerance limit with 95% confidence. Arrows show the time when WDT was calculated according to MRLs. [Correction added after online publication: 25-November-2009, Legend for Fig. 1 replaced].

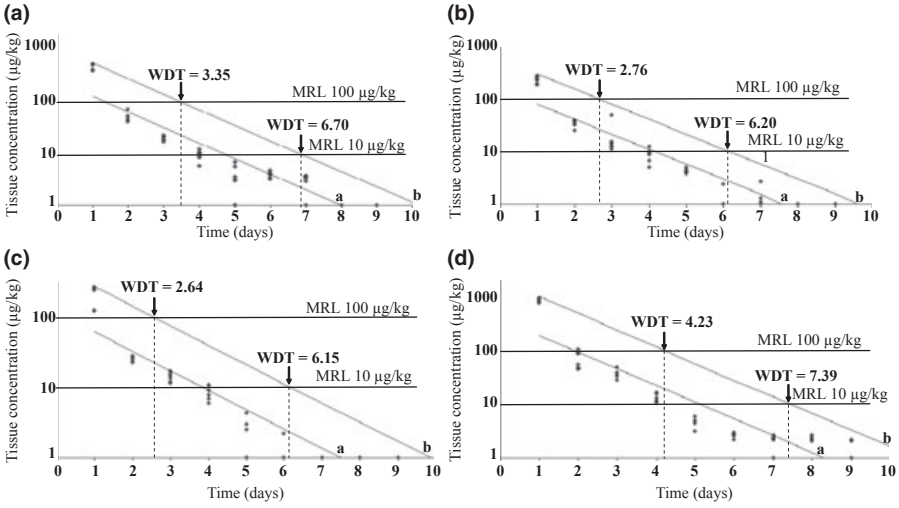

Fig. 2. Plot of muscle depletion of enrofloxacin plus ciprofloxacin following the oral administration of different formulations of enrofloxacin. A: 10% enrofloxacin (r = −0.907); B: 20% enrofloxacin (r = −0.903); C: 80% enrofloxacin (r = −0.917); D: 16% enrofloxacin (r = −0.918). a: linear regression line; b: 95% tolerance limit with 95% confidence. Arrows show the time when WDT was calculated according to MRLs. [Correction added after online publication: 25-November-2009, Legend for Fig. 2 replaced].

Table 4. Withdrawal periods calculated for muscle and liver from broiler chickens treated with enrofloxacin (10 mg/kg/bw once-a-day during five consecutive days) according to MRLs

Enrofloxacin formulation	Withdrawal period (days) MRLs (μg/kg)			
	Muscle		Liver	
	100	10	200	10
10%	4	7	3	7
20%	3	7	3	7
80%	3	7	4	7
16%	5	8	4	8

In this study, WDTs of different pharmaceutical formulations of ENRO were determined after daily oral dose in broilers chickens (10 mg/kg bw for five consecutive day), considering MRLs established by regulatory agencies of different countries.

During the tissue depletion study ENRO+CIPRO concentrations in liver reached higher values and depleted slower than muscle through all the experiment. This suggests that the liver could be the target tissue to evaluate the WDTs in broilers chickens. According to the EMEA (2002), target tissues for ENRO+CIPRO are muscle, liver and kidney in poultry. On the other hand, the FDA has established that the edible tissue from which residues deplete most slowly could be considered as the target tissue (Ellis, 2004).

Concentrations of CIPRO were lower than those detected for the parent drug ENRO. This metabolite was less than 2 μg/kg in all the experimental groups at 6 and 8 days after the last dose in muscle and liver, respectively. When formulations of 10% and 16% were administrated, ENRO was detected until 9 days post-treatment in both matrices. In the 20% and 80% formulations, this drug was detected until the day 8 and 6, respectively.

For the calculations of WDTs the EMEA (1995) recommends to perform a linear regressions analysis of the logarithmic transformed concentrations during the tissue depletion phase of the drugs and/or their metabolites. In this study, when the linear regressions analyses were performed, we considered the sum of ENRO and CIPRO in the different sampling times. Using the model of the EMEA, the WDTs of each formulation was determined as the point at which the upper 95% tolerance limit for the residue is less than MRLs with 95% confidence.

When different formulation of ENRO (16%, 20% and 80% premix powder and 10% solution) were analyzed, differences between 24 and 48 h were observed in the WDTs. For example, considering the liver as a target tissue and an MRL of 200 μg/kg, two pharmaceutical formulations showed WDTs of 4 days and two formulations showed a WDT of 3 days. If muscle is considered as target tissue and a MRL of 10 μg/kg, the WDTs of the different formulations fluctuated between 7 and 8 days.

As all the birds used in this study were from the same genetic line, same age were maintained under same conditions during all the experiment, and received same treatment (dose, length, and administration route), the variability observed could be attributed to the different pharmaceutical formulation ingredients of ENRO, even when the labels do not contain this information. On these regard KuKanich *et al.* (2005) pointed out that the formulation can have profound effects on the pharmacokinetics and depletion of a drug. Although the differences founded in our work are short (24–48 h), our data confirm that differences between different pharmaceutical formulations of the same drug can result in variations of the tissue depletion times.

On the other hand, in agreement with the Codex Alimentarius Comission (1995), the lengths of the WDTs are defined by MRLs of each veterinarian drug. In this study, the WDTs of the four pharmaceutical formulations were determined considering the MRLs defined by the EU, Chile and Japan. On this regard, based on the MRLs of the EU and Chile (100–200 μg/kg for muscle and liver respectively), and considering the liver as target tissue, the WDTs did not exceed 5 days for all the studied formulations. When the lower MRL was considered (10 μg/kg defined by Japan), the WDTs increased up to 8 days.

In conclusion, based on our results we can suggest that when slight differences in the WDT are observed between formulations, as in this study, the larger WDT could be assigned as a precautionary principle for public health, without a significant economical impact for the producer. On the other hand, these results indicate that for WDTs determination, the differences among pharmaceutical formulations of a drug must be considered as well as the MRLs.

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