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Disposition and Residues of Marbofloxacin in Eggs of Laying Hens

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Abstract

Marbofloxacin belongs to the group of fluoroquinolones, used exclusively in domestic animals. This study was carried out with the purpose of studying the temporal disposition of marbofloxacin in edible compartments of the egg after administration of 1, 52 mg/kg of marbofloxacin in drinking water for 11 consecutive days in hens in posture peak and average weight of 1,91±0,25 kg. Birds were housed in individual cages under controlled light conditions, ambient temperature, relative humidity, water and balanced feed ad libitum. After the administration, eggs were collected daily and immediately separated into albumen and yolk, identified per bird/day and stored at-20°C until analysis. The preparative assay consisted in extraction of the analyte using 200 mg of yolk or albumen, as appropriate, in deionized water, homogenizing solution and enrofloxacin solution as internal standard. Separation and quantification were performed by HPLC by reverse phase isocratic elution with fluorescence detector, mobile phase composed of water, acetonitrile and triethylamine adjusted to pH 3. According to the peak areas of known concentrations, the concentrations of the test samples were calculated by simple linear regression. The established marbofloxacin levels are higher and more persistent in the yolk than in albumen, reaching 8 and 9 days, respectively. The disposition characteristics observed with marbofloxacin are compatible with the physico-chemical properties of the antimicrobial with the respective compartments of the egg and the time required for its formation. Data obtained from marbofloxacin depletion in egg compartments were analyzed using the EMEA WT 1.4 software and conjecturing a rigorous MRL (0,001 µg/kg), a withdrawal period of 13 and 17 days was estimated, for albumen and yolk, respectively.

Keywords: Egg; Marbofloxacin; Residues

Introduction

Second generation fluoroquinolones are antimicrobial resources used to control enteric and respiratory infectious diseases in birds [1,2], result by its spectrum towards Gram negative germs and mycoplasmas, their bactericidal activity blocking DNA gyrase A subunit and/or topoisomerase IV enzymes, essential for DNA duplication [2,3] and also because fluoroquinolones can be administered in drinking water, making it possible to dose a large number of birds in a short time [4].

However, in several countries the application in birds is restricted and maximum residue limits (MRLs) in birds were established for different tissues and for various members of the group [2,5], due to the increase in resistant hospital strains, an aspect related to oral application and the risk of ineffectiveness of these substances to treat infections in humans caused by Campylobacter spp and Salmonella spp. [2,5].

In order to protect the food consumer, the administration of fluoroquinolones in animals destined for human consumption is subject to regulation; maximum residue limits (MRLs) were established in birds for different tissues and for several members of the group [2,5], in order to limit residues impact, for each drug and animal species treated, a withdrawal period estimated at 5-7 days was stipulated for fluoroquinolones and its application in laying birds is not allowed [1].

When antimicrobials are administered in chicken production, transfer and residual accumulation of these drugs is associated with their physiology and egg formation processes [5,6], although the physicochemical characteristics of each group of drugs also influence, especially when they are very fat soluble [5,6]. The distribution of xenobiotics to the egg compartments is complex, is conditioned by the physicochemical properties of the egg compartments, mainly the yolk and pH, in relation to the physicochemical properties of the drug: many residues can be deposited in the yolk or the albumen, according to the affinity for plasma proteins, the hydrophobic or lipophilic character and its influence to cross biological membranes [6].

Marbofloxacin is a second generation fluoroquinolone exclusively used in Veterinary Medicine. This drug was authorized in the US and Europe for its administration in domestic animals in presence of infectious enteric diseases and respiratory diseases and in bovine mastitis caused by E. coli [7]. Escherichia coli MIC90, one of the most relevant avian pathogens, stands at 0.01 µg/mL [7].

Marbofloxacin exhibits amphoteric properties, although the physicochemical characteristics of each group of drugs also influence, especially when they are very fat soluble has an isoelectric point of 6.85 that allows its solubility in both acidic and basic solutions (pKa=5.7; pKb=8) [3,7,8]. The weak acid character is provided by the carboxylic acid, while the weak basic character is provided by the piperazine cycle [7]. Under these conditions, it undergoes minimal ionization at a physiological pH of 7.4, an attribute that facilitates tissue distribution [7,8]. An increase in elimination half-life is also observed and good bioavailability, due to the incorporation of the oxadiazine ring [7].

In birds, previous studies indicates that marbofloxacin oral administration provides 56,8% bioavailability, moderate permanence in the body and wide tissue diffusion [9,10]. As with other fluoroquinolones, the use of marbofloxacin was not approved in laying birds, however it can be applied extra-label with the purpose of saving life of the animals. In this context, assuming the complexity of the egg matrix [11], it is proposed to validate an analytical methodology to determine marbofloxacin in egg compartments.

Material and Methods

Animals

Hens (N=11), ISA-Bovans Brown genetic line 30-35 weeks of age, clinically healthy, in laying peak (83±16% at the beginning of the trial) and average weight of 1.91±0.25 kg. The birds were housed in individual cages in controlled light conditions for a total of 17 hours under conditions of environmental thermoneutrality; thermal comfort stabilized at 20.5±1.9°C, with 60-70% humidity and controlled light for a total of 17 hours [12]. Ventilation of the environment allowed permanent air renewal and the elimination of possible toxic gases.

Two weeks before and during the development of the experience, the birds received water ad libitum according to the productive needs of the animals [12], feeded with commercial balanced meal that provides 19.5% crude protein, 5.6% fiber, 4-6% fat, 3.2-3.6 g of calcium and 2950 kcal/kg of metabolizable energy. Consumption of balanced food was ranged between 105-116 g per day.

After a two-week setting period, birds were weighed and received commercial marbofloxacin (Marbocyl ® solution 2%, Vétoquinol S.A., Spain) in the drinking water at a rate of 20 ppm for 11 consecutive days. After suppressing the administration of marbofloxacin, the birds were weighed again in order to estimate the average weight during the administration of the drug and the total consumption of medicated water in the 11 days. With the data obtained, it was determined that each bird received a daily dose of 1.52 mg/kg of marbofloxacin. Immediately, non-medicated water administration was restored ad libitum, until the end of the trial.

Sampling

Eggs were collected daily between 11 a.m. and 1 p.m., from the last day of the application of the antimicrobial and up to three weeks after application, each unit was identified indicating the day of laying and number of animal, and it was kept in a refrigerator at 4°C, until its analysis.

At the end of the collection, albumen and yolk were separated manually. The samples obtained were identified and conditioned in sterile Eppendorf tubes, without additives, and were labeled with the extraction time and the number of animal sampled, and were stored at -70°C until their analysis by HPLC.

Experimental protocol, was approved by the Ethics Committee of the National University of Río Cuarto (Disposition 160/16), which was included in the program

"Disposal and residues of marbofloxacin in compartments of edible eggs and tissue of birds", Secyt, UNRC, 2016-2019.

Sample Treatment

Extraction of the analyte was carried out using 200 mg of yolk or albumen, as appropriate, 200 µL of deionized water, 800 µL of homogenizing solution made up of methanol: water: perchloric acid: phosphoric acid 50:50:2:1 v/v/v/v and 20 µL of a 20 µg/mL solution of enrofloxacin as internal standard. The whole was vortexed for 30" and then centrifuged at 13.500 rpm at 4°C for 25 minutes [13].

Marbofloxacin Separation and Quantification

The separation and quantification was carried out by HPLC by means of isocratic elusion in reverse phase using a C-18 column, 5 µm, continuous flow of 0.8 mL/min and reading in a fluorescence detector established at 295 nm of excitation and 500 nm of emission and mobile phase composed of water, acetonitrile and triethylamine (79:20:1 v/v/v) adjusted to pH 3.0 [13].

Concentration Calculations

With the chromatogram obtained in the run of the albumen and yolk samples separately and with the known concentration standard, a quotient was obtained taking the value of the peak area of the drug and the corresponding internal standard. A quotient of both was obtained and used for the preparation of the calibration curve and to establish plasma concentrations, in albumen and yolk of marbofloxacin by simple linear regression [14].

Chromatographic Method Validation

Calibration Curve: Known marbofloxacin concentrations between 0.0048 to 1.25 µg/mL, incorporated in the preparation of the egg yolk and egg albumen samples, were used, according to the procedure described above.

Linearity test was performed by linear regression (Graph Pad Prism software) with the areas under the curve presented in the peaks of the chromatogram in the samples of the analyte under study and the internal standard, determining the regression coefficient, the slope, the intercept, the limits of detection (LD) and quantification (LQ), from antimicrobial-free egg albumen and yolk samples.

Inter-Day, Intra-Day Trials and Recoverability (%): To establish precision, the intra-day and inter-day tests were used, which refers to the elution of marbofloxacin calibration standards for albumen from (0.156 to 0.625 µg/mg) and for yolk (0.156 to 1.25 µg/mg) six times and for 6 days. It was considered acceptable when the coefficients of variation (CV) between elusions average was ≤ 3%.

The "relative" recovery consisted in determining the variation experienced by the concentration of the analyte under study, when the sample containing it was subjected to extraction. Three marbofloxacin calibration standards were diluted by triplicate for albumen (0.156-0.312-0.625 µg/mg) and yolk (0.156-0.312-1.25 µg/mg) that were compared with fortified samples and the percentage of recoverability was calculated (% R).

Detection limit (DL) was carried out using the formula recommended by EMEA [15], which considers the surface area in the chromatograms originated after the elution of the smallest concentrations of marbofloxacin by means of which a calibration curve was established, and β value was obtained, which corresponds to the SD of the values obtained in the calibration curve.

$$DL = \text{average DS} \times 3.3 / \beta$$

Quantification limit (QL) was determined with the same formula and values, only that the average of the areas obtained was multiplied by 10:

$$QL = \text{average DS} \times 10 / \beta$$

The repeatability test relied on the elution of the calibration standards six times. It was considered acceptable when the CV between elusions, as the area index of the chromatograms was ≤ 1.5%.

The reproducibility test consisted in eluting the calibration standards in tests carried out on 6 different days. It was considered acceptable when the CV of the area indices of the chromatograms was ≤ 3%.

Withdrawal Period Calculation in Eggs: Residual concentrations versus yolk and albumen time were analyzed separately using the EMEA WT 1.4 program [16], which allowed estimating a withdrawal period for egg consumption, conjecturing an MRL of 0.001 µg/kg, because it was not established for marbofloxacin in eggs.

Statistical Analysis: The antimicrobial concentrations during the 9 days post application were compared through the Mann-Whitney test with the Graph Pad Prism software to evaluate significant differences between marbofloxacin concentrations in egg albumen and yolk [17].

Results

Tables 1&2 indicate the results of the linearity, precision and recoverability tests for marbofloxacin in each egg compartments. Marbofloxacin chromatogram retention

times for both albumen and yolk, were 5.57±0.17 minutes and 7.87±0.47 minutes for internal standard (enrofloxacin).

The detection limits (DL) established for albumen and yolk were 0.012 and 0.010 µg/g, respectively. The quantification limits (QL) for marbofloxacin obtained were 0.037 µg/g in albumen and 0.031 µg/g in yolk. Table 3 indicates the average concentrations (± SD) obtained per day in albumen and yolk. EMEA WT 1.4 program [16], used for the analysis of the residual concentrations in each compartment versus time, estimates a withdrawal period of 11.92 days (12 days) for albumen and 16.53 days (17 days) for yolk.

Matrix	µg/g (*)	A	B	r^2
Albumen	0.0048 a 0,625	0.009853	1.286	0.995
Yolk	0.0024 a 1.25	-0.00726	0.692	0.997

Table 1: Linearity of marbofloxacin in egg compartments. (*) Marbofloxacin concentration range. A: Intercept, B: Slope, r2: adjustment coefficient.

Matrix	Recoverability	Intra-day (%CV)	Inter-day (%CV)
Albumen	94.7±15.3	1.59±0.88	1.05±0.92
Yolk	87.8±6.9	1.68±0.72	0.96±0.26

Table 2: Assays of recoverability (% + SD) and intraday and interday precision of marbofloxacin in white and yolk.

Day	Albumen (µg/g)	Yolk (µg/g)
1	0.2692±0.1080	0.6250±0.1773
2	0.1189±0.0842	0.3707±0.1143
3	0.0809±0.0791	0.2786±0.0946
4	0.0406±0.0764	0.2322±0.1003
5	0.0378±0.0802	0.1614±0.0775
6	0.0267±0.0804	0.1130±0.0773
7	0.0200±0.0805	0.0912±0.0422
8	0.0143±0.0805	0.0411±0.0091
9	--	0.0355±0.0084

Table 3: Average concentrations (Mean±SD) per day in albumen and yolk.

Discussion

The development of highly sensitive and selective analytical techniques to quantify fluoroquinolone residues in food samples is a requirement to ensure food safety and understand its risk to public health [18]. As the egg compartments are complex and chemically different [11,19], difficulties often arise for the implementation of sensitive and specific analytical methods, consequently the samples requires different pretreatments, such as spraying, homogenization, deproteinization and filtration [11,18).

Due to its polar nature, fluoroquinolone extraction process usually requires a large amount of organic solvents such as dichloromethane [20], acetonitrile [20], methanol [13] or trichloroacetic acid [21], followed by liquid or solid phase extraction and by denaturation with perchloric acid [20,22].

In this experience, the extraction procedure in both matrices contemplated the use of a homogenizing solution where methanol was administered to separate soluble substances from insoluble ones [13,23], similar to the acid solution made up of acetic acid and acidified ethanol (1:99 v/v) for albumen and acetonitrile and acidified ethanol for yolk [24]. Sample preparation is critical in analysis and there is a need to minimize the number of steps to reduce both time and sources of error. There is currently a trend towards environmentally friendly techniques, which use less solvent and smaller sample sizes to avoid consumer protests. Optimal sample preparation can reduce analysis time, improve sensitivity, and allow unequivocal identification, confirmation, and quantification [19].

HPLC coupled to the fluorescence detector (FD) provides an increase in sensitivity against the ultraviolet detector 20-30 times greater of and can be somewhat more sensitive than GC/MS in some cases. HPLC/FD is even more interesting from an analytical point of view. Fluorescence detection by is favored by the piperazinil group of marbofloxacin, similar to that reported with other members of the group [23,24].

Fluorescence is of particular interest because of its high sensitivity and selectivity; marbofloxacin exhibits a characteristic absorption wavelength that facilitates its detection; the highest sensitivity was obtained with a detector reading established at 295 nm excitation and 500 nm emission, in contrast to other lengths used in other studies with fluoroquinolones [24].

In this study separation was carried modifying the original composition of the mobile phase, composed this time of water, acetonitrile and triethylamine (79:19:1 v/v/v) at pH 3.0 [13], where the incorporation of acetonitrile favors selectivity, triethylamine and acidic pH modulate retention and avoid interferences of the mobile phase in the chromatogram [25], different from the combination of acetonitrile and trifluoroacetic acid at pH 2.5 to quantify other fluoroquinolones in eggs [26].

Optimal separation of the analyte demands not only the composition of the mobile phase but also, pH adjustment, this time to the value of 3.0 [13,27], was adequate, in a suggested range of 2.5 to 4.5 [28].

The analytical conditions implemented were sufficiently specific and precise with advantages over microbiological methods considered less specific [11]. In residue monitoring plans, the implemented HPLC method is a simple option, with low solvent requirements, low-cost since the extraction applied with respect to the solid phase does not require the use of extraction cartridges; it is versatile and adaptable to different matrix [13].

The adjustments obtained in albumen and yolk linearity tests (r2=0.99), validate the reliability of the analytical method implemented. In both matrices, the repeatability tests, assessed by repeated intraday and interday analyzes carried out within the same laboratory, show the precision of the results obtained as they did not exceed the coefficients of variation of 3%.

The high percentages of recoverability achieved in both compartments of the egg, without the need for a second cleaning of the sample after extraction prior to analysis, favor less manipulation of the same. The results obtained in the validation tests corroborate the reliability of the implemented method [15].

In this experience, sufficient absorption of the antimicrobial in the digestive tract was confirmed to generate plasma levels with the ability to diffuse in the avian organism according to the kinetic antecedents available in birds after the administration of 2 mg/kg orally through a commercial formulation [9,10].

The experimental design was adequate to analyze the disposition characteristics of marbofloxacin in edible compartments of the egg, which justifies the results achieved not only by the applied dose and the implemented administration model of 11 days of continuous exposure of the antimicrobial in drink water, with a clear impact on the time of formation of yolk, since to reach residues in the yolk a previous exposure of 8-10 days is generally required [29], but also in terms of the number of animals; the 11 laying hens in laying peak assigned to the experimental group to obtain 9-10 eggs per day, according to the European guidelines for the harmonization of withdrawal periods [30], for HPLC analysis during the period of depletion and, the collection of daily samples in a determined hourly range.

Marbofloxacin levels were more significant and durable in the yolk compared to albumen ($p<0.05$). Drugs tend to be distributed in different ways in the two compartments of the egg, depending on their physicochemical properties. In the case of quinolones and fluoroquinolones, there is no consensus on this point. Some authors describe that these substances are stored mainly in the yolk, being sequestered and incorporated into the eggs during their development [31,32].

Extensive tissue distribution of fluoroquinolones verified in mammals [3] and birds [9,10], associated to the physical-chemical characteristics [3], low affinity for plasma proteins, due to the fact that only the free drug has the ability to diffuse [7,8], but also the elimination-if it is assumed that the egg represents a mode of elimination of xenobiotics-[33], agree with the marbofloxacin levels established in this experience up to 8 and 9 days after application in egg albumen and yolk, respectively [34].

The results obtained support the available information on disposition in eggs with enrofloxacin [26,35], ciprofloxacin [26,35], sarafloxacin [26] and danofloxacin [36], but they disagree with those reported with oxonilic acid and with enrofloxacin [37,38], the last one was supplied for only 5 days, insufficient time to achieve significant concentrations in yolk.

Without ignoring the complexity of the processes that determine the entry of xenobiotics into the egg [34,39], an additional important factor in the disposition is the pH of the different phases; yolk pH is approximately 6.0, while is close to 7.6 in albumen [34]. Quinolones are reported to be slightly more soluble at alkaline pH, so that differences in pH between egg phases can influence the partition of drugs between these compartments.

Levels of marbofloxacin residues in eggs involve some risks to human health; therefore it is necessary to apply an egg withdrawal period after administration in laying birds. This experience exposes the potential risk; the antimicrobial levels obtained in egg albumen and yolk exceeds MIC on E. coli [7], up to days 8 and 9 after application, respectively.

The application of a rigorous MRL (0.001 µg/kg) due to the fact that it was not established for marbofloxacin and the residual concentrations of the antimicrobial in the edible compartments of the egg, evaluated with the WT 1.4 program [16], allowed to estimate a withdrawal period of 13 and 17 days for white and yolk, respectively, which corroborates the yolk as a marker for this antimicrobial.

The established withdrawal values exceed those suggested for the set of fluoroquinolones for their application in poultry [1] and in particular for eggs with enrofloxacin and its metabolite ciprofloxacin [38] and are significantly lower than those estimated for danofloxacin [36], although

too extensive to discourage the poultry producers from using marbofloxacin in producing birds.

Conclusions

The analytical HPLC method used is simple, reliable and requires a low amount of solvents. The analytical methodology applied is useful to quantify marbofloxacin in chicken egg compartments. In the egg for consumption, different authors have shown that the antimicrobials used in poultry industry are transferred to this food during its formation process, with the risk that they reach concentrations that exceed the maximum limits allowed by different governmental and intergovernmental organizations. Marbofloxacin disposition is complex: is related to the egg formation, although the chemical characteristics of the antimicrobial and the chemical composition of the egg also intervene. The extra label application of marbofloxacin in chickens in production requires a withdrawal period of 17 days.

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